# Towards the development of a sustainable low-cost bio-active amendment for the *in situ* remediation of urban brownfield top soil contaminated with Lead (Pb) and Polycyclic aromatic hydrocarbons (PAH)

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## Summary

Urban soils present distinctive characteristics resulting from high anthropogenic pressure. Contamination levels (e.g. heavy metals and organic compounds) tend to be higher even in the absence of heavy industry, and can pose a hazard to health.

Brownfield land has been defined as any land which has been previously developed, which may or may not be contaminated. Sustainable development of brownfield land encompasses sustainable (e.g. nature-based) methods of restoration-rehabilitation.

In the historically industrial city of Glasgow, there are significant areas with derelict land. This not only acts as a key detractor for investment but also denies usability for residents, posing a challenge for the city council.

An example of this it the Old Glasgow Meat market site, in the east end of Glasgow, which in preliminary site investigations showed issues with lead (Pb) and polycyclic aromatic hydrocarbon (PAH) contamination.

The aim of this PhD project is hence to develop a sustainable, bioactive, low-cost amendment that can both immobilize Pb and support biodegradation of PAHs in urban brownfield top soil, particularly applicable for sites with moderate contamination and no immediate development strategy.

Different approaches have been studied for the *in situ* remediation of Pb contaminated soil, but the immobilization of Pb as pyromorphite ($Pb_5[PO_4]_3X$; X= F, Cl, B or OH) with phosphate amendments has shown the most promise for the reduction of Pb bioavailability. There are limitations to this method in the urban environment, however, as acidic soil pH is required for Pb and P to react forming pyromorphite.

Recent studies have shown that the fungus *Paecilomyces javanicus*, isolated from Pb contaminated soils in Scotland, is able to induce the biomineralization of Pb as pyromorphite in laboratory cultures.

Biodegradation is one of the main processes responsible for the reduction of PAH concentrations in soil. For this reason, both bioaugmentation with allochthonous microorganisms and biostimulation of native microorganisms, with the addition of nutrients, have been used for the remediation of PAH contaminated soil.

Biodegradation of PAHs has been extensively researched with bacteria. However, fungi have also been proposed as important promoters of PAH biodegradation. Biosorption of PAHs to microbial biomass may also occur in the environment.

Brewery spent grain is an important agro-industrial waste in Scotland, and has a high nutrient content. As a lignocellulosic material it also has potential to stimulate PAHs biodegradation through enzymatic co-metabolism. The use of unprocessed brewery spent grain for bioaugmentation strategies in the context of soil remediation, has not been studied before, to the knowledge of the author. For these reasons, brewery spent grain was selected to be tested as a carrier matrix for the cultivation and subsequent inoculation of *P.javanicus* in soil. The Pb-biomineralizing capability of *P.javanicus* combined with the use of brewery spent grain as an inoculum carrier and soil amendment, was studied as a bioaugmentation/biostimulation strategy for the co-remediation of Pb and PAHs in soil.

Liquid culture experiments were carried out as a first experimental stage. In cultures spiked with Pb and an organic P source, the ability of *P.javanicus* to biomineralize Pb as pyromorphite was corroborated with x-ray diffraction (XRD) and energy-dispersive X-ray (EDX) spectroscopy. An initial capture of Pb by *P.javanicus* as Pb-oxalate, during the first 10 days of incubation, was followed by an increase in pH and a transformation in pseudomorphism to pyromorphite.

*P.javanicus* showed tolerance to the presence of both benzo(a)pyrene (B(a)p) and phenanthrene (Phen) in spiked culture media. With high-performance liquid chromatography (HPLC) analysis of culture media, no Phen was detected in solution after 10 days. Gas chromatography–mass spectrometry (GC-MS) analysis of fungal biomass, showed that biosorption was the main process responsible for the removal of Phen from solution.

These liquid culture experiments showed that the use of active *P.javanicus* biomass has potential for the removal (remediation) of Pb and Phen-PAHs in aqueous matrices.

Out of eleven samples collected from the Old Glasgow Meat market, for use as a matrix in co-remediation experiments, three had concentrations of Pb above selected reference guideline values, and none had detectable PAH concentrations. For this reason, Meat market samples were used in preliminary qualitative experiments and spiked soils in quantitative microcosm experiments.

Sterile spent grain proved to be a suitable matrix for the cultivation of *P.javanicus*, and the preparation of inoculum carriers. *P.javanicus* appeared to be successfully introduced in soil in microcosm and pot experiments, with the use of spent grain as an inoculum carrier and soil amendment to encourage fungal colonisation of soil, indicating that the use of spent grain as an inoculum carrier and soil amendment has significant potential as a bioaugmentation strategy for soil remediation.

In microcosm experiments, sorption of Pb and other metals to added brewery spent grain occurred. This could have positive effects in reducing environmental mobility of metals. A reduction of the P concentration in spent grain used as a soil amendment indicated that P may have been released from the brewery spent grain into the soil system, providing an additional positive benefit of spent grain amendments as an organic P source in soils.

In microcosm experiments with treated Pb-spiked soils, no significant changes in Pb UBM-bioaccessibility or BCR-residual Pb (sequential extraction) were observed, indicating that the limitations of P amendments in soils with neutral to alkaline pH, were not overcome with *P.javanicus*-bioaugmentation and that the ability of *P.javanicus* to biomineralize pyromorphite in liquid cultures was not easily transferred to a soil matrix.

While the inoculation of *P.javanicus* in microcosms with Phen and B(a)p spiked soil did not appear to have an effect in their concentration over time, the amendment of spiked soil with sterile or non-sterile spent grain produced a significant decrease in B(a)p concentrations after 150 days of incubation.

In pot experiments with poor quality urban soil, the use of brewery spent grain as an amendment, non-sterile and as a *P.javanicus* inoculum carrier, significantly increased above ground and root biomass of a native grass and wildflower mixture.

A combination of the proposed bioaugmentation/biostimulation strategy with the growth of native grass and wildflower species, may help enhance the biodegradation of PAHs and availability of P for the biomineralization of Pb as pyromorphite, and is hence recommended for future studies on the co-remediation of Pb and PAHs in urban brownfield soils. The use of nature based approaches such as the latter, may be acceptable best practice for brownfield land with poor soil quality and discrete, moderate contamination. Although they may not completely resolve all pollution issues, they improve soil health and may have added benefits such as the provision of ecosystem services.

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**Statement of Originality to Accompany Thesis Submission**

**Name:** Felipe Esteban Sepúlveda Olea

**Registration Number:**

I certify that the thesis presented here for examination for a PhD degree of the University of Glasgow is solely my own work other than where I have clearly indicated that it is the work of others (in which case the extent of any work carried out jointly by me and any other person is clearly identified in it) and that the thesis has not been edited by a third party beyond what is permitted by the University's PGR Code of Practice.



I declare that the thesis does not include work forming part of a thesis presented successfully for another degree [unless explicitly identified and as noted below].

I declare that this thesis has been produced in accordance with the University of Glasgow's Code of Good Practice in Research.

I acknowledge that if any issues are raised regarding good research practice based on review of the thesis, the examination may be postponed pending the outcome of any investigation of the issues.

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## List of abbreviations

| | |
|---|---|
| ABA | Absolute bioavailability |
| AG | Above ground |
| ASE | Accelerated solvent extraction |
| B(a)p | Benzo(a)pyrene |
| BCR | Sequential extraction method |
| CI | Confidence interval |
| CRM | Certified reference material |
| EDX | Energy-dispersive X-ray spectroscopy |
| G2P | Glycerol 2 phosphate |
| Gc | Gastric (UBM bioaccessibility phase) |
| GC-MS | Gas Chromatography - Mass Spectrometry |
| GR | Germination rate |
| GS | Garden soil |
| HPLC | High performance liquid chromatography |
| ICP-OES | Inductively coupled plasma atomic emission spectroscopy |
| Ist | Intestinal (UBM bioaccessibility phase) |
| LOD | Limit of detection |
| LOI | Loss on ignition |
| MCD | Modified Czapek-Dox media |
| MEA | Malt extract agar |
| MM | Meat market |
| MMS | Meat market soil |
| MR | Main root biomass |
| P amendments | Phosphate amendments |
| PAH | Polycyclic aromatic hydrocarbons |
| Pb | Lead |
| PDb | Potato dextrose broth |
| Phen | Phenanthrene |
| PI | Prediction interval |
| Pj | *Paecilomyces javanicus (P. javanicus)* |
| pXRF | Portable X-ray fluorescence |
| RBA | Relative bioavailability |
| RefMtx | Reference matrix |
| Res | Residue |
| RMIst | Intestinal (UBM bioaccessibility phase) normalized to the reference matrix |
| S | Soil |
| SEM | Scanning electron microscope |
| SG | Spent grain |
| SMMS | Sterile Meat market soil |
| SP | Superphosphate |
| SSG | Sterile spent grain |
| UBM | In vitro bioaccessibility assay method |
| XRD/pXRD | Powder X-ray diffraction |

# CHAPTER 1 Defining a research problem

## 1.1 Introduction

Urban soils present distinctive characteristics, compared to rural soils, resulting from high anthropogenic pressure (1-4). Contamination levels of urban soils tend to be higher, even in the absence of heavy industry, showing significant increases in the concentrations of a number of metals and other compounds that can pose a hazard to health (2, 4, 5). The changes and physico-chemical parameters of these soils are dominated by their past and current uses, which can lead to compaction of deeper layers, low biodiversity, presence of fragments of various materials and high concentrations of pollutants (2-4). Commonly found contaminants in urban soils include heavy metals (lead, zinc, copper, cadmium and chromium); organic pollutants such as PAHs and pesticides; asbestos and microbial pathogens (1, 4, 6-8).

Heavy metals and organic contaminants present numerous health dangers to higher organisms and are also known to decrease plant growth, ground cover and to have a negative impact on soil microflora (8). Direct ingestion of dust and soil particles via hand-to-mouth contact strongly contributes to exposure of contaminants in children (2, 7). Consumption of home-grown produce containing elevated concentrations of potentially toxic substances, inhalation of contaminated particles or direct skin contact with contaminated soil, water or airborne particulates, are also common pathways for human exposure (1, 7, 10).

Sources of contamination in the urban context are multiple: industrial uses, demolition of buildings, atmospheric deposition from motor vehicles, domestic and industrial burning of fossil fuels, incinerators, particles of paint from houses and other structures (1, 4, 6, 7). Historic use of waste (ash, building rubble, tar, sludge and mineral materials) as fill in sites, for levelling, has also been an important source of contamination (1, 12). Even when a cosmetic layer of topsoil is placed over building rubble, some contaminants can re-contaminate the topsoil (1). In addition to the higher degree of contamination, the presence of concrete and building rubble, and absence of the original topsoil, can also cause extreme physico-chemical properties such as high pH and low water holding capacities (1). Due to the latter, urban topsoil and subsoil tend to be largely artificial and complex matrixes (6).

Since World War II, there has been a steady decline of manufacturing and an exodus of industry from urban areas to the suburbs, exurbs (e.g. commuter towns) and emerging economies (10, 15). This phenomenon has been a feature of most developed economies (10, 15), and the consequent deindustrialization of cities has left behind extensive tracts of underutilized and vacant land, commonly referred to as brownfields (10).

In the UK, brownfield land has been defined as any land which has been previously developed, including derelict and vacant land, which may or may not be contaminated (10, 16). Despite several decades of urban policy initiatives, many former industrial cities in the UK still possess large areas of disused land and buildings (17). In Scotland, the total amount of derelict and urban vacant land has decreased by 716 hectares (6%) between the years of 2017 and 2018, from 11,753 hectares to 11,037 hectares respectively (18). Amongst City Authorities, however, Glasgow City has the largest urban vacant land area, with 1,005 hectares, 9% of the Scotland total (18). Data from 2019 in England, showed up to 18200 registered brownfield sites covering more than 26000 hectares (19).

Not only is the natural environment of brownfield land potentially contaminated and hazardous for human health, but they also have negative socioeconomic impact to their surroundings, contributing to depressed real estate markets, increasing crime rates and providing a general sense of community depreciation (10, 17).

Brownfields can provide available space for new developments within the urban environment, however they often persist for longer than desirable (20). In the UK, as well as in many countries, over the past few decades planning policy has prioritised the redevelopment of brownfield areas in an effort to bring vacant land back into use, encouraging urban regeneration and preservation of greenfield land (12, 15, 16, 21-23).

The concept of sustainable development, has gained strength over the past decades (16). A widely accepted definition of sustainable development is: development that meets the needs of the present without compromising the ability of future generations to meet their own needs (Brundtland Commission, 1987) (16). In other words, sustainable development promotes economic growth, maintaining social inclusion and minimising environmental impact (12, 16, 20, 24).

Circular land management is a concept that refers to sustainable and repeated use of development land: avoiding new brownfields, recycling existing brownfields and compensating the effects of land consumption, particularly aiming at the reduction of greenfield development and urban sprawl (15, 16, 20, 23, 24).

Soft re-use of brownfields describes intended temporary or final re-uses of brownfield sites, which are not based on built constructions or infrastructure (hard-development), such as public green space. These are uses where the soil is not sealed, remaining biologically productive for agriculture, habitat, forestry, amenity or landscaping (20). Hard and soft use are not necessarily mutually exclusive (20). While historically there has been a preference for hard redevelopment of brownfield land, regeneration for soft re-uses is now used in a

number of countries including the United Kingdom (20). In urban environments, greenspace can offer important aesthetic improvements, encourage inward investment, provide facilities for recreation, improve air quality and have an effect on decreasing the urban heat island effect (20, 25). Green infrastructure can also help create habitat for migrating birds and other species in urban areas, as well as aid in the mitigation of climate change impacts (20). A renaissance of new forms of urban gardening, community gardens and urban farming has also increased the demand for adapting brownfields for green uses (20).

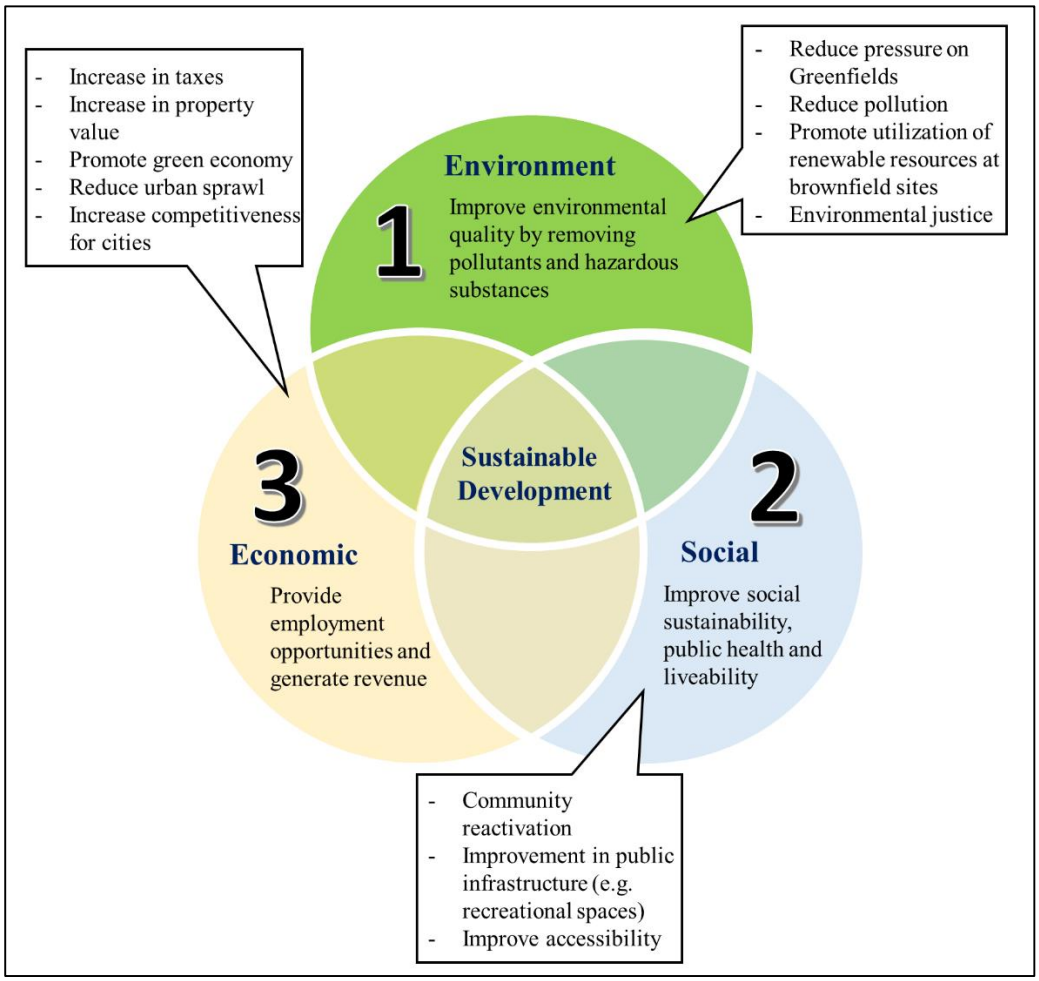



*Figure 1.1. Schematic representation of the benefits of sustainable brownfield redevelopment. Modified from Ahmad et al. (2018) (26).*

As brownfield sites represent a range of previous land-use types (4), that may contain soil contamination, many sites must be rehabilitated to allow re-development (8). The growth of plants and hence the viability of soft re-uses is also dependent on a suitable level of soil functionality (20).

Sustainable development encompasses sustainable methods of brownfield restoration-rehabilitation (20). A paradigm shift is occurring to accommodate ecological approaches to what was formerly achieved through rigid engineering and a general avoidance of any reliance on natural systems (27). The EU Landfill Directive has also driven developers

towards more sustainable methods of remediation, to avoid the use of 'dig and dump' as a clean-up method (16). Dig and dump refers to contaminated soil, with recalcitrant pollutants, which is excavated and landfilled in a secure hazardous waste disposal facility (10). Besides having a high cost, this method is no longer considered to be environmentally sustainable (10, 16).

An alternative that has emerged in situations where site environmental conditions are such that pollutants are deemed to be unlikely to reach receptors, other than by direct ingestion or inhalation of contaminated soil, is covering a contaminated brownfield site with layers of clean soils (10).

Other *in situ* technologies involving the removal of contaminants from contaminated locations without displacing soil or water from the site, however, are viewed as more sustainable (10). Bioremediation is a good example of sustainable *in situ* remediation (10). Phytoremediation, which uses plants, is an *in situ* decontamination approach that has shown promise for addressing both organic and inorganic contaminants (8). Another example of bioremediation is the introduction or stimulation of indigenous pollutant-degrading microorganisms (i.e. bacteria and fungi) (10). Essential nutrients such as nitrogen and phosphorus are supplied to promote biodegradation (10). The advantages of bioremediation are that it is relatively inexpensive, it does not require the addition of harsh chemicals and it has the potential to completely transform organic pollutants into non-toxic products (10).

These methods can be applied in synergy, combining processes on site to deliver several useful services and improvements in the long term, for example combining gentle bioremediation and biomass cultivation, or some other form of intervention such as green infrastructure (20).

The benefits that humans derive from nature are known as ecosystem services (20). Ecosystem services can be divided into four categories: provisioning services, regulating services, habitat or supporting services, and cultural services (20). Examples of regulating ecosystem services are the mitigation of contamination through biological processes *in situ* (20) and waste re-use and recycling (e.g. beneficial re-use of composts or agro-industrial residues) (20, 28). In the context of sustainable development and remediation, combining these two examples for the improvement of brownfield land has hence significant promise.

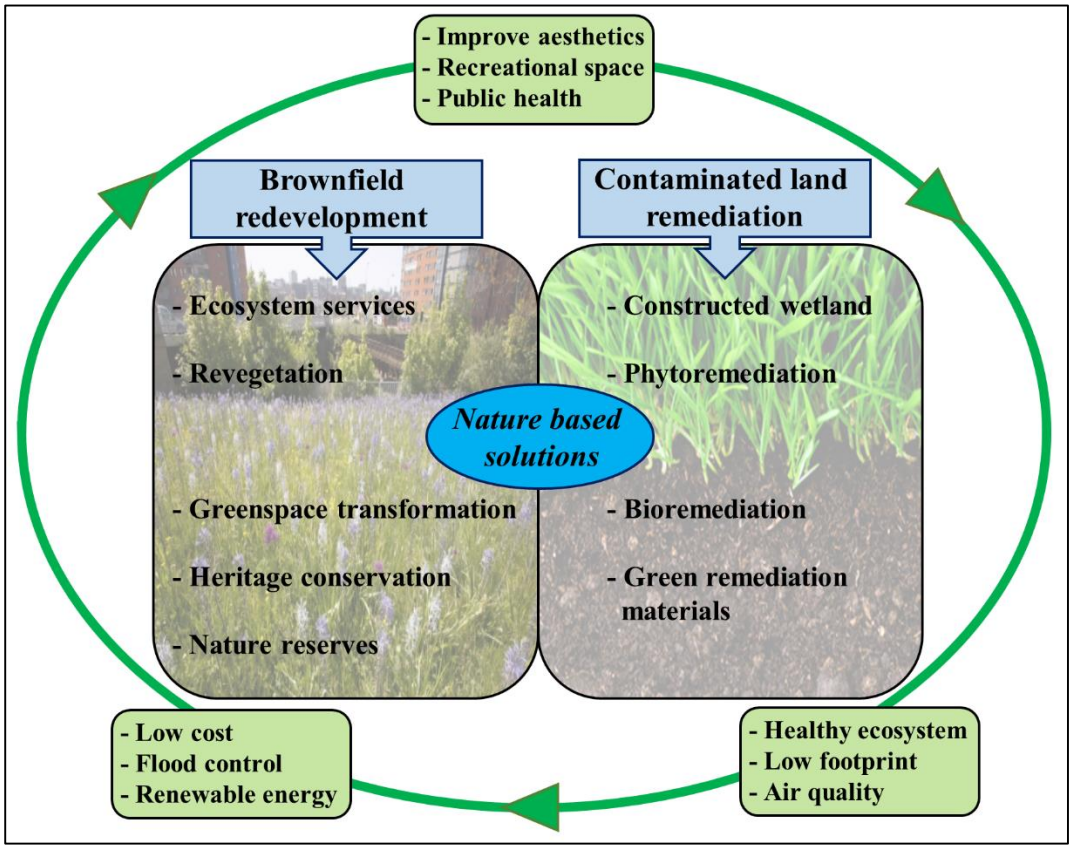



*Figure 1.2. Schematic representation of the benefits of nature based solutions for the redevelopment and remediation of brownfield land. Modified from Song et al. (2019) (29).*

## 1.2 Collaboration with Glasgow City Council

The Glasgow City Region City Deal is an agreement between the UK Government, the Scottish Government and eight local authorities across Glasgow and the Clyde Valley, which aims to supply funding and support the development of the local area though the following (30, 31):

- Improved infrastructure: funding to support the delivery of an improved transport network across Glasgow and the Clyde Valley, key development and regeneration sites, bring large tracts of land back into use, and improved public transport (30, 32).
- Growth in life sciences: establishment of world class research and development and commercialisation facilities (30).
- Supporting business innovation: providing additional business incubator and grow-on space for entrepreneurs across the region enabling more small and medium enterprises to grow (30).
- Tackling unemployment: creation of thousands of new jobs and establishment of programmes to provide targeted support to 16-24 year olds and vulnerable residents,

and testing new ways of boosting the incomes of people on low wages to make them more self-reliant (30).

The Collegelands Calton Barras (CCB) project, is one of the main projects within the City Deal, and aims to deliver an integrated, attractive and resilient neighbourhood within the inner east end of Glasgow (33).

The Calton area of Glasgow lies immediately east of Glasgow Cross in the city centre and is bisected by Gallowgate, London Road and main rail lines (34). In the past, Calton was an independent Burgh with a rich history and identity related to its role as a centre for weaving and pottery (35). Over time, the area has changed following the arrival of the railways and the related heavy industries, the founding of the Barras trading market in the 1920s, industrial decline and subsequent shifts in the local population (35).

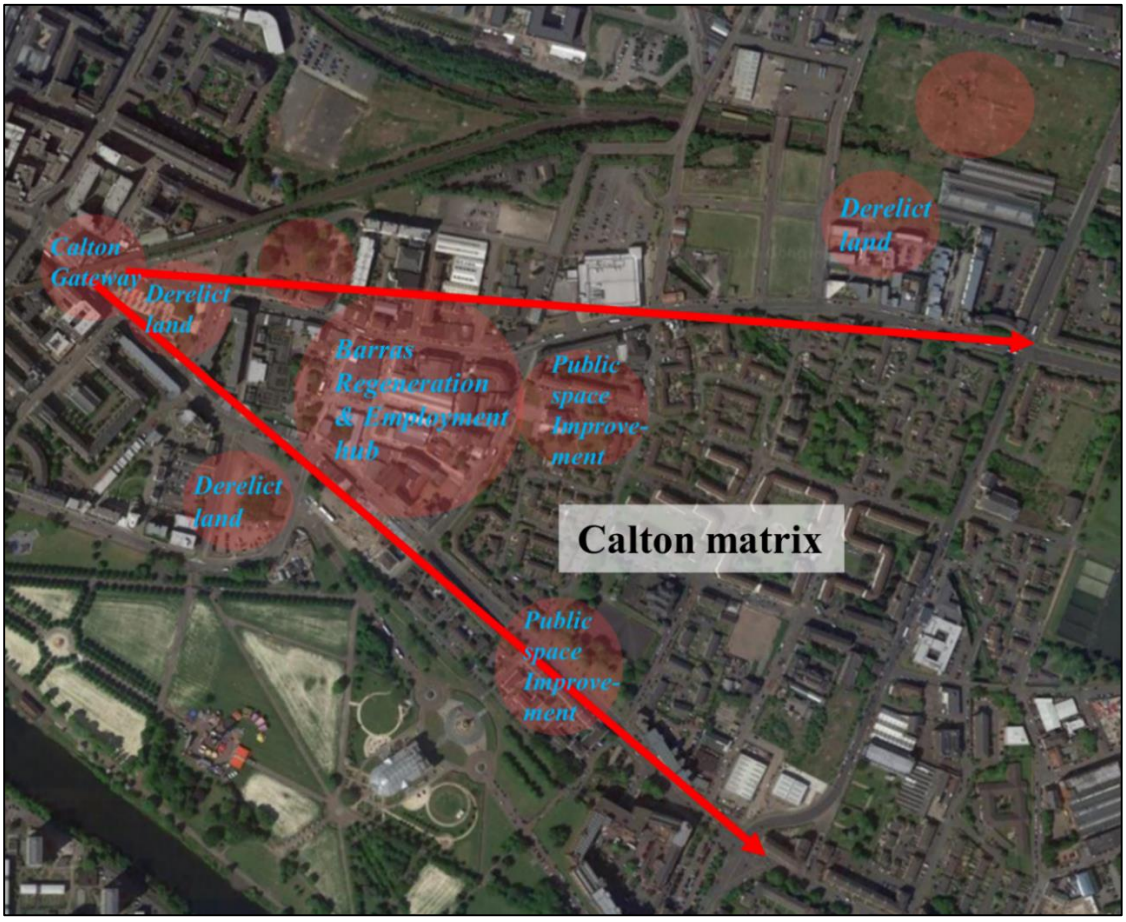



*Figure 1.3. Schematic overview of the Calton-Barras action plan project. Modified from: (34) with satellite image extracted from Google Maps, 2020.*

The development project in this area responds to identified interlinked social, economic and environmental problems, with a third of the built up area being vacant at the time, and much of the rest in poor or part derelict condition (34). This not only acts as a key detractor for investment but also denies usability for residents and visitors alike (34). In consequence

encouraging development is a main objective of the project, through critical enabling works to remediate these sites and, where necessary, the use of Council powers to pro-actively encourage positive development (34). In cases where it is determined that no development is likely to take place in a short timescale, stalled sites or underutilised open spaces can be environmentally remediated and taken forward to community groups and local organisations to develop temporary projects (34, 36). For instance, the Stalled Spaces Glasgow project (36), encourages use of sites as growing spaces, pop-up gardens, wildlife areas, urban gyms and natural play places, amongst others. In developing and delivering value-added projects in partnership with community groups, such initiatives aim to improve the local population's quality of life, by improving their social, economic and environmental well-being (37). These temporary uses also encourage more long term development by making sites more marketable or as longer term provision of a community asset, whilst minimising any liabilities on maintenance of the sites by the Council (34).

In this context, and in an effort to encourage research in the subject of sustainable remediation, with a particular focus on low cost *in situ* bio- and/or phytoremediation of contaminated top soil, Glasgow City Council acted as a collaborator in this PhD project. The City Council facilitated access to an exemplary case site, that presented characteristics commonly found in urban derelict brownfield land in Glasgow, and the UK in general. This permitted the collection of environmental soil samples for the study of soil contamination issues and use as a matrix in the experimental research carried out in this PhD project.

### 1.3 The Meat market site

The old Glasgow Meat market vacant site is located within the Calton area of Glasgow, in the North-East limit (Duke Street and Bellgrove Street). The site of ~ 5.17 ha, at the time mainly consisted of overgrown land in the central and northern portions, and open side structures in its southern portion, which are classified as Listed Buildings and other statutory Heritage Features (38).

Over the last 150 years, different areas of the site had undergone a variety of uses. From 1814 towards the south of the site, a cattle market livestock sale yard had been functioning, and in the late 1851 a slaughterhouse was established (39). In the 1870s the land to the north end was purchased and a new meat market was opened in 1878 (39). This development continued to expand, with the addition of a new modern slaughterhouse where around 33 wholesalers and 150 slaughtermen operated on site (39). In the years from the 1870s to the 1930's the Calton area became possibly the busiest area in the whole of Glasgow, and the Meat market saw further development on the site over this time (39). However, the late 50's

and early 60's saw a marked deterioration in the housing stock in the area (39). Many of the residents of the area began to receive offers of alternate housing in newly emerging schemes towards the outer suburbs of the city (39).

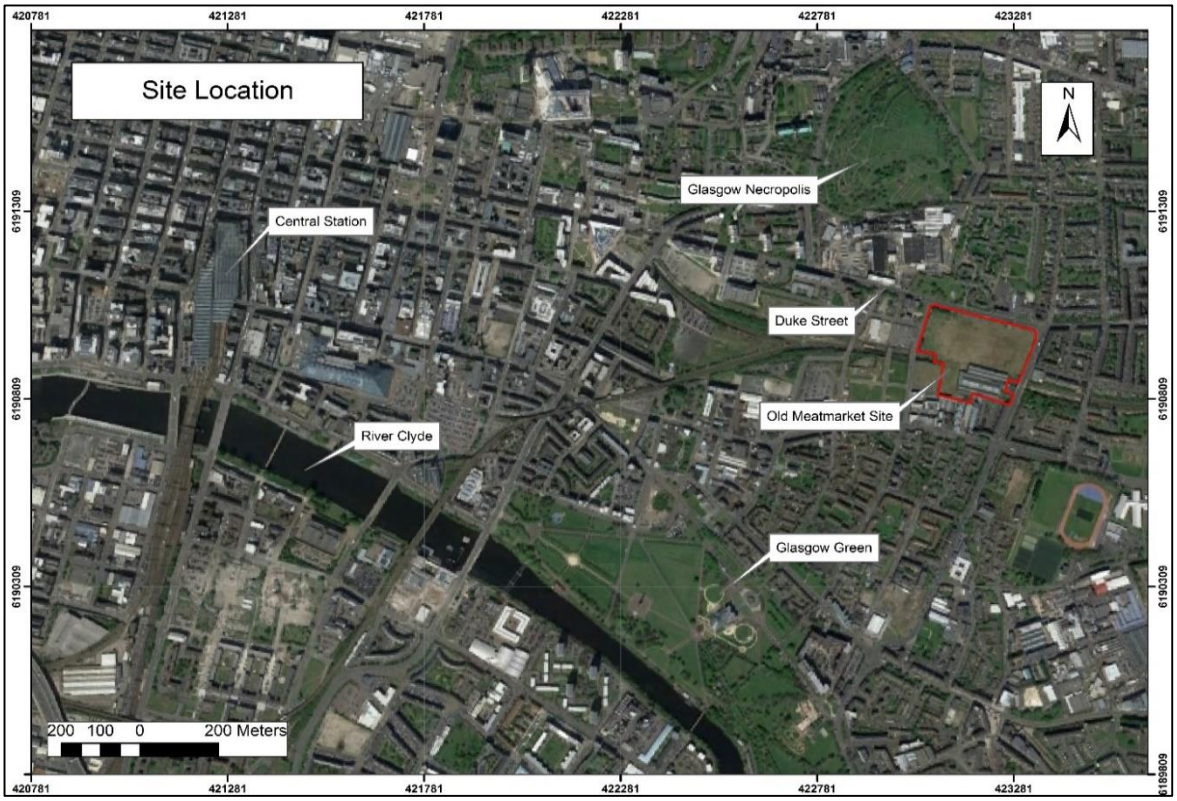



*Figure 1.4. Satellite image (Google Maps, 2017) with the location of the Meat market site and other landmarks in Glasgow.*

From this period on there was a slow degradation of the district (39). In the early 1970s the original Meat market closed and a New Glasgow Abattoir opened on the site, with modernised standards in line with the worldwide meat trade (39). It was expected that the market would flourish and smaller slaughterhouses close down, due to strict new hygiene regulations (39). However, with the support of Common Market grants, smaller businesses were also able to modernize and continue functioning (39). The New Glasgow Abattoir managed to stay open until 2001 and was demolished in 2007 (39). Sheds were subsequently operated as a car market before falling into disuse after 2015 (38, 39). In summary, over the period of time the market functioned, the following uses were undertaken in different portions of the site: grinding mills, cattle market/abattoir, hide, skin and tallow market, chemical works, and lastly builder's yard and tanks (38).

Ground investigations with exploratory boreholes, carried out on site while derelict, indicated that made ground composed the uppermost layer across the site (38, 39). Ranging in thickness from 0.4 – 6.0m, made ground was described as sandy gravelly clay and gravelly sand, with inclusions of brick, concrete, burnt shale, rootlets, metal, glass, plastic, litter, ash,

*Table 1.1. Summary of concentrations of selected heavy metals and organic contaminants obtained from preliminary chemical data of the Meat market site, compared to compiled generic assessment criteria and background values.*

| | Mean (mg kg$^{-1}$) | Range (mg kg$^{-1}$) | C4SL Res. whgp[1] (mg kg$^{-1}$) | n (%) | C4SL Allot.[2] (mg kg$^{-1}$) | n (%) | C4SL POS[3] (mg kg$^{-1}$) | n (%) | CLEA SGV (2011) Res.[4] (mg kg$^{-1}$) | n (%) | Dutch Soil Intervention Value[5] (mg kg$^{-1}$) | n (%) | Proposed UK Ecological SGV[6] (mg kg$^{-1}$) | n (%) | Glasgow Background BGS mean[7] (mg kg$^{-1}$) | n (%) | SH UK (USM)[8] (mg kg$^{-1}$) | n (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **As** | 7.140 | 1.580-20.50 | 37.00 | 0 | 49.00 | 0 | 79.00 | 0 | 32.0 | 0 | 76.00 | 0 | | | 10.90 | 12.0 | 7.540 | 34.3 |
| **Cd** | 0.630 | 0.184-1.280 | 26.00 | 0 | 4.900 | 0 | 220.0 | 0 | 10.00 | 0 | 13.00 | 0 | 1.150 | 1.9 | 0.380 | 86.1 | 0.280 | 89.0 |
| **Cr** | 34.19 | 9.920-427.0 | | | | | | | | | 180.0 | 0.9 | 21.10 | 80.6 | 125.0 | 0.9 | 38.30 | 22.2 |
| **Cu** | 47.37 | 5.750-153.0 | | | | | | | | | 190.0 | 0 | 88.40 | 13.0 | 76.20 | 16.7 | 34.80 | 51.0 |
| **Ni** | 37.74 | 11.90-256.0 | | | | | | | 130.0 | 0.9 | 100.0 | 0.9 | 25.10 | 70.4 | 54.90 | 13.9 | 26.20 | 67.6 |
| **Pb** | 119.5 | 7.100-1300 | 200.0 | 16.7 | 80.00 | 45.4 | 630.0 | 1.9 | | | 530.0 | 3.7 | 167.9 | 19.4 | 180.1 | 16.7 | 118.0 | 31.5 |
| **Zn** | 148.7 | 23.30-2750 | | | | | | | | | 720.0 | 0.9 | 90.10 | 50.9 | 201.0 | 13.9 | 99.9 | 44.4 |
| **B(a)p** | 7.490 | 0.021-464.0 | 5.000 | 11.1 | 5.700 | 8.3 | 70.00 | 5.6 | | | | | | | | | 0.236 | 57.4 |
| **PAH** | 50.24 | 0.248-2220 | | | | | | | | | 40.00 | 16.7 | | | | | | |

**Columns with "n (%)" correspond to the percentage of samples with concentrations that surpass the respective guideline or background value. [1] Category 4 Screening Level for residential use with home-grown produce (DEFRA (9)); [2] Category 4 Screening Level for allotment use (DEFRA (9)); [3] Category 4 Screening Level for public open space use (DEFRA (9)) [4] CLEA 2011 soil guideline value for residential use (Fordyce et al., 2014 (11)); Dutch Soil Intervention Value for soils (VROM (13)) [6] Proposed UK Ecological Soil Guideline Value (considering any adverse effects on wildlife such as birds, mammals, plants, soil invertebrates or on the microbial function of soils) (Fordyce et al., 2014 (11)); [7] (Fordyce et al., 2014); [8] Soil and Herbage UK mean background value for urban areas in Scotland (EA (14)).*

sandstone, dolerite, mudstone and coal (38, 39). The underlying natural soils were found to comprise soft laminated sandy clays and silts, underlain by glacial clay (39).

Available chemical data, resulting from the ground investigation performed by environmental consultancy WSP Parsons Brinckerhoff, was provided to the author by Glasgow City Council. Data from a total of 108 samples from 40 locations, was compared to available generic assessment criteria and the urban soil geochemical background of Glasgow (11) (Table 1.1). Within the metals investigated, lead (Pb) stood out as a potential contaminant of concern, as 16.7 % of the samples surpassed Category 4 Screening Level defined for a residential end use (200 mg $kg^{-1}$ (9)), 45.4 % the value for allotments (80 mg $kg^{-1}$ (9)), and 1.9 % the value for open public space (near residential, 630 mg $kg^{-1}$ (9)), with a maximum concentration of 1300 mg $kg^{-1}$. Regarding other heavy metals, 80.6 %, 70.4 % and 50.9 % of samples, had concentrations above a proposed UK Ecological Soil Guideline Value (11) for Chromium (Cr), Nickel (Ni) and Zinc (Zn) respectively. In comparison with the soil geochemistry background for Glasgow (11), Cadmium (Cd) was present above the mean soil value in the greatest amount of samples (>86.1 %).

Regarding organic contaminants, total PAHs (16 priority contaminants USEPA) with a mean of 50.2 mg $kg^{-1}$ and a total range of 0.248 – 2220 mg $kg^{-1}$ were found in concentrations above the Dutch Soil Intervention Value of 40 mg $kg^{-1}$ (13) in 16.7% of the samples. This directly correlated with high concentrations of Benzo(a)pyrene (B(a)p) (correlation coefficient of 0.944), which surpassed in 11.1 % of the samples the Category 4 Screening Level value for residential use with home-grown produce of 5 mg $kg^{-1}$ (9), in 5.6 % the value for public open space (near residential) of 10 mg $kg^{-1}$ (9), and in 57.4 % the mean value for urban soils in Scotland of 0.236 mg $kg^{-1}$ (14).

Overall, the main contaminants of concern, particularly regarding human receptors, that arose from the available chemical data of the Meat market site were Pb and B(a)p/PAHs. The latter, though present in moderate concentrations, surpass some of the mentioned guidelines, and would consequently still require intervention measures. For this reason, the combination of Pb and PAHs in moderate concentrations was defined as the mixed-contamination profile of interest.

The redevelopment planning statement by Glasgow City Council (39) proposed a mixed use (residential, commercial and open space). Remedial options considered for contaminated made ground that coincided with proposed garden and landscape areas, included made ground removal or implementation of a cover system (39), with potential relocation/encapsulation of impacted materials at depth or below proposed hardstanding

(39). Soil washing was also considered as a suitable technology to be undertaken within pre-development enabling works, to reduce the thicknesses of cover materials utilised, thus presenting cost savings in the overall remedial solution (39).

In the same document, the Council also indicated interest in encouraging active, community based temporary uses of the development plots once the site remediation and infrastructure works were completed and prior to final development (39).

### 1.4 General aims of the PhD project

With the characteristics of the Meat market site, as well as the Calton area in general, and the motivations of the Council in mind, the following was defined as the general aim of this PhD project:

To develop a sustainable bioactive low-cost amendment that can both immobilize Pb and support biodegradation of polycyclic aromatic hydrocarbons (PAHs) in urban brownfield top soil.

This would be particularly applicable for sites with moderate contamination and no immediate development strategy and reduce the amount of material that would eventually require removal and/or replacement with a cover material. Overall, aiming to maximise the reutilization of soil, reducing costs, and ideally allowing for potential temporary uses in value-added projects.

In the urban context both human and ecological receptors were considered.

In the following chapter, a literature review of topics relevant to the general aims is made, concluding with identified specific objectives and tasks to be carried out in order to achieve this general aim.

# CHAPTER 2 Literature review

## 2.1 Lead (Pb) soil contamination and remediation

### 2.1.1 Lead (Pb) general properties and provenance

Pb is a non-essential heavy metal, ubiquitous in soil. It commonly exists in three oxidation states, Pb(0) (metallic lead); Pb(II); and Pb(IV). In the environment Pb(II) is the most stable and hence abundant (40). Pb(IV) is only formed under extremely oxidizing conditions and inorganic Pb(IV) compounds are not found in ordinary environmental conditions (40). Metallic lead exists in nature, but its occurrence is rare (40).

The average Pb concentration on the Earth crust is 16 mg $kg^{-1}$, while in uncontaminated soil it commonly found in concentrations around 40 mg $kg^{-1}$(41). Though natural weathering of rocks is not an uncommon source (42), most of the lead found in soils has an anthropogenic provenance, both historic (leaded gasoline emissions, colouring pigments) and current/active (mining and smelting, lead-acid batteries, bullets and shot, weights, solder, pewter, and fusible alloys) (41-43) . From the 1930s to 2000 in the UK, Pb was added in the form of alkyl Pb additives to petrol, with the car-exhaust emissions of inorganic Pb peaking in the 1970s (44). The resultant urban Pb contamination, including that of Glasgow soils, has been reported in several publications since then (44). Pb released to the environment as a result of leaded gasoline combustion, commonly is in the form of Pb halides (e.g. PbBrCl), though Plumbogummite ($PbAl_3(PO4)_2(OH)_5$* $H_2O$) is another form of Pb that has also been identified in soils adjacent to highways (45).

Pb is one of the less mobile heavy metals and is the reason why it tends to accumulate in the top soil layers (41, 46). Its chemistry in soils is affected by three main factors: specific adsorption to different solid phases, precipitation of sparingly soluble compounds, and the formation of relatively stable complexes or chelates with the soil organic matter (41, 45). At the same time, soil grain size and pH influence these factors, and with them the distribution and accumulation of lead in the different soil phases such as metal hydroxides, metal carbonates, phosphates, organic compounds, Fe and Al oxides and clay (41). An inverse relationship exists between Pb concentration and grain size (which implies surface area), for example the wind transportable soil fraction (<100 μm) is usually found to be associated with elevated Pb concentrations in contaminated sites (43).

Organic matter can adsorb Pb and aid its immobilization, but when organic matter is soluble, especially in alkaline environments, complexation with Pb can sometimes allow mobilization and leaching down the soil column (46).

In natural soil environments Pb is present mainly as carbonates (cerussite-hydrocerussite) and sulfates (anglesite) in oxidized conditions and sulfide (galena) in reduced conditions (Figure 2.1) (45, 47-49). Though more minor, Pb oxides such as litharge (PbO) and minium ($Pb_3O_4$) may also form when metallic Pb is exposed to oxidizing environmental conditions (50).

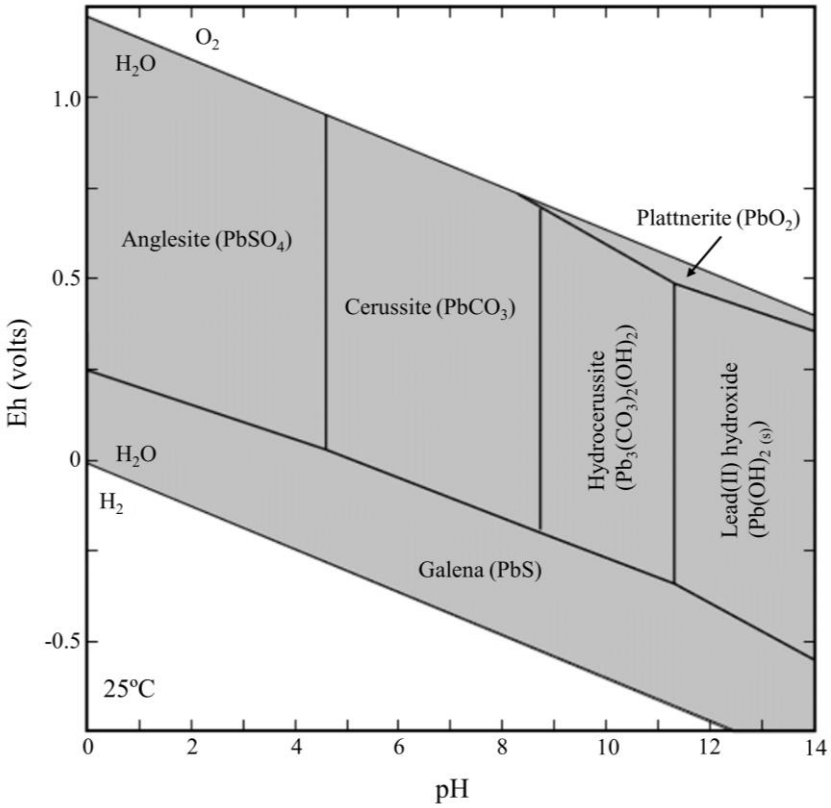



*Figure 2.1. Eh-pH diagram for Pb. Modified from Wilkin et al (2007) (49). Total Pb = $10^{-5}$ mol $kg^{-1}$ (2 mg $kg^{-1}$), total C = $10^{-3}$ mol $kg^{-1}$(12 mg $kg^{-1}$), total S = $10^{-3}$ mol $kg^{-1}$(32 mg $kg^{-1}$). All organic lead complexes are suppressed and activity coefficients for all species are set equal to 1. Grey indicates solid phase.*

### 2.1.2 Human health issues associated to lead (Pb)

The main pathway of exposure to Pb for humans is direct ingestion (43). Children are more susceptible to exposure to Pb contaminated soil, due to the frequency of hand-to-mouth behaviour (pica), and a greater rate of intestinal absorption and retention (43, 45, 51-54). It has been reported (43) that children age 1–6 years can ingest up to 100 mg $day^{-1}$ of soil via hand-to-mouth activity. Inhalation of high-Pb dust may also be an important pathway for human exposure (55).

Previous published research (45, 55, 56) indicates that Pb does not tend to phytoaccumulate. The translocation from soil to roots, and roots to plant shoots appears to be minimal, so consumption of home grown produce does not seem to be a highly relevant pathway for

humans (45, 55, 56). A model elaborated by Clark *et al*., (2008)(43) suggests that consumption of home-grown produce accounts for only 3% of children's daily exposure of Pb, while ingestion of fine grained soil (<100 µm) accounts for 82% of the daily exposure.

Once in the organism, approximately 10% of Pb can be excreted and the remainder is transported and absorbed by organs such as the brain, liver, kidneys or bone, where it can begin to harm cells (45, 52). It is likely that Pb acts on several cellular and subcellular sites to cause harmful effects (45, 52).

Pb in the organism, has a different effect depending on where it is accumulated. Pb stored in the skeleton is mostly inactive physiologically, but in blood or soft tissue is associated with negative biological effects (45, 52).

Effects of Pb to human health, depending on the dose, can be acute or chronic, and can be quite severe (Table 2.1). Blood levels higher than 80 µg $dl^{-1}$ can cause coma, convulsions, and even death in children. Lower levels cause problems in the central nervous, kidney, and hematopoietic systems, as well as cognitive and behavioural problems in children, particularly on verbal IQ and hearing (45, 51). The effects on IQ development in children, can occur with concentration increases as low as 1 to 10 µg $dl^{-1}$ (45, 54). Other effects observed are immune dysfunction, depression, fatigue, muscle weakness and aches, anaemia, skin rashes, high blood pressure and memory loss (52).

*Table 2.1. Blood levels associated with specific biological responses in children and adults (45).*

| **Blood lead level (µg/dL)** | **Effect in children** | **Effect in adults** |
|---|---|---|
| 8 | Subtle neurological impairment | |
| 10 | δ-Aminolevulinic acid dehydratase inhibition | δ-Aminolevulinic acid dehydratase inhibition |
| 15 | | Erythrocyte protoporphyrin elevation |
| 15-20 | Erythrocyte protoporphyrin elevation | |
| 20-25 | | Chromosomal abnormalities |
| <25 | Verbal IQ, mental development, physical size, and age at physical milestones such as first step, hearing thresholds, and postural sway | |
| 30 | | Toxicity to foetus |
| 30-40 | | Reduced fertility (to women) and altered spermatogenesis (to men) |
| 40 | Increased urinary ALA excretion, anaemia, coporporphyrin elevation | |
| 40-50 | | Anaemia |
| 40-60 | | Psychological, sensory, and behavioural changes |
| 50 | | Impaired kidney function |
| 50-60 | Cognitive (central nervous system) deficits. Peripheral neuropathies | Peripheral neuropathies |
| 80-100/120 (acute levels) | Encephalopathic symptoms (also at lower blood Pb levels) | Encephalopathic symptoms (also at lower blood Pb levels) |

The US Centres for Disease Control and Prevention has recognised that there is no identified blood Pb level without deleterious health effects in children, while setting a blood Pb reference value of 5 µg $dl^{-1}$ (56).

### 2.1.3 Remediation of lead (Pb) contaminated soil

Lead (Pb) contaminated land has been dealt with from three main approaches: (i) extraction and disposal of contaminated soil; (ii) capping; (iii) in situ treatment (56).

Excavation and replacement of contaminated soil is cost prohibitive and highly disruptive. Both the removal of clean soil from its origin, and the disposal of contaminated soil can have negative environmental/ecological impact (56).

Capping with clean soil, biosolids, grass, raised gardens, gravel or concrete; intends to reduce direct contact exposure (56). However, the long term effectiveness of capping is limited because there can be disturbances with re-exposure of the contaminated soil or contamination of the barrier in itself, over time (43, 45, 51, 56).

*In situ* amendments intend to reduce the bioavailability of Pb by the precipitation of complexes with low solubility, formation of organic complexes, or adsorption into organic and inorganic soil components (45). Different materials have been tested for this purpose (57) and are discussed below.

Examples of the use of biosolids such as compost for Pb remediation, have shown site and/or material specific effects (53, 55, 57-59). Bioaccessibility reduction is limited (53, 55, 57-59) and has poor long-term effectiveness, due to organic matter decomposition over time. The dilution of the concentration of Pb, through the addition of material, is thus the main effect (53, 56, 58, 60). In some cases, however, enhanced mobilisation of Pb has been shown to occur by complexation with solubilised organic matter (46, 57, 58, 60), the effectiveness of biosolids as an in-situ amendment to reduce the bioavailability of Pb is thus difficult to predict.

Alkaline compounds (calcium hydroxide, $CaCO_3$, CaO, coal fly ash) increase soil pH and surface area available for adsorption. The effect, however, is not long lasting (57) and does not reduce human availability by direct ingestion, because Pb is mainly shifted to the carbonate phase, which is unstable in the physicochemical conditions of the digestive system (59, 61).

Adsorption of Pb can be irreversible in the soil environment, and Mn hydroxides appear to be the most effective adsorbents, due to the formation of strong inner and outer sphere

complexes on octahedral vacancy sites of $MnO_x$ crystal structure, as well as ternary complexes with oxide surface-metal-ligand (organic or inorganic) (45, 62). $MnO_x$ mining industry by-products and wastes have been tested as *in situ* amendments. A large Pb adsorption and immobilization capacity was demonstrated in soil, but the shift of Pb towards the adsorbed phase did not produce changes in the human bioaccessibility of Pb, as shown by sequential extraction and *in vitro* tests (62).

#### 2.1.3.1 Phosphate amendments

Changing Pb from common soil phases such as cerussite, hydrocerussite, PbO and $PbO(OH)_2$ to less soluble inorganic phases, is another mechanism of in situ amendments. The strongest evidence for Pb bioavailability/bioaccessibility reduction, has been shown for pyromorphite (45, 56, 57, 63).

Pyromorphite ($Pb_5[PO_4]_3X$; X= F, Cl, B or OH) is the most stable form of Pb in soils, under a wide range of physicochemical conditions (e.g. pH and Pb concentration) (42, 45, 48), and can be formed by the weathering of other Pb minerals under oxidizing conditions (42). Chemical precipitation of metals depends on the solubility products of the solids formed (Melamed *et al*., 2003) (46). The solubility product constant (Ksp), is the equilibrium constant for a solid substance dissolving in an aqueous solution (64). It represents the level at which a solute dissolves in solution (64). The more soluble a substance is, the higher the Ksp value it has (64). The solubility product of chloropyromorphite was calculated by Scheckel and Ryan (2003)(52) as $Ksp=10^{-25}$; Lindsay (2001, in (65)) as $10^{-25.05}$; and Yang *et al*. (2001 in (65)) as $10^{-24.04}$. Most authors (42, 48, 50, 66, 67) however, cite the value $Ksp=10^{-84.4}$, calculated originally by Nriagu, 1973 (in (65)). For other minerals in the pyromorphite family, proposed Ksp values are: $10^{-71.6}$(F-pyromorphite); $10^{-78.1}$(Br-pyromorphite); $10^{-76.8}$(OH-pyromoprphite) (42).

Regardless of the chosen Ksp, *in vivo* and *in vitro* tests show that in the gastrointestinal system, pyromorphite tends to remain insoluble while most Pb inorganic phases or Pb adsorbed phases release Pb (44, 45, 52, 56, 59). The US EPA with the University of Missouri, studied the *in vitro* bioaccessibility (see definition in section 2.1.5) of Pb for different phases, with results showing 95-100% for Pb-oxides, 85-100% for cerussite, 15-25% for anglesite, 1-5% pyromorphite, 0-1% galena (45).

Different products have been used as phosphate amendments to promote pyromorphite formation in contaminated soils. Phosphate fertilizers such as superphosphate ($Ca(H_2PO_4)_2$) and triple-superphosphate, (mainly $Ca(HPO_4)_2$*H2O), phosphate salts and phosphoric acid, are inexpensive soluble sources, and can be quite effective in reacting with soil Pb to form

pyromorphite, but are more likely to have secondary effects in phosphate leaching, and also soil acidification (46, 56, 57).

Phosphate rock (e.g. $Ca_5(PO_4)_3F$, $Ca_{10}(PO_4)_6(OH)_2$), with a lower solubility, can provide a prolonged formation of pyromorphite in soil, due to long term supply of a continuous source of $PO_4^{3-}$ (46, 68), reducing the risk of eutrophication. At neutral to alkaline pHs however, P soluble concentration is low, resulting in a relatively slow Pb immobilization, while at lower pHs phosphate rock dissolves and Pb immobilization is instantaneous (46).

Tang *et al*. (54), according to in vitro accessibility tests, proposed the following order in effectiveness for bioavailability reduction for phosphate amendments: hydroxyapatite > phosphate rock > hydroxyapatite + single super-phosphate > single superphosphate. Chen *et al*. (69) based on phytoavailability by *Brassica campestris*, proposed the order: hydroxyapatite > phosphate rock > diammoinum phosphate > triple-superphostphate.

Zhang and Ryan (1998 and 1999 in (45, 70, 71)) studied the reactions between mixtures of synthetic hydroxyapatite, anglesite, cerussite, and galena in suspensions of 0.001 M NaCl and 0.1 M NaNO3, at varied pHs (pH 2 to 7). A complete transformation of anglesite to chloropyromorphite was observed at pH 4 and 5; and above pH 5, the transformation of anglesite to chloropyromorphite was incomplete due to reduced dissolution of hydroxyapatite (45, 70, 71). A complete transformation of cerussite to chloropyromorphite was observed at and below pH 4 (45, 70, 71). However, the transformation was incomplete at or above pH 5 due to reduced dissolution of both hydroxyapatite and cerussite (45, 70, 71). These results indicate that the rate of formation of pyromorphite is controlled by the dissolution of apatite and the pre-existing Pb-phases which is pH dependent.

This dependency on slightly acidic to acidic pHs for the dissolution of P amendments and pre-existing Pb phases to form pyromorphite has also been observed by several other studies and phosphate sources (46, 57, 68, 72). Mignardi *et al*. (2012 (72)) noted that despite hydroxyapatite and phosphate rock amendments being effective in reducing Pb solubility by metal complexation and possibly precipitation of other heavy metal-phosphates, they did not induce the formation of pyromorphite when the pH conditions only allowed a very limited dissolution of the amendment and pre-existing Pb-phases.

Consequently, and besides a general consensus, there has been questioning on the effectiveness of phosphate amendments. Hashimoto *et al*. (2009) (47) tested the effectiveness of different P sources. Hydroxyapatite amendment was the most effective, with a maximum of 31% of Pb in the form of chloropyromorphite after 380 days, equivalent to the reduction in organically bound Pb, Pb oxides and Pb-carbonates in relation to the control

soil (47). Hashimoto *et al* (2009) (47) argued that the much lower rate of formation of pyromorphite in relation to previous studies, was attributed to the use of EXAFS-LCF (extended X-ray absorption fine structure spectroscopy coupled with linear combination fitting) to quantify pyromorphite, instead of sequential extraction. The authors suggested sequential extraction can overestimate the results, due to the formation of pyromorphite during the analytical procedure (47). It should however be noted that the P/Pb rate at which amendments were added was lower in this case than in other studies (46, 57, 68).

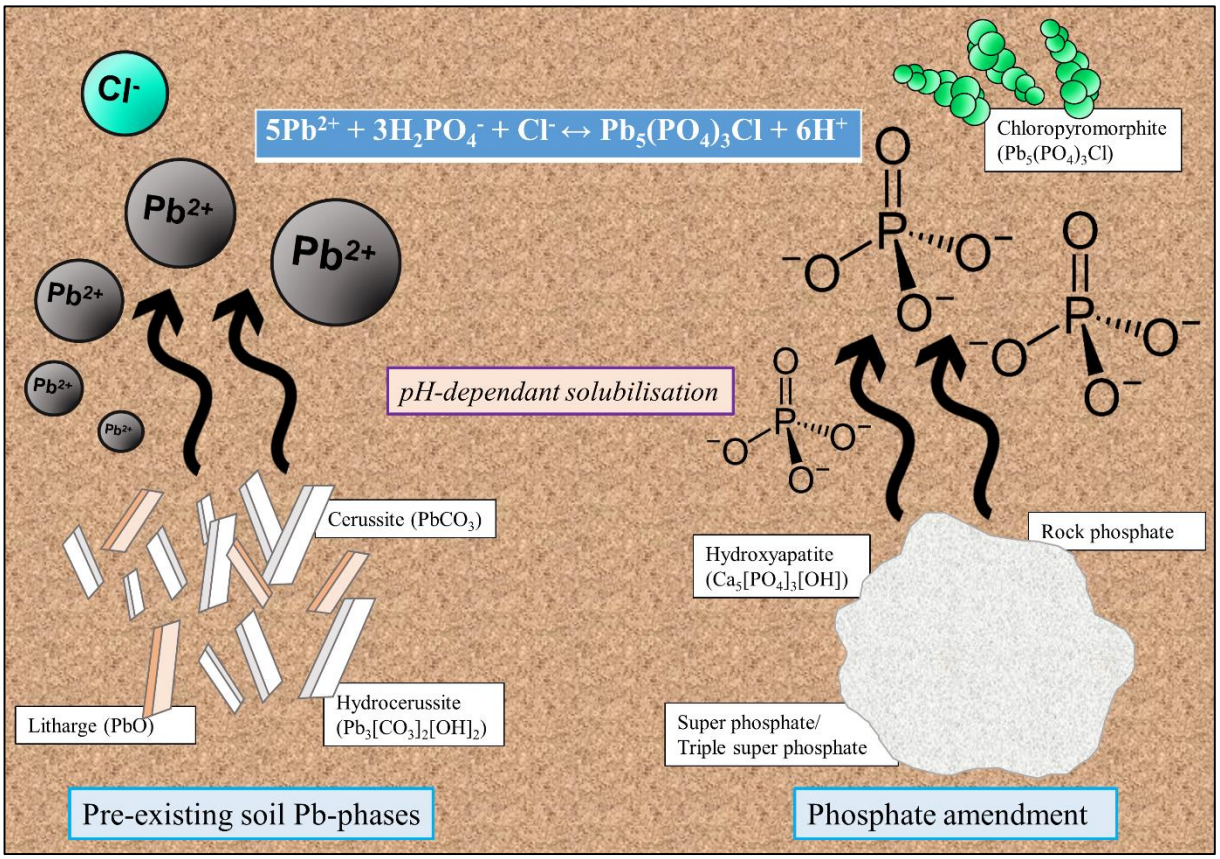



*Figure 2.2. Schematic representation of in situ soil remediation of Pb contaminated soil with phosphate amendments.*

### 2.1.3.2 Phosphate solubilizing microorganisms

Available P can be fixed in soil, by insoluble forms which are not easily available, such as iron (Fe) and aluminium (Al) at low pH and calcium (Ca) at high pH (73). Different types of soil microorganisms (fungi, bacteria and actinomycetes) are capable of solubilising various forms of P, by causing changes in the pH of the soil microenvironment and by producing chelating substances (73, 74). A decrease of pH in soil or medium microenvironments, caused by the growth of phosphate solubilizing microorganisms (PSM), is due to the production of organic acids, such as citric, gluconic, fumaric, malic, oxalic, lactic, 2-ketogluconic and malonic acids (73). PSM are also generally considered to contribute a significant part of the total soil phosphatase activity (73).

In general, fungi exhibit greater P-solubilizing ability than bacteria in both liquid and solid media (73). Fungi are also able to penetrate deeper in soils and more easily than bacteria, and hence may be responsible for more P solubilisation (73). A large number of fungi have been reported to solubilize insoluble forms of P through the production of organic acids (73). These include the genera *Aspergillus, Candida, Penicillium, Rhizopus, Cladosporium* and *Paecilomyces* (73).

Recent research has suggested that PSM can be used in union with rock phosphate (RP) so that phosphorus in the RP can be made available in the soil (73).

#### 2.1.3.3 Pb biomineralization

A soil's microbial population has a direct effect on Pb speciation, toxicity and mobility. Microorganisms may counteract or contribute to Pb immobilization (56, 74). Biologically induced mineralization occurs when an organism modifies its local microenvironment creating conditions such that there is extracellular chemical precipitation of mineral phases, with microbial surfaces providing chemically reactive sites for sorption, which can lead to the nucleation and formation of mineral precipitates around biomass (74).

Fungi generally acidify their microenvironment through a variety of mechanisms, including the excretion of protons via the proton-translocating plasma membrane ATPase, uptake of nutrients in exchange for protons, excretion of organic acids, and carbonic acid formation from respiratory $CO_2$. In addition, fungi excrete a variety of metal-complexing metabolites (e.g. sideophores, carboxylic acids, amino acids and phenolic compounds) (67, 74). The nature and amount of acids excreted by fungi are mainly influenced by the pH and buffering capacity of the environment, the carbon, phosphorus and nitrogen sources, and the presence or absence of certain metals and other trace elements (67). For example, acidification tends to occur with $NH_4^+$ as nitrogen source, rather than $NO_3^-$ (42). The acidification of the microenvironment may then result in (i) formation of surface metal complexes through adsorption; (ii) alteration of the saturation state of the solution; (iii) changes in metal speciation; (iv) metal hydrolysis and complexation (42).

Soils can be inoculated (bioaugmented) with microorganisms exhibiting high affinity for metals and can biosorb/precipitate toxic heavy metals by various mechanisms, immobilising them and consequently reducing toxicity to organisms, even though the metals remain in the soil (75).

Rhee *et al*. (48, 50, 76) studied Pb biomineralization, in a liquid medium, by *Paecilomyces javanicus*, a fungus isolated from mining related Pb-contaminated soil in Wanlockhead,

Scotland. *Paecilomyces javanicus* (*P. javanicus*) is a filamentous fungi that according to the Myco-bank database (77) belongs to the phylum *Ascomycota* and family of *Trichocomaceae,* which contains other genera such as *Penicillium* and *Aspergillus*.

*P. javanicus* was shown to be able to grow in the presence of metallic Pb, and biomineralize and deposit chloropyromorphite on the Pb surfaces (10). The formation of chloropyromorphite, which was also accompanied by the formation of Pb carbonates and oxides, was detected after 1 month by scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX) and X-ray powder diffraction (XRD), and continued to increase significantly after 3 months (50). Abiotic controls, on the other hand, showed only formation of Pb carbonates and oxides (minium $Pb_3O_4$, hydrocerussite $Pb_3(CO_3)_2(OH)_2$ and litharge PbO after 2 months and cerussite after 3 months) (50). The authors suggest that the fungal metabolites, by acidolysis and complexation, were responsible for the liberation of mobile Pb species, leading to the formation of pyromorphite with available chloride and phosphate (48, 50).

In a later study, however, Rhee *et al*. (2016 (78)), using fungal pellets (aggregated hyphal structures rather than in dispersed form) of *P.javanicus,* detected the formation of Pb-carbonates and oxalate ($PbC_2O_4$) in cultures, but not pyromorphite. The absence of pyromorphite was attributed to differences in the microenvironment and the pellet form of growing fungi. The differing physicochemical and biotic conditions inside or at the surface layers of the fungal pellet and in the medium may not have promoted pyromorphite formation (78).

Liang, *et al*. (2016 (42)) however, were able to detect *P.javanicus* induced pyromorphite formation; they studied the secondary minerals found in fungal hyphae of *P. javanicus* grown in Pb spiked liquid cultures with an organic P source with EDXA and XRD (42). Results showed the presence of Pb-oxalate and chloropyromorphite. They proposed that the mechanism was the phosphatase-mediated hydrolysis of the organic phosphate source and the consequent phosphate-metal precipitation (42). In addition to inorganic sources, phosphatase enzymes can liberate phosphate from phosphate containing organic compounds by cleaving C-O-P ester bonds (42).

Liang, *et al*. (42) further proposed a theoretical model, relevant to the *P.javanicus* -Pb-oxalate.-pyromorphite system, showing that pyromorphite dominates at pH 2-11 in the absence of oxalic acid (Figure 2.3-b), while in its presence, pyromorphite's stability is reduced to pH 4.8-8 (Figure 2.3-a).

Other authors have suggested (42, 66, 67) that fungi may also aid in the dissolution of pyromorphite. The need of fungi to obtain phosphate in a soil with a poorly soluble pool of phosphorus can result in the dissolution of inorganic phosphate. The presence of toxic metal minerals may also induce the production of some organic acids that can produce their dissolution (67). Experimental results obtained by Fomina *et al*., (2004) (67) with cultures of different fungal strains, showed that only one culture (*Beauveria caledonica*) was able to produce substantial solubilisation of pyromorphite (21%) by the excretion of oxalate and subsequent complexolysis (ligand-induced metal dissolution), and metal accumulation by the biomass. It also has been observed that the species *Aspergillus niger* induces the transformation of pyromorphite into Pb-oxalate dehydrate, but only when pyromorphite is the only available P source (42, 65, 66). In general, the fungal dissolution of pyromorphite occurs when and if it is the sole source of P (42).

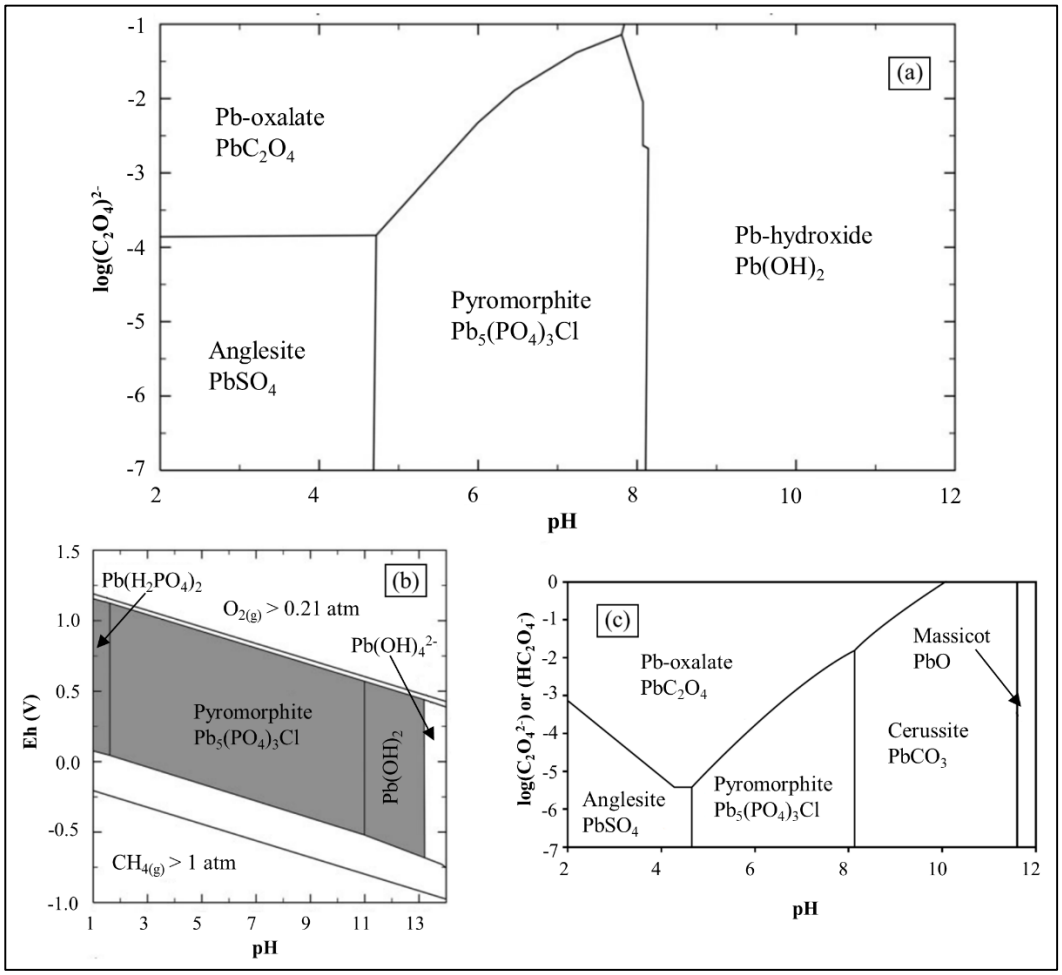



*Figure 2.3. Pb-minerals stability diagrams: (a) pH vs log oxalate concentration, with total concentrations for Pb and P in the system of 5 and 30 mM respectively, modified from Liang et al (2016) (42); (b) stability field of pyromorphite in the system Pb, Mg, S, K, Cl, N, C, Na, Fe, P, H and O, modified from Liang et al (2016) (42); stability fields in the system Pb, P, Cl, C, O, S and H, as a function of pH and oxalate activity, modified from Sayer et al (1999) (66).*

Furthermore, even though most crystalline metal oxalates are highly insoluble, the conversion of Pb-oxalate back to pyromorphite in the presence of P, at a wide pH range normal for soils, is not unlikely (42, 66).

### 2.1.4 BCR sequential extraction

The way an element, such as Pb, is bound to the solid components of soil influences its mobility and bioavailability, and consequently toxicity to organisms (79). Sequential extraction methods rely on the application of a series of reagents to a sample to sub-divide the total content of one or various metals into fractions according to their mobility (79, 80). In general, in a given sequential extraction procedure, the strength of each extraction increases in subsequent steps, targeting the element fractions which are most weakly bound to the solid phase, and hence are more potentially mobile, early in the process (79). In turn, metals released later in the process may have less environmental impact (79).

While classical speciation refers to specific chemical compounds or oxidation states of an element (e.g. cerussite [$PbCO_3$] versus pyromorphite), sequential extraction delivers an operational speciation or fractionation (79-81). The latter refers to a fraction or species defined by the extraction reagent used (e.g. 'acetic acid soluble') (79).

There are various single sequential extraction methods, with different methods using different reagents and number of sequential steps. In order to create a unified standardised sequential extraction procedure which would allow meaningful comparisons between results obtained in different studies, The Community Bureau of Reference of the Commission of the European Communities (BCR) commissioned research which led to the development of a harmonized, three-stage sequential extraction protocol (79-81). A summary table with the steps of the BCR sequential extraction and corresponding target phases is shown in Table 2.2.

*Table 2.2. Regents and nominal targets for each BCR stage* (81).

| **Extraction step** | **Reagent (s)** | **Volume** | **Nominal target phase** |
|---|---|---|---|
| 1 | $CH_3COOH$ (0.11 mol $l^{-1}$) | 40 ml | Soil solution, carbonates, exchangeable metals |
| 2 | $NH_2OH$*HCl (0.1 mol $l^{-1}$) adjusted to pH 2 with $HNO_3$ | 40 ml | Iron/manganese oxyhydroxides |
| 3 | $H_2O_2$ (8.8 mol l-1) then $CH_3COONH_4$ (1.0 mol $l^{-1}$) adjusted to pH 2 with $HNO_3$ | 20 ml and 40 ml respectively | Organic matter, sulphides |
| 4 | Aqua regia (1:1 37% HCl: 70% $HNO_3$) | 10 ml | Residual phase: remaining, non-silicate bound metals |

Sequential extraction has since been widely accepted and adopted to study the behaviour of potentially toxic elements in environmental samples, and the assessment, characterization and management of potential risks from environmental contaminants (79, 81). Environmental interpretation of BCR sequential extraction, however, varies in respect to defining a correspondence with the bioavailable/mobile pool (79). Some authors consider only the first and most easily extracted fraction (BCR1) as bioavailable, while others consider all fractions except the residual and final step (BCR4) to be bioavailable (79).

Caution is recommended when using this method (79, 81), as elements in a sample will be divided into portions soluble in certain reagents under particular conditions, and hence direct association of an element to specific minerals should not be drawn only from BCR sequential extraction results (79). The latter is justified by certain methodological/ operational effects that have been shown to occur (79, 81, 82):

- The re-distribution of analytes among phases during extraction
- Non-selectivity of reagents of defined target phases
- Incomplete extraction of a particular element
- Post-extraction re-adsorption of released metals
- Precipitation of 'new' mineral phases during and as because of the extraction

Re-distribution may be a consequence of the formation of mineral phases not originally present in the sample, during the extraction process (81). In the case of Pb contaminated soils, sequential extraction has been used to assess the effectiveness of phosphate amendments in transforming Pb from non-residual to residual forms, through the hypothesized precipitation of pyromorphite (68, 83-85). It has been shown that pyromorphite remains mostly stable over subsequent sequential extraction stages, remaining by the most part in the residual portion (82, 86). Scheckel *et al*. (63, 82), however, showed that in heavily phosphate-amended soil, insoluble lead phosphate (pyromorphite) can form during the sequential extraction process itself, rather than in the soil. Pb-spiked samples were extracted with and without added P amendment, and while soils examined in the absence of phosphate resulted in the expected operationally defined fractions, soil extracted with added phosphate showed a significant shift of Pb to the residual phase (82). The formation of pyromorphite during the extraction procedure was further confirmed by X-ray diffraction and X-ray absorption spectroscopy analysis of the soil, before and after extraction (82). Since pyromorphite formation is exceptionally quick, it is very conceivable that pyromorphite could form in solution after adding an environmental sample that contained independent sources of soluble Pb and P (82).

Despite these limitations, BCR sequential extraction has proven useful for 'before and after'-type applications, to assess the effectiveness of remediation methods in contaminated sediments and soils (e.g. confirmation of metals being bound in less extractable forms after amendment of the soils) (79, 81). The strength of sequential extractions in this kind of application, is that a general change in contaminant metal reactivity can be identified, and consequent implications for changes in bioavailability/mobility inferred (79). For example, Ryan *et al.* (2001) (86) studied a sequential extraction procedure in Pb-contaminated soil, with and without added hydroxyapatite (phosphate amendment). At time zero (before incubation), it was observed that soil residual Pb increased from 11 % to 49 % just by adding the hydroxyapatite amendment immediately prior to beginning the sequential extraction procedure (86). This indicates that the increase of residual Pb in time zero amended soil is an artefact of the chemical extraction procedure itself (86). After incubating the amended soil for 240 days, however, a further increase of residual Pb to 60% was detected, indicating the occurrence of reactions in the soil itself during incubation (86).

For these reason, BCR sequential extraction was selected in this thesis as an appropriate methodology, in combination with a complementary method (section 2.1.5), to assess effects/changes on Pb mobility-bioaccessibility when treating contaminated soil with selected amendments. By using this method under equal conditions to compare the same soil matrix before and after treatment, changes can be identified despite operational artefacts.

### 2.1.5 UBM bioaccessibility assay

Bioavailability has been defined as the extent to which a chemical can be absorbed by a living organism (87, 88). Oral bioavailability, specifically, as the fraction of an administered dose that reaches the central (blood) compartment from the gastrointestinal tract (88, 89), or that crosses the gastrointestinal epithelium and becomes available for distribution to internal target tissues and organs (90). Oral bioaccessibility, on the other hand, in respect to direct ingestion of soil, refers to the fraction of a substance that is released from a soil matrix in the gastrointestinal tract and is available for absorption (44, 88). The latter depends on soil edaphic properties (e.g., pH, granulometry) and the soil metal speciation (90). Bioaccessibility reflects the bioavailability of a soil contaminant and allows the estimation of an exposure concentration respective to the total soil concentration of the contaminant (90). Low values of bioaccessible Pb have been attributed to the presence of more stable Pb minerals such as sulfate, sulfide, and phosphate phases (91).

In order to assess bioaccessibility and potential harmful effects on human health due to the intake and subsequent adsorption of trace metals from direct ingestion of contaminated soil

(44), various chemical extraction tests have been devised, simulating the conditions of the gastrointestinal tract (44). Tests vary from only mimicking the acidic (pH ~1.5) conditions of the stomach (44, 92), to more complex physiologically based procedures, using artificial digestive juices, enzymes and bile acids capable of simulating the chemical conditions of both the stomach and gastrointestinal tract (pH ~6.3) (44, 90, 93-95). As the different existing tests produced a wide range of bioaccessibility values, the Bioaccessibility Research Group of Europe (BARGE) was created to produce a harmonised in vitro physiologically based ingestion bioaccessibility procedure for soils, known as the Unified BARGE Method (UBM) (44, 90, 96). The unified method was developed as a progression of a pre-existing method (Dutch Institute of Public Health, RIVM (93)) that closely mimicked the physiochemical conditions in the human gastrointestinal tract.

The UBM method comprises two phases, a stomach or gastric phase and a subsequent gastric + intestinal (or simply intestinal) phase (88, 90, 96). Validation of the method was carried out with inter-laboratory trials (96) and *in vivo* validation (90).

For the inter laboratory-trial (96), arsenic (As), cadmium (Cd) and lead (Pb) contaminated soils), as well as two National Institute of Standards and Technology (NIST) standard reference materials, were used. The UBM procedure was carried out in 7 different laboratories and subsequently analysed in the BGS Analytical Geochemistry Laboratories (96). The obtained median relative standard deviation repeatability (same laboratory) for Pb in the stomach/gastric phase was 3.6 %; and median relative standard deviation reproducibility (different laboratories) 22.8 % (96). For Pb in the intestinal phase, the median relative standard deviation repeatability was 14.6% and median relative standard deviation reproducibility 81.4% (96), indicating better quality results for the gastric phase.

A correlation between in vitro bioaccessibility and in vivo bioavailability is considered necessary, for both regulatory and scientific acceptance (90). The UBM in vivo validation study (90) included arsenic (As), cadmium (Cd), lead (Pb), and antimony (Sb), and compared relative bioavailability in soil using a juvenile swine model, for 16 contaminated soils, measured in kidney, liver, bone, and urine (90). The juvenile swine model is considered to be a good physiological model for gastrointestinal absorption of contaminants in children (90). Relative bioavailability was used, referring to the uptake of an element in the target organ when the swine was fed with the contaminated soil matrix, relative to the uptake of the same element when the swine was fed with a readily soluble salt (reference matrix) (90). Pb-acetate ($(CH_3COO)_2Pb$) was used as a reference matrix for Pb (90). The same reference matrix was then used in the UBM assay to calculate the relative bioaccessibility of the soils.

While direct or absolute bioaccessibility corresponds to a % of the total soil Pb that is soluble in each UBM phase, relative bioaccessibility is recalculated in reference to the % of total Pb that is soluble when adding the reference matrix (Pb-acetate) in each UBM phase, in other words the bioaccessibility of the reference matrix. Pb-acetate showed bioaccessibility of 99 ± 2 % in the gastric phase and 66 ± 3 % in the intestinal phase. The reduction of bioaccessibility of the reference matrix in the intestinal phase was explained by the higher pH environment causing metals to precipitate from solution, be reabsorbed onto the soil and/or be complexed by pepsin (2, 44, 90, 97, 98). Soils used contained 1460 to 40214 mg $kg^{-1}$ (mean of 15411 mg $kg^{-1}$) of Pb, with pHs ranging from 6.6 to 8.1 (mean 7.32) and organic matter content of 0.3 to 13.6 % (mean 6.1 %). Absolute bioaccessibility of Pb in soils was significantly lower in the intestinal phase (6.1 to 59.4 %, mean 31.7 %) in respect to the gastric phase (10.5 to 81.2 %, mean 50.1 %). Relative bioaccessibility in the intestinal phase (9.2 to 90 %, mean 48.0 %), however, was not significantly different (t-statistics) to that of the gastric phase (10.6 to 82.6 %, mean 50.6 %). No correlation of bioaccessibility (in %) with total Pb concentration, soil pH or organic matter content was apparent, but a high correlation between gastric and intestinal bioaccessibility (coefficient of 0.955) was noted. The study concluded that the UBM produced bioaccessibility data was a very good analogue of juvenile swine bioavailability measurements for As, Cd, and Pb (90). Furthermore, given that relative bioaccessibility appeared to be equal in both the gastric and intestinal phases, they suggested that the gastric phase alone may be a good analogue of in vivo bioaccessibility, but confirmation with further research was recommended (90).

For health risk assessment of Cd and Pb, the absolute gastric bioaccessibility was deemed a more appropriate conservative estimate than intestinal bioaccessibility, even though in the human gastrointestinal tract, absorption of trace elements occurs predominantly at near-neutral pH, such as those of the intestinal phase (91).

A study in Glasgow soils using the UBM method to assess Pb bioaccessibility (44) obtained stomach (gastric, Gc) bioaccessibility ranging from 23 % to 77 % with a mean of 52 % (or 46 to 1580 in mg $kg^{-1}$ with a mean of 354 mg $kg^{-1}$) relative to soil total Pb concentrations, ranging from 126 to 2160 mg $kg^{-1}$ (with a mean of 659 mg $kg^{-1}$) (44). In the intestinal phase, lower bioaccessibility was obtained, ranging from 2 % to 42 % with a mean of 22% (or 6 to 623 in mg $kg^{-1}$ with a mean of 149 mg $kg^{-1}$) relative to soil total Pb concentrations (44). A high correlation between gastric and intestinal bioaccessibility was found, when evaluated in concentrations. When assessed in percentage, however, no correlation was found between gastric bioaccessibility, intestinal bioaccessibility and total Pb concentrations, indication that

the total quantity of Pb in soil does not necessarily correlate to the proportion of Pb that is bioaccessible (44).

Other studies using the UBM method have reported Pb gastric bioaccessibility of 33–76% (mean 62±11%) in urban soils (2) and 16–69% (mean 55±14%) in agricultural smelter-contaminated soil (91). For intestinal bioaccessibility, the same studies reported respectively 14–63% (mean 32±11%) (2) and 2–42% (mean 20±10%) soil (91).

The UBM bioaccessibility method was selected in this thesis as an appropriate methodology, in combination with BCR sequential extraction, to assess effects/changes on Pb bioaccessibility when treating contaminated soil with selected amendments.

## 2.2 Polycyclic aromatic hydrocarbons (PAHs) soil contamination and remediation

### 2.2.1 Polycyclic aromatic hydrocarbons (PAHs) general properties and provenance

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds. The group includes several hundred chemically related compounds with various structures, that tend to be environmentally persistent, with different levels of toxicity (99). All PAH compounds are complex hydrocarbons formed by carbon and hydrogen atoms, organized in two or more benzene rings fused in linear, cluster, or angular arrangements (99, 100). They are commonly classified in environmental chemistry into two groups: low molecular weight (LMW-PAH), having two or three aromatic rings; and high molecular weight PAH (HMW-PAH), with four or more rings (101).

PAHs have a high melting and boiling point, low vapour pressure (lighter PAHs tend to be more volatile) and very low aqueous solubility, which decreases with each additional ring. They are relatively hydrophobic and generally lipophilic, hence soluble in organic solvents. With increasing molecular weight, the resistance to oxidation and reduction also increases (99, 100, 102).

PAHs can be formed during biological processes, but the major source is the incomplete combustion of organic materials, either natural (forest and brush fires) or anthropogenic (coal, oil and wood combustion, automobile emissions, cigarette smoke) (99, 100, 102). Sources have been classified in three types (99). Pyrogenic PAHs are formed by pyrolysis when organic substances are exposed to high temperatures under low oxygen or no oxygen conditions, for example by destructive distillation of coal into coke and coal tar; thermal cracking of petroleum residuals into lighter hydrocarbons; incomplete combustion of motor fuels in cars and trucks; incomplete combustion of wood in forest fires and fireplaces; and

incomplete combustion of fuel oils in heating systems (99, 100). Petrogenic PAHs are formed at lower temperature over long time periods, implying mainly crude oil formation (over million years) and its subsequent anthropogenic dispersal in the environment (99, 100). Finally, biological PAHs are synthesized by certain plants (plant derived terpenoids) and bacteria or formed during the degradation of vegetative matter (99, 100).

Air, soil, sediment, water and vegetation all act as environmental sinks for PAHs (102). The atmosphere is the most important means of PAH dispersal, where they can be found in two separate phases, vapour and sorbed onto particulate matter (99). From the atmosphere they become deposited in soil by dry or wet deposition processes. This can occur with PAHs from nearby sources (e.g. automotive exhaust from adjacent roadways) or with PAHs from distant sources, carried through the air. Otherwise, they can be added to soils through industrial activity, spills and contaminated fill materials (99). A preliminary inventory of PAHs in the UK environment showed that soil was the main repository for PAHs (102).

In soils, PAHs have a tendency to partition into organic matter, particle surfaces and biological lipids and out of the aqueous phase (103).

Benzo(a)pyrene (B(a)p) is a relatively large PAH (molecular weight of 252.3 g $mol^{-1}$), composed of five unsubstituted rings, that result in the chemical formula $C_{20}H_{12}$. B(a)p's main source, as other PAHs, is incomplete combustion or pyrolysis of organic matter. At ambient temperature it is found as a pale yellow solid and is not readily volatile or reactive. It has low solubility in water (1.62 µg $l^{-1}$ at 25ºC). The octanol-water partition coefficient (log $K_{ow}$) for B(a)p has been found to range from 5.97 to 6.35 (104). B(a)p has been well studied due to its toxic effects on human health and is commonly used as an indicator of and reference for general high molecular weight PAH contamination.

### 2.2.2 Human health issues associated to Polycyclic aromatic hydrocarbons (PAHs)

PAHs may interfere in cellular membrane functions and enzymatic systems and some have been proven to cause carcinogenic and mutagenic effects and act as immune-suppressants and teratogens (99).

Though the health effects of different PAHs are not entirely the same, 16 of them have been identified, and grouped, as being of greatest concern for human health (99). The International Agency for Research on Cancer classified some PAHs as known, possibly, or probably carcinogenic to humans (99). PAHs with 2 or 3 aromatic rings (e.g. naphthalene, acenaphthene, acenaphthylene, fluorene, anthracene, phenanthrene) have a low tendency to bind to the Ah-receptor (responsible for mediating toxicity), possibly because they do not

meet the structural requirements (100). B(a)p, on the other hand, has been studied in detail for its carcinogenicity (100). Other PAHs are evaluated in comparison/equivalence to B(a)p (100).

The bioavailability of PAHs after ingestion is quite high because of their lipophilic behaviour (99). Once in the organism, PAHs undergo a multistep metabolic activation by specific enzymes (99). Rather than un-metabolized PAHs, the major toxic effect is exerted by reactive metabolites, such as epoxides and dihydrodiols, that bind to cellular proteins and DNA leading eventually to mutations, tumours, and cancer (99).

In addition to the carcinogenic effects, other registered effects from long-term exposure to PAHs are cataracts, kidney and liver damage, breathing problems, asthma-like symptoms, and lung function abnormalities. Acute exposure can have negative effects during pregnancy, generating low birth weight, premature delivery, heart malformations, and IQ development problems (99).

Plants appear to exhibit very limited ability to accumulate PAHs from soils, and to translocate PAHs from root tissue (99, 100). This consequently implies that plant consumption is not a significant pathway, versus direct ingestion and inhalation of soil particles (99, 100).

### 2.2.3 Remediation of Polycyclic aromatic hydrocarbons (PAHs) contaminated soil

Different techniques have been developed to treat PAH contaminated soil *in situ*: electrokinetic remediation, solvent extraction, chemical oxidation (e.g. ozone), photooxidation, photolysis and thermal technologies, amongst others (99, 100, 105). Compound that can react with PAHs are $NO_x$, $SO_2$ (100) and oxides like $TiO_2$ and ZnO if they are combined with UV irradiation (99). Adsorption and volatilization processes have also been used to deal with PAH contamination (99, 103).

However, in the soil environment, one of the more promising techniques for successful elimination/reduction of PAHs over time, in an intermediate to long term, is microbial degradation (100, 105-108). Microorganisms can be used to reduce, eliminate, contain, or transform contaminants present in soils into non-toxic forms (107, 109). Major advantages of bioremediation compared to chemical and physical methods of remediation, are that it is generally more environmentally friendly and has lower costs (110).

Several factors determine the rate of biodegradation: the properties of the PAHs present, temperature, soil characteristics, pH, and the microbial population, particularly its pre-

conditioning to encourage the presence of PAH-degrading types. Pre-exposure of a microbial community to hydrocarbons, increases its hydrocarbon oxidizing potential through adaptation (100, 103).

A bioremediation system generally requires an energy source (electron donor), an electron acceptor, and nutrients (109). Electron acceptor may be oxygen, nitrate, manganese, iron (III) and sulfate (109). Under aerobic conditions, bacteria and fungi utilise oxygen for both ring activation and cleavage of the aromatic nucleus and as the electron acceptor for its complete degradation (102).

The time-efficiency of this process is reduced with an increase in the number of rings and molecular weight of PAHs, due to resistance to biodegradation, which occurs slowly in structures with more than 4 aromatic rings (100, 103).

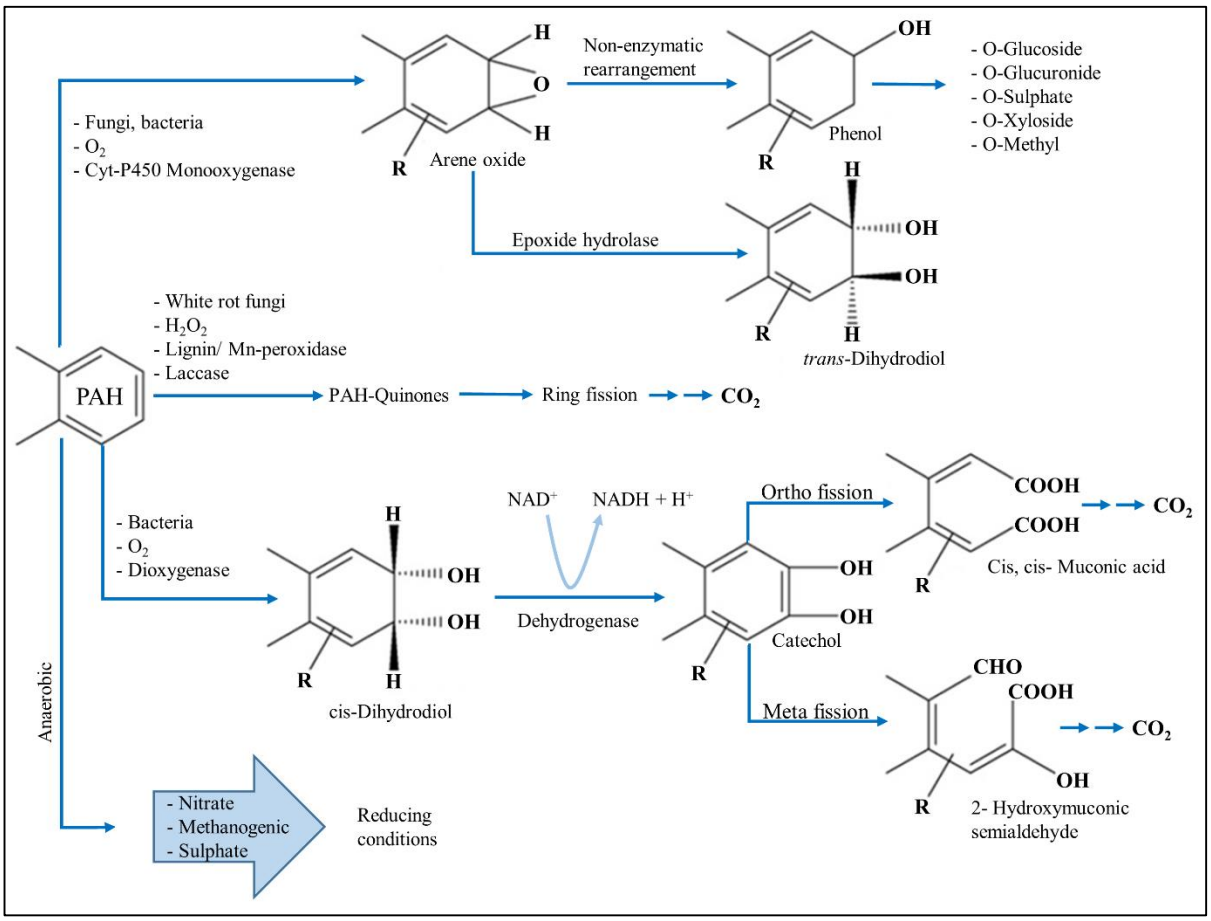



*Figure 2.4. Proposed pathway for microbial catabolism of polycyclic aromatic hydrocarbons. Modified form Cerniglia (1992) (111).*

The chemical partitioning and degree of sorption of PAHs to soil phases, particularly organic matter, is an important factor in biodegradation (100, 103). In order for microbes to degrade any given PAH, it must be made available for uptake, in the dissolved phase (99). This can

be achieved by the use of surfactants, such as organic solvents or biosurfactant-producing microbes (i.e. *Pseudomonas aeruginosa*) (103).

Bioremediation of PAHs, and other contaminants, can be achieved via biostimulation or bioaugmentation (106, 112).

#### 2.2.3.1 Biostimulation

Many soils contaminated with PAHs contain PAH-degrading microorganisms. These microbes are often limited in their degradation capability because of limiting environmental factors like low aqueous solubility of PAHs, low bioavailability of PAHs, nitrogen or other nutrient limitation, high co-contamination levels that can inhibit PAH biodegradation, amongst others (113, 114).

Biostimulation involves the modification of the environment to stimulate existing bacteria capable of bioremediation (100, 102, 103, 109, 110, 115-119). This can be done by addition of various forms of nutrients (e.g. nitrogen and phosphorus) and electron acceptors, which are otherwise available in quantities low enough to constrain microbial activity (102, 109, 114, 116-118, 120). Nutrients may be added in the form of fertilizers and organic amendments such as animal manure, sewage sludge and compost (115, 116, 121). Enzyme diversity becomes greater in high organic-containing soils (117).

It is estimated that 140 billion metric tonnes of biomass are generated globally every year from agriculture (122). As the accumulation of these wastes is detrimental, concerns are rising and there is a need to develop eco-friendly technologies for the minimization of green wastes (122). Although there is an emerging trend in the utilization of green wastes, they are still largely under-utilized and accumulated in landfills, especially in developing countries (101, 122). These large volumes of green waste have attractive potential for remedial utilization, as they are widely available, virtually free and are renewable resources (116, 122, 123).

Several studies have reported bioremediation of contaminated soils by addition of lignocellulosic agroindustrial wastes for biostimulation, such as chopped wheat straw, pine wood chips, mushroom spent, sugarcane bagasse, hay, corn cobs, peanut shells, fruit peelings and rice straw biochar, amongst others (101, 105, 106, 116, 124-129). Agroindustrial wastes contain nutrients and are generally rich in phosphorus, nitrogen, and carbohydrates, which can promote the growth of organic pollutant degraders and in consequence reduce contaminant concentrations (123, 125, 127, 128, 130).

Lignocellulosic materials mainly consist of 38–50% of cellulose, 23–32% hemicellulose, and 15–25% lignin (131). With their high lignin content, these materials may act as co-substrates contributing to the biodegradation rate of recalcitrant compounds through co-metabolism (101, 126, 127). In co-metabolism microorganisms do not use the hydrocarbons as the main source of energy for their metabolism, obtaining their energy from other transformable compounds (e.g. lignocellulosic amendments) and co-metabolising/ co-oxidising the contaminant in the process (123). The latter specially enhances the elimination of heavier compounds (e.g. high molecular weight PAHs) and produces less long-term accumulation of extractable polar and more available intermediates (101, 103, 119).

The addition of agroindustrial residues to soil can also increase its physical properties, like bulk density, aggregation, porosity, water holding capacity, oxygen diffusion and improve pH and ion exchange capacity (115, 116, 121, 125, 130). This implies not only aiding with specific contaminant biodegradation, but an overall effect of reclaiming degraded soils and contributing to its fertility (106, 115, 126).

Another form of biosimulation is combining the provision of oxygen and nitrogen (e.g. fertilizer) with physical mixing of the soil to distribute the contaminants over a greater surface area of soil particles and bring them into contact with the microbes, enhancing the degradation capabilities of natural microbial communities (113).

#### 2.2.3.2 Bioaugmentation

It is possible that the native microorganism population, though adapted to the climatic, physicochemical and nutrient conditions, is not large enough or does not have the ability to degrade a specific contaminant, particularly those with high molecular weight. In such case bioaugmentation is a good alternative (102, 126, 130, 132, 133). Bioaugmentation involves the introduction of microorganisms that have been cultured to perform a specific remediation task in the soil system, such as biodegradation (107, 117, 118, 126, 132, 133). It is through the introduction of excess, active microorganisms that remediation can occur at a faster rate than would otherwise be possible by means of the indigenous microorganisms (102).

Possible sources to obtain exogenous microorganisms are remediated or contaminated sites, commercial suppliers and genetic engineering (133). The inoculated microorganisms must have the ability to withstand different soil environmental conditions and to survive in the presence of other microorganisms, and contaminants such as heavy metals (132).

If the latter is achieved, positive interaction between the inoculated consortium and the autochthonous microbiota of each soil, may enhance the bioremediation process (130).

Studies have reported an increased rate of PAH removal when using bioaugmentation with microorganisms, compared to biostimulation alone (113, 132).

### 2.2.3.3 Combined biostimuation and bioaugmentation

At times, nutrient application alone or augmenting with microbes is not sufficient for remediation. In these cases a combination of bioaugmentation and biostimulation may further enhance microbial enzymatic activity (101, 109, 117, 121, 130, 132, 134). Both indigenous and exogenous microorganisms can benefit from biostimulation by the addition of energy sources, allowing metabolic cooperation and co-metabolic relations (101, 117). Survival of inoculated microorganisms as well as their bioremediation performances can be enhanced by the supply of carbon substrates with the inoculum (117, 123, 130).

For example Straube *et al*. (2003) (113) compared biostimulation with rice hulls and dried blood to biostimulation plus bioaugmentation with *Pseudomonas aeruginosa* (a biosurfactant-producer on a vermiculite carrier). After 11 months the biostimulated microcosms showed a 34% decrease in total PAHs and a 57% decrease in total BaP toxic equivalents (TEQs, toxicity expressed relative to B(a)p as a reference standard: TEQ = ∑(PAH i × TEF i), where PAH i and TEF i are the concentration and toxicity equivalency factor, respectively, for individual PAH congeners (135)), while the biostimulated and bioaugmented microcosms revealed an 87% decrease in total PAHs and a 67% decrease in total BaP toxic equivalents (113).

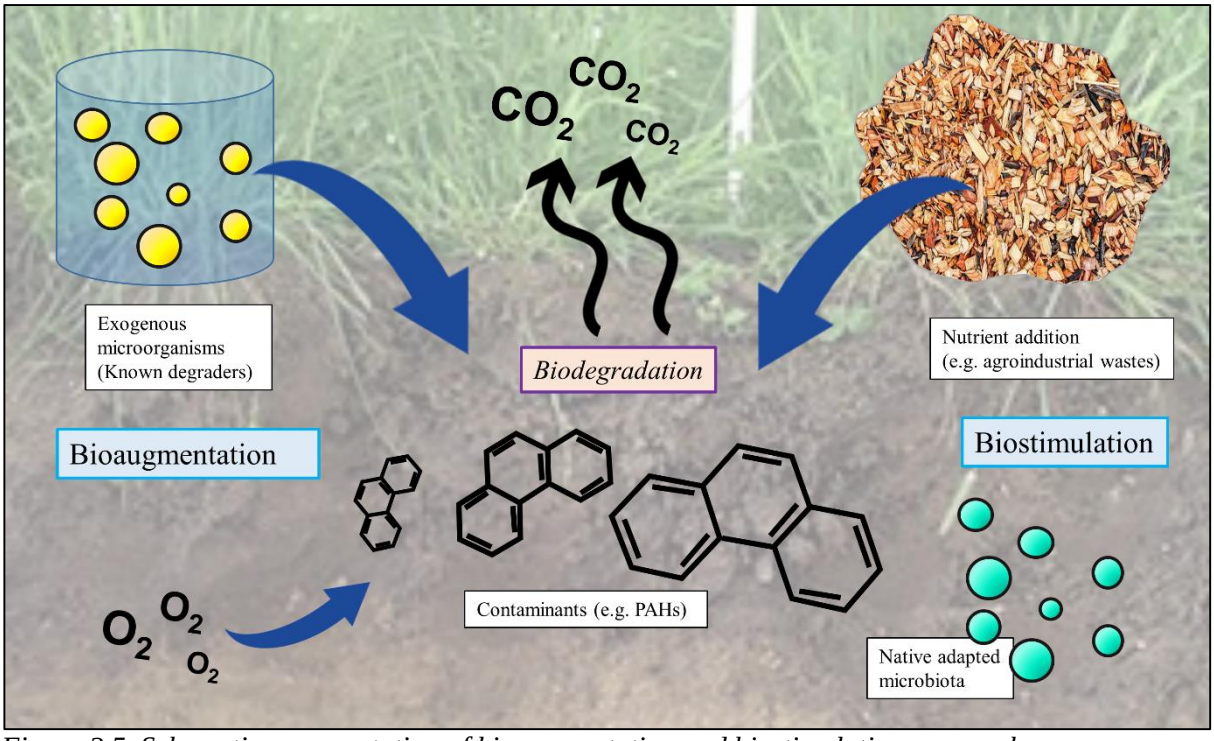



*Figure 2.5. Schematic representation of bioaugmentation and biostimulation approaches.*

Biostimulation strategies with organic amendments, may in themselves produce bioaugmentation, as they can be rich in microorganisms, which could potentially participate in pollutant removal (106, 116, 121, 127, 128, 130).

#### 2.2.3.4 PAHs biodegradation by fungi

Biodegradation of PAHs has been extensively studied, mainly with bacteria (105, 108). Fungi, and particularly white rot fungi have been proposed as promoters of PAH biodegradation and of general importance in detoxification of the environment (102, 105, 108, 112, 113, 119).

Compared to bacteria, some fungi species are better able to colonize soil and to compete with the autochthonous microflora (105, 112, 119, 132). Moreover, the ability of fungal hyphae to reach the pollutants by penetrating contaminated soil, even at low nutrient concentrations, low humidity and acidic pH, combined with the production of extracellular enzymes with high metabolic versatility, gives fungi a significant advantage over bacteria (102, 105, 112, 119, 132, 136). They also possess a larger surface area for adsorption and are generally believed to play a more important role in degradation of organic matter-sorbed contaminants than bacteria (102, 119).

Lignin-degrading fungi, most commonly found within white rot fungi, are known to produce extracellular lignin-modifying enzymes with low substrate specificity (105, 106, 119, 132, 136). The extracellular ligninolytic enzyme system of fungi consists of peroxidases (e.g. Mn-peroxidase, versatile peroxidase and lignin-peroxidase), phenoloxidases (lacases, tyrosinases), $H_2O_2$-producing enzymes, and has been directly linked to biodegradation of a wide range of organic pollutants, including complex and more recalcitrant toxic compounds with high molecular weight, such as both low and high molecular weight PAHs (103, 105, 106, 108, 112, 132, 136). Research has shown that ligninolytic enzymes produced by fungi have an advantage in comparison to bacteria when it comes to biodegrading heavy molecular weight PAHs (106). The intracellular cytochrome P-450 enzymatic system has also been identified as capable of PAH degradation (137).

Furthermore, it has been observed that most of the fungal transformation products formed are less mutagenic than the parent compound (102).

The disadvantage of white rot fungi is that even if they show a good degradative capacity in liquid culture conditions, they are not well adapted, and hence ineffective to colonize soil (105, 119).

Soil fungi have also been studied for PAH bioremediation. Silva *et al*. (2009 in (103)) studied the ability to degrade PAHs and produce ligninolytic enzymes of soil fungi under microaerobic and very low oxygen conditions. For high molecular weight PAHs (4-7 rings), maximum degradation was observed in *Trichocladium canadense*, *Aspergillus sp*., *Verticillum sp*. and *Achremonium sp.* Fungal extracellular enzymes catalyse radical formation by oxidation to destabilize bonds in a molecule. Their optimum activity occurred at temperatures ~45ºC for laccase (103).

Mao and Guan (119) studied the fungal strain PZ-4 *Scopulariopsis brevicaulis* (isolated from an aged PAH contaminated soil). After 30 days, the observed removal efficiency was of 82.1% benzo(a)pyrene, 64.3% pyrene, 61.9% fluoranthene and 60% phenanthrene. Though the higher efficiency of removal was for benzo(a)pyrene, the rate of growth of the fungi in a liquid medium with this compound, was the lowest. The experiment also showed that the inoculated strain was able to survive in non-native soil and compete with indigenous microbial population, even stimulating the growth of soil bacteria.

Benzo(a)pyrene, in particular, has been found to be biodegradable by bacteria, algae and fungi. Fungal biodegradation by *Cunninghamella elegans* in cultures metabolized into trans-7, 8-diol, trans-9, 10-diol, 3,6-quinone, 9-hydroxybenzo(a)pyrene, 3-hydroxybenzo(a)pyrene and 7,8-hydroxybenzo(a)pyrene (100).

Fungi are capable of partially or completely degrading PAHs by co-metabolism (102). A wide range of fungi have the enzymatic capacity to oxidise PAHs when grown on an alternative carbon source (102).

Most of the soil bioaugmentation approaches with fungi use mushroom growers and consist in the production of fungal inoculum with lignocellulosic wastes (137). Fungi can be grown on inexpensive lignocellulosic substrates, such as agroindustrial wastes, used as inoculum carriers which is mixed with the polluted soil (123, 125, 137). Lignocellulosic waste inoculum carriers enhance enzyme production of fungi and can easily overcome the lack of nutrients and allow soil colonization (105, 137).

#### 2.2.3.5 Biosorption

It is well-known that soil organic matter has a major influence on the transport and fate of PAHs (and other hydrophobic organic compounds) in the environment (138). As an important original source of soil organic matter, microbial biomass also plays a significant role through biosorption and biodegradation of PAHs (138, 139).

In the environment (e.g. soils and surface water), biosorption of organic pollutants by microbial biomass is a common phenomenon (138-140). In the context of remediation, however, biosorption of PAHs by microorganisms has received less attention than biodegradation (138, 139). The remediation applications of biosorption have mainly been studied in aqueous environments, using inactive or live biomass from various origins (e.g. fungi, bacteria, and algae) (141).

Biosorption is a physic-chemical process in which PAHs (and other chemical substances) accumulate by sorption on a biological matrix/surface (solid phase) (139). Sorption of organic pollutant to biomass may involve simultaneous surface sorption, partitioning processes and chemical reactions (138, 140).

Cell surfaces contain many hydrophobic groups, such as biolipid alkane chains and polysaccharides, providing biosorption sites for hydrophobic organic contaminants (140, 141).

It has been said that the primary mechanism of PAHs (and other hydrophobic organic compounds) biosorption by fungi, is partitioning into the biomass, particularly in the case of inactivated biomass (138, 140). Ding *et al*. (2013) (139) reported that the biosorption mechanism of phenanthrene and pyrene by dead white-rot fungi was dominated by partition into fungal biomass and that the biosorption-desorption was reversible.

With live bacterial biomass, the biosorption of PAHs may involve surface sorption and chemical reactions rather than non-specific partitioning, as the physiology of the biomass itself influences the sorption processes (138). An advantage of live biomass versus inactivated biomass, is that as it continues to grow, binding sites continue to be created, allowing increased removal of the compound (139).

It has been suggested that biosorption can decrease the rate of PAHs biodegradation in the short term, yet contribute to the immediate removal of PAHs from polluted solution (139). The extent of biosorption and biodegradation may, however, vary with nutrient conditions (138, 139). Furthermore, microorganisms with higher sorption affinity for organic pollutants may have an advantage, as capturing the compound may result in higher biodegradation efficiency, especially for those pollutants with relatively low water solubility, and thus, bioavailability (141).

The relative contribution of biosorption and biodegradation is necessary to precisely understand the fate of PAHs in the environment, optimizing bioremediation practices (139). The latter is most readily achieved in liquid culture than in soil systems, however, because

it is difficult to distinguish biosorption of PAHs to the soil microbial body and sorption to other soil components in the complex system (139).

### 2.3 Brewery spent grain

The use of carrier materials for the inoculation of microorganisms in soil has shown promise in remediation as they provide a protective niche for the organism's settlement in soil, nutrition, reduction of the toxic effect of the contaminants and improve the competitive ability of the fungi added to the resident microbiota (75, 105, 112, 118). Bioremediation of soils by ligninolytic fungi can be approached by inoculating with fungus grown on lignocellulosic agroindustrial wastes. This form of inoculation encourages the production of ligninolytic enzymes known to be involved in PAH degradation (105, 137). In this sense lignocellulosic materials can be used not only as inoculum carriers but also as a soil amendment (112).

Scotland's brewing and distilling sectors play a vital role in the Scottish economy, with over a 100 brewing enterprises as of 2018 (142). The beer brewing process has three main by-products: spent grains, spent hops and surplus yeast (143). Spent grains is the most abundant of the three residues (~ 85% of total) (143). An estimated 200 t (wet weight) is produced per 1000 $m^3$ beer (143), hence the importance of reuse and obtainment of value-added products.

In the UK, a study of by-product disposal methods of craft breweries showed that rural brewers and larger urban brewers dispose of spent grain mainly as animal feed (143-145). Medium and small urban craft breweries sometime utilize alternative routes of spent grain disposal, such as composting or direct fertilizing. Some of the smaller breweries however, dispose of their spent grain in landfill (143-145). Other minor uses are bio-recycling and anaerobic digestion for production of biogas, and in some cases as a supply for bakeries (145). Urban brewers tend to dispose of waste using external companies while rural brewers through direct relationships with farmers. Regardless of the route of disposal spent grain remains largely available at no cost (145).

Spent grain results from the mashing stage of the brewing process. Milled barley malt is mixed with water in a mash tun (vessel with a stirring mechanism), and the temperature is slowly increased (from 37 to 78°C) to promote enzymatic hydrolysis of malt constituents (143). During this process, malt starch is converted to fermentable and non-fermentable sugars; and proteins are partially degraded to polypeptides and amino acids (143). The residual solid (spent grain) is then allowed to settle and separated by filtration from the resulting liquid (wort) (143).

Spent grain is mainly constituted of barley grain husks, but has other components such as residual starch endosperm and residues from other cereals that can be used in the brewing process (e.g. wheat, rice, corn, oats, rye) (143). Barley malt husk is a lignocellulosic material, and hence rich in cellulose, hemicellulose, lignin, and proteins (143). Cellulose and hemicellulose constitute around 50% of spent grain composition, and contain a significant amount of sugars (e.g. xylose, glucose and arabinose) (143). Other components of spent grain are minerals, particularly silicon; as well as waxes, lipids, gums, starches, resin, tannins, essential oils and vitamins. Spent grain is considered to be rich in nitrogen (N) owing to its high protein content, with recorded carbon C: N ratios of 8–25 (146). P is also found in relatively high concentrations (Table 2.3) (120, 143).

*Table 2.3. Chemical composition of spent grain (143).*

| **Components** | **% dry weight** | **SD** |
|---|---|---|
| **Cellulose (glucan)** | 22.35 | 3.500 |
| **Hemicellulose** | 30.43 | 7.260 |
| **Xylan** | 20.25 | 0.350 |
| **Arabinan** | 8.750 | 0.250 |
| **Lignin** | 19.58 | 5.879 |
| **Acetyl groups** | 1.200 | 0.100 |
| **Proteins** | 21.30 | 4.250 |
| **Ashes** | 3.200 | 1.463 |
| **Extractives** | 7.650 | 1.850 |
| **Minerals** | **mg $kg^{-1}$ dry weight** | |
| **Ca** | 3515 | - |
| **Na** | 309.3 | - |
| **K** | 258.1 | - |
| **Mg** | 1958 | - |
| **Al** | 36.00 | - |
| **Fe** | 193.4 | - |
| **Ba** | 13.60 | - |
| **Sr** | 12.70 | - |
| **Mn** | 51.40 | - |
| **Cu** | 18.00 | - |
| **Zn** | 178.0 | - |
| **P** | 5186 | - |
| **S** | 1980 | - |
| **Cr** | 5.900 | - |
| **Si** | 10740 | - |

**Modified from Mussatto, 2009(143). % dry weight are averages of up to 4 separate values shown in (143).*

The specific chemical composition of spent grain varies according to the barley variety, harvest time, malting and mashing conditions and other cereal grains used in the brewing process (143). An example of spent grain chemical composition, obtained from Mussatto (2009) (143), is shown in Table 2.3.

Because of its chemical composition and nutritional content, spent grain has been studied for use in various biotechnological processes (143). Research has shown success on the use of spent grains (pre-treated or with no previous treatment) as a substrate for the cultivation of microorganisms and production of several extracellular enzymes, with species such as the fungus *Pleurotus ostreatus*, *Agrocybe, Lentinus, Aspergillus* and *Trichoderma*, and the bacteria *Bacillus* and *Streptomyces* (a soil actinobacteria) (147-151). Studied enzymes were sought for applications in the pharmaceutical, food and paper industry (147). Variation in fungal biomass production was observed in a study with *Pleurotus ostreatus (151)* and non-processed spent grain, depending on the type of malt, baking degree of malt, additive type, and ratio of mixture with other substrates; though growth occurred in all cases (151). This fungus also showed the ability to degrade part of the lignin present in the spent grain used as substrate for its growth (151).

Other biotechnological uses that have been studied are the extraction of value-added compounds such as sugars, proteins, acids and antioxidants (143).

A few studies have evaluated the potential of spent grain as a soil amendment, mainly for agricultural purposes, to improve soil nutrition (organic carbon and nitrogen), soil texture-aggregation and water holding capacity (152). Field trials with spent grain in combination with NPK fertilizer, for the growth of maize, showed a significant increase in plant height, stem girth and number of leaves in comparison to soil treated with only fertilizer or untreated soil (152). Organic carbon and total nitrogen content of soil, was also increased by spent grain with fertilizer or on its own (152).

Crosier (2014) (153), also in field trials, studied the effect of composted spent grain with sawdust as well as directly applied non-composted spent grain, on yield of kale, beets and peas; using horse manure-sawdust compost for comparison. Neither compost treatment significantly improved yields on a first round of crop cultivation, while spent grain-sawdust compost decreased yield in kale. In a second season/round of crop cultivation with kale, yield was increased by the addition of non-composted spent grain and horse manure compost (153). Although the composts used provided additional nutrients to the soil and improved some physical properties (e.g. water holding capacity), an insufficient maturity of the composts was proposed as an explanation for the failure to increase yields. Immature

composts may have an unstable C/N balance, causing a reduced N availability (153). On the other hand, on un-composted spent grains the C/N ratio (~9) may have provided more available N to the plants. Nevertheless, the increases in soil organic matter and gradual nutrient provision/availability produced by non-composted spent grain was questioned for the long term (153).

A similar study with composted and non-composted spent grain in a farm environment (154), looked to overcome the "shelf life" of non-composted spent grain, which tends to quickly become anaerobic due to high moisture and N content, when in storage (154). Studied plant species increased yields in 26% and 29% respectively for composted and non-composted spent grain in comparison to unamended soil.

A recent pot-experiment study using spent grain and compost for the amendment of calcareous soil and growth of squash (*Cucurbita pepo* L.) resulted in increased soil water holding capacity, organic matter, macronutrients, micronutrients, germination parameters, and reduced soil pH (155). Other studies have also characterized spent grain as an acidic material (pH 5–6.4) (146).

In terms of remediation of heavy metals, the sorption of cadmium (Cd) and Pb from aqueous solutions by spent grain has been studied (156). Maximum sorption capacities of 17.3 and 35.5 mg $g^{-1}$ for Cd and Pb respectively were obtained with NaOH pre-treated dry spent grain, ground to < 1.0 mm. Maximum sorption capacities were attained within 2 hours in aqueous solutions with single Pb and Cd concentrations between 50 and 450 mg $l^{-1}$ (156). When binary Cd-Pb solutions of concentrations higher than 50 mg $l^{-1}$, spent grain sorption sites showed preference for Pb ions (156).

Few studies have assessed the suitability of spent grain for organic contaminants remediation. A research group based in Malaysia (114, 120) studied the biostimulation of soil artificially contaminated with used lubricating oil, using banana skin (BS), brewery spent grain (BSG) and spent mushroom compost (SMC), for a period of 84 days under laboratory conditions. 42.05 % total petroleum hydrocarbons (TPH) loss was recorded in the unamended control soils, while 68.73 %, 62.03 % and 57.01 % oil loss were recorded in soil amended with BSG, BS and SMC respectively (120). Hydrocarbon utilizing bacterial (HUB) counts were high in all the organic wastes amended soil ranging between $10.2*10^6$ CFU $g^{-1}$ (colony forming unit per gram of soil) to $80.5*10^6$ CFU $g^{-1}$ compared to unamended control soils ($1.0*10^6$ CFU $g^{-1}$ to $3.5*10^6$ CFU $g^{-1}$) throughout the 84 days of study (114, 120). The count of HUB in soil amended with BSG was about 5% higher than those amended with BS and SMC (114). A higher amount of liberated $CO_2$ was also measured in soils amended with

organic wastes, indicating utilization of hydrocarbon fractions as a source of energy by microbial community (120).

A second experiment showed TPH reductions of 95 %, 93 %, 92 % and 68 % for BSG, BS, SMC amended soils and the unamended control respectively after 84 days (114).

The higher biodegradation efficiency shown by BSG was associated to a higher nitrogen (necessary nutrient for bacterial biodegradative activity) and P content and possible enhanced nutrient availability to microorganisms (114, 120, 157)

A follow up study combined the same organic wastes (10% BS, BSG and SMC) with the sowing of the flowering plant species *Jatropha curcas* in soil artificially contaminated with 2.5 and 1 % (w/w) of waste lubricating oil (157). The experiment was carried out for a period of 180 days under room temperature condition (157). 56.6 % and 67.3 % loss of TPH was recorded in Jatropha remediated soil without organic amendment for 2.5 % and 1 % contamination respectively. Contaminated soil treated with BSG recorded the highest loss of oil with a removal of TPH with 89.6 % and 96.6 % in soil contaminated with 2.5 % and 1 % oil, respectively. Control soils without Jatropha plant showed 36.9 % and 51% oil loss in 2.5 % and 1 % contaminated soil respectively at the end of 180 days. 11.6 % and 14.2 % oil loss in soil contaminated with 2.5 % and 1 % oil was attributed to non-biological factors like evaporation, measured in autoclaved soil treated with sodium azide (157).

It was also noticed that Jatropha plants amended with BSG grew taller (~20%) than other treatments (157).

Contaminated soil treated with BSG and Jatropha remediation showed high counts of HUB, with $240*10^5$ CFU $g^{-1}$ and $193*10^5$ CFU $g^{-1}$ in soil contaminated with 2.5% and 1% oil respectively, compared to the treatment with only Jatropha plant and no organic wastes, which showed $48*10^5$ and $45*10^5$ CFU $g^{-1}$ in 2.5 % and 1 % pollution respectively (157).

The mechanism of oil degradation was interpreted by rhizodegradation or phytovolatilization as Jatropha plant roots did not appear to accumulate hydrocarbons from the soil (as demonstrated by Soxhlet extraction and GC MS analysis) (157).

As only total petroleum hydrocarbon were determined in these studies, rather than individual petroleum components (114), the fate and effect on specific recalcitrant compounds like PAHs, caused by the addition of spent grain remains unknown.

A pilot study (158) in mycoremediation of soil contaminated with diesel range hydrocarbons (DRO) in the form of on-site biopiles with Oyster Mushrooms (*Pleurotus Ostreatus*

*Columbinus*) used non-spent grain for the preparation of soil inoculation spawn. Sawdust and wood shavings were also added in the soil as a nutrient source for the growth of the fungus. Although preliminary results after one growth season were unable to prove a reduction in the contamination, a successful inoculation was achieved, with growth of visible fruiting bodies in the soil surface, and establishment of an improved soil ecosystem that could potentially support the degradation of DRO in further seasons of growth (158).

All mentioned re-utilization studies and properties of spent grain, paired with the abundance and easy access to this agro-industrial waste product in Scotland, lead to its selection in this thesis as a potentially suitable matrix for use in bioaugmentation approach for soil remediation. As a lignin rich matrix, the use of spent grain as an amendment in soil may also have potential in stimulating the activity of lignin degrading microorganisms, known to play a role in the degradation of PAHs, already present in the soil.

### 2.4 Specific research questions derived from the general aims and literature review

In basis of the set general aims of this PhD thesis (section 1.4), opportunity gaps were identified from the literature review, which in the defined context of this research project (sections 1.1 to 1.3) allow specific research questions/ objectives to be defined.

While the use of P amendments has been widely studied and accepted as a suitable method for in situ remediation of Pb contaminated soils (section 2.1.3.1), in the context of urban brownfield soils that often have a neutral to alkaline pH, the effective formation of pyromorphite is likely to be limited, as the solubilisation of an added P source/amendment and the pre-existing Pb-phases may not occur. Harnessing the ability of P solubilizing organisms (section 2.1.3.2), may aid to overcome this limitation. The solubilisation of P however, must be paired with a tolerance of these microorganisms to Pb contamination in their environment.

The fungus *P.javanicus* has been identified in previous research (section 2.1.3.3) for its tolerance to Pb contamination, and ability to solubilize P and biomineralize available Pb as pyromorphite in laboratory culture experiments. The occurrence of pyromorphite biomineralization by *P.javanicus*, however, has not been studied in a soil matrix.

Certain fungi have also been identified as capable of biodegrading organic contaminants, such as PAHs, in soil (section 2.2.3.4), and studied for bioaugmentation remediation approaches. While bioaugmentation for the biodegradation of PAHs has been studied taking in consideration the presence of other contaminants such as heavy metal (section 2.2.3.2), to

the author's knowledge, co-remediation of PAHs and Pb with bioaugmentation, and more specifically with fungi, has not been studied before.

Agroindustrial wastes have shown promise (sections 2.2.3.1, 2.2.3.3 and 2.2.3.4) as inoculum carriers for the bioaugmentation of contaminated soils, as well as for the biostimulation of both naturally occurring and introduced microorganisms, in order to remediate PAHs contaminated soils.

Brewery spent grain is an agro-industrial waste available in abundance in Scotland (section 2.3). Its potential as a suitable matrix for bioaugmentation of soil with *P.javanicus* and biostimulation of naturally occurring soil microorganisms for the co-remediation of Pb and PAHs in soil can hence be studied.

From these observations, the following have been defined as specific research questions or objectives:

- Replicate laboratory culture experiments with *P.javanicus* to corroborate its ability to biomineralize Pb as pyromorphite (Chapter 3).
- Study the tolerance of *P.javanicus* to PAH contamination in laboratory cultures (Chapter 3).
- Assess the potential of biodegradation and/or biosorption of PAHs in *P.javanicus* - liquid culture experiments (Chapter 3).
- Characterize Pb and PAH content of soil samples from the Old Glasgow Meat market site, for potential use as a real environmental matrix in soil-matrix experiments with *P.javanicus* (Chapter 4).
- Test the suitability of brewery spent grain for the preparation of *P.javanicus* inoculums (Chapter 5).
- Study the feasibility of *P.javanicus* inoculation, propagation and growth in soils with the aid of brewery spent grain at a laboratory scale in microcosms (Chapter 5).
- Understand the exchange of transition metals and P between soil and spent grains when the latter is used as an amendment (Chapter 5).
- Using BCR sequential extraction and the UBM bioaccessiblity assay, assess Pb mobility and bioaccessibily in Pb and PAH contaminated soils, with neutral to slightly alkaline pH, and the effects that treatment of soil with brewery spent grain, *P.javanicus*-bioaugmentation and a traditional phosphate amendment have on Pb mobility and bioaccessibily (Chapter 6).
- Study the effects of soil treatment with brewery spent grain and *P.javanicus*-bioaugmentation on PAH concentrations (Chapter 7).

- Assess the effect on vegetation growth and root development caused by the amendment of poor quality urban soil with brewery spent grain and inoculation of *P.javanicus* in a pot experiment (Chapter 8).
- Using soil plate cultures, study qualitative changes in the soil microbial population caused by the amendment of poor quality urban soil with brewery spent grain and inoculation of *P.javanicus* in a pot experiment (Chapter 8).

# CHAPTER 3 Lead (Pb) biomineralization and Phenanthrene (Phen) biodegradation/biosorption culture experiments

## 3.1 Introduction

A common contamination profile found in urban soil is that of Pb and PAHs (1, 4, 6-8). The Old Glasgow Meat market is an example of this type of contamination, with Pb, total PAHs (16 priority contaminants USEPA) and B(a)p surpassing selected soil guideline values (section 1.3).

Pyromorphite ($Pb_5[PO_4]_3X$; X= F, Cl, B or OH) is the most stable form of Pb in soils, as it remains insoluble under a wide range of physicochemical conditions (e.g. pH and Pb concentration), as well as in the human gastrointestinal system (42, 45, 48, 52). Recent studies with the fungal species *P.javanicus* (42, 48, 50, 78) have shown its ability to biomineralize Pb as pyrmorphite in laboratory cultures. Fungi have also shown promise for PAH remediation through biodegradation and biosorption (102, 105, 108, 112, 113, 119, 138, 139).

It was therefore important to reproduce the growth of *P.javanicus* in liquid media to assess its potential for the biomineralization of Pb as pyromorphite, in addition to investigating *P.javanicus'* ability to tolerate PAH toxicity and its capacity for remediating PAHs through the mechanisms of biodegradation and/or biosorption. Experiments in liquid cultures were carried out as a first step, for proof of concept, before moving to a more complex soil matrix.

Three samples of *P.javanicus*, in the form of malt extract agar (MEA) culture plates, were provided by Prof. Geoffrey Gadd and his team at the Geomicrobiology group, University of Dundee (42, 48, 50, 78).

*P.javanicus* growth trials were initially carried out in non-contaminated MEA plate cultures, liquid potato dextrose broth (PDb) and modified Czapek-Dox media (MCD (78)), in order to become familiar with the culturing process and optimal incubation conditions.

Once growth of *P.javanicus* in optimal conditions was attained, exposure to Pb and PAHs, represented by B(a)p, was carried out in liquid cultures, to corroborate the tolerance to Pb described in previously published work on *P.javanicus* (42, 48, 50), and determine if a PAH contaminated environment would allow for the growth and reproduction of *P.javanicus*. B(a)p was utilised in this stage as it represents the main PAH of concern in the exemplary case site of the Old Glasgow Meat market (section 1.3).

A detailed Pb-spiked culture experiment was then carried out to confirm and study the ability of *P.javanicus* to biomineralize Pb as pyromorphite.

To investigate processes of biodegradation and biosorption, the PAH phenanthrene (Phen) was used as a model PAH in two rounds of culture experiments (with different time scales), and its presence in solution in the culture media and in fungal biomass was monitored over time.

Although B(a)p is one of the main PAHs that causes concern in regards to its toxicity, its low water solubility ($1.62*10^{-3}$ mg $l^{-1}$ at 25 °C (159)) only allows for very low concentrations of dissolved B(a)p in culture media. This hinders the quantification of concentration variations overtime in the culture supernatant. Phen, on the other hand, has a higher water solubility (~1.10 – 1.15 mg $l^{-1}$ at 25°C (160)), facilitating liquid culture media spiking at a concentration that allows more detailed measurement of concentration variations over time. Phen has often been used as a model substrate for studies on metabolism of carcinogenic PAHs because analogues of its structure (e.g. bay-region and a K-region) are found in carcinogenic aromatic hydrocarbons (102). For these reasons Phen was selected as a more suitable compound for use in culture experiments encompassing the quantification of dissolved PAHs concentration in *P.javanicus* cultures over time.

## 3.2 Objectives

- Define appropriate conditions and methods for the culturing of *P.javanicus*.
- Study the tolerance of *P.javanicus* to B(a)p contamination in laboratory cultures.
- Corroborate the tolerance of *P.javanicus* to Pb contamination in laboratory cultures
- Carry out laboratory culture experiments with *P.javanicus* to corroborate its ability to biomineralize Pb as pyromorphite.
- Assess the potential of biodegradation and/or biosorption of PAHs in *P.javanicus* - liquid culture experiments.

## 3.3 Methodology

### 3.3.1 Culturing of *P.javanicus*

#### 3.3.1.1 *P.javanicus* MEA plate cultures

In 1 l of Milli-Q water, 50 g of malt extract agar (MEA, Merck KGaA, Darmstadt, Germany) was dissolved. The mixture was then autoclaved at 121°C for 15 minutes. After removing from the autoclave, the media was poured into sterile polystyrene Petri dishes, and allowed to cool at room temperature.

From the original MEA plates sent from the University of Dundee, circular plugs were cut on the edge of growing colonies, using previously sterilized glass Pasteur pipettes, and transferred to the newly prepared MEA plates with the assistance of sterile syringe needles. Agar plates were then sealed with Parafilm® M (Sigma, Saint Louis, Missouri, USA) and placed in an incubator (static), lid facing up, at 20°C in the dark.

#### 3.3.1.2 *P.javanicus* PDb liquid cultures

Potato dextrose broth (PDb) was prepared by dissolving 24 g of PDb powder (Sigma-Aldrich, Saint Louis, MO. USA) in 1 l of Milli-Q water, and autoclaving at 121°C for 15 minutes. Once at room temperature, 150 ml of broth was transferred into 250 ml sterile Erlenmeyer flasks. Flasks were inoculated as described for MEA plates in the previous section (section 3.3.1.1) and placed in an orbital incubator at 20°C and 150 rpm, in the dark.

#### 3.3.1.3 *P.javanicus* MCD liquid cultures

MCD media (Modified Czapek-Dox media (78)) was prepared according to the following: in 0.95 l of Milli-Q water, 3.0 g $NaNO_3$ (Sigma-Aldrich, St. Louis, MO. USA); 0.5 g $MgSO_4*7H_2O$ (Sigma-Aldrich, St. Louis, MO. USA) and 0.5 g KCl (Sigma-Aldrich, St. Louis, MO. USA) were added; this solution was then autoclaved at 121°C for 15 min. In a separate solution: 30 g of D-Glucose ($C_6H_{12}O_6$, Sigma-Aldrich, St. Louis, MO. USA); 1.0 g $Na_2HPO_4$ (Sigma-Aldrich, St. Louis, MO. USA) and 0.01 g $FeSO_4*7H_2O$ (Sigma-Aldrich, St. Louis, MO. USA) were dissolved in 50 ml Milli-Q water, then added by filter sterilization (Minisart® 0.2µm syringe filter, Sartorius, Gottingen, Germany) to the first solution to create the final MCD media.

Once at room temperature, 150 ml of media was transferred into 250 ml sterile Erlenmeyer flasks. Flasks were inoculated as described for MEA plates in section 3.3.1.1 and placed in an orbital incubator at 20°C and 150 rpm, in the dark.

#### 3.3.1.4 Glycerol stock preparation

For long term preservation, four *P.javanicus* glycerol stocks were prepared from two 7 day old PDb cultures, as follows.

From the 7 day *P.javanicus* PDb culture, 1.0 ml of media was transferred to sterile 2.0 ml screw cap cryogenic tubes. 1 ml of sterile 50% Glycerol (Sigma-Aldrich, Saint Louis, MO. USA) in Milli-Q water solution, was then added. The tube was sealed and gently shaken by hand (end over end) 5 times. The four resulting stocks were stored at -80°C.

### 3.3.2 Growth of *P.javanicus* on contaminated media: preliminary qualitative experiments

A preliminary growth experiment was carried out with spiked liquid media to assess the tolerance of *P.javanicus* to Pb and B(a)p contamination.

#### 3.3.2.1 Experimental set up

Six PDb liquid cultures were prepared, two spiked with $Pb(NO_3)_2$ (Sigma-Aldrich, St. Louis, MO. USA), two spiked with B(a)p (Supelco, Bellefonte, PA, US), and two controls with no spike. PDb culture preparation was carried out as described in section 3.3.1.2.

Pb spiked cultures were prepared by adding 0.192 g of $Pb(NO_3)_2$ to 300 ml of PDb before autoclaving, giving a final Pb concentration of 400 mg $l^{-1}$.

B(a)p spiked cultures were prepared by adding 0.5 ml of a 3.0 mg $ml^{-1}$ B(a)p in toluene (Fisher Scientific, Loughborough, UK) stock solution (30 mg of B(a)p in 10 ml of toluene) to the sterilized Erlenmeyer flasks, before the addition of the media to give a final concentration of 10 mg $l^{-1}$ of B(a)p in culture media. The flasks were then left sealed, only with the added stock solution, until no liquid toluene was visible. Before adding the PDb media in the flasks, the seals were opened in a sterile environment, to allow the volatilized toluene to escape the flask. With a solubility of $1.62*10^{-3}$ mg $l^{-1}$ at 25 °C (159), B(a)p remained as precipitated solids in the bottom of flasks, after the addition of media.

Inoculation was made from an 8 day old PDb culture, by transferring 0.5 ml of media into the new cultures, with sterile pipettes.

Culture flasks were placed in an orbital incubator at 20°C and 150 rpm, in the dark, for 30 days.

### 3.3.3 Lead (Pb) biomineralization culture experiment

#### 3.3.3.1 Experimental set up

The experiment was setup with three replicate cultures for three harvesting times (10, 20 and 30 days). An abiotic (negative) control with spiked media but no fungal inoculum and a positive control with no Pb spike were also included for each harvesting time, giving a total of 12 fungal cultures and 3 abiotic controls.

Potato dextrose broth (Sigma-Aldrich, St. Louis, MO. USA) was used as culture media. For the spiked media, 2.556 g of $Pb(NO_3)_2$ (Sigma-Aldrich, St. Louis, MO. USA) was added to

2.0 l of media, to give a final concentration of 800 mg $l^{-1}$ Pb. 9.455 g of Glycerol 2 phosphate (G2P, $C_3H_7Na_2O_6P*5H2O$, Sigma-Aldrich, St. Louis, MO. USA) was then added, as an organic phosphate source, to give a molar ratio of 1Pb:4P (46, 57, 68). Media was sterilized by autoclaving at 121°C for 15 minutes. pH was adjusted to 7.5 before sterilization with a 10 mol $l^{-1}$ NaOH solution, giving a final pH after sterilization of 7.10 ± 0.04.

For the control, 500 ml of media was prepared as above, with an equivalent amount of Glycerol 2 phosphate (2.364 g) and adjusting pH to 7.5 before sterilization with NaOH (10 mol $l^{-1}$ solution), giving a final pH after sterilization of 7.82 ± 0.05.

150 ml of spiked media was added to twelve 250 ml Erlenmeyer flasks. Nine of those flasks were inoculated from a 10 days old *P.javanicus* liquid culture (prepared as in section 3.3.1.2), by adding 1.0 ml of media from the culture with a sterile pipette. The three remaining flasks were kept as abiotic controls. In three more flasks, 150 ml of the non-spiked media was added and inoculated in the same way. The resulting 15 culture flasks were then left in an orbital incubator at 20°C and 150 rpm in the dark.

PDb media was chosen, as other media (i.e. MCD media) when spiked with Pb at the chosen concentration, caused instant precipitation, which prevented a known and homogenous Pb concentration to be maintained amongst the culture replicates.

Excess spiked media was stored at -80°C for further processing.

Sacrificial harvesting of three *P.javanicus*-Pb spiked cultures and one of each type of control was carried out every 10 days. Cultures were harvested by centrifugation. The content of flasks was transferred to 50 ml polypropylene centrifuge tubes and centrifuged at 4000 rpm for 30 minutes to separate fungal material and precipitates from the supernatant. The pH of the supernatant was measured (Orion 5 star Thermo Scientific pH meter with an Orion 8156BNUWP ROSS Thermo Scientific ultra-combination pH electrode) and all harvested materials were stored at -80°C until further processing.

### 3.3.3.2 Analytical techniques and respective sample processing

#### 3.3.3.2.1 Pb in solution in the supernatant

**Sample preparation**

Supernatants were digested with a modified version of USEPA method 3010A (161). 100 ml of supernatant harvested from each culture, as well as 100 ml of the excess time zero spiked media was transferred into two separate 50 ml DigiTUBEs ® (SCP Science, Quebec H9X 4B6 Canada). 1.5 ml of $HNO_3$ (ARISTAR®, VWR chemicals, Leuven, Belgium) was

added to each tube, which were then placed in a DigiPREP Jr. ® (SCP Science, Quebec H9X 4B6 Canada) digestion block at 95°C, until the volume had reduced to < 5.0 ml. Tubes were removed from the digestion block, and allowed to cool to room temperature. 3 ml of $HNO_3$ was added to each tube and they were covered with watch glasses and returned to the digestion block, at 95°C for an hour. Watch glasses were then removed and the solution allowed to evaporate, until < 3.0 ml was left.

The content of the two tubes were combined and transferred to a single 100 ml volumetric flask, by repeatedly adding 5% $HNO_3$ to the tubes and transferring to the volumetric flask, until the volume reached ~100 ml. The flask was then made to volume with 5% $HNO_3$.

All resulting solutions (other than those corresponding to non-spiked controls) were diluted 1:10 and 1:100 with 5% $HNO_3$. Both dilutions and the undiluted solution were analysed by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES).

**ICP-OES analysis**

Analysis was performed at SUERC with assistance of technical staff using a Perkin Elmer Optima 7300 DV ICP-OES. A calibration line (1, 3, 5, 10, 15 and 20 mg $l^{-1}$) was prepared with a Specpure® 1000 µg Pb $ml^{-1}$ in 5% $HNO_3$ standard (Alfa Aesar, Zeppelinstr. 7 76185 Karlsruhe). A 1 mg $l^{-1}$ standard was placed approximately every 9 samples, and a blank every 15. Two wavelengths were measured for Pb (182.205 nm and 216.999 nm), but wavelength 182.205 nm was selected for concentration calculations, as it had a better fitting (R2) calibration line, and consistency of standards throughout the run (Appendix CH3 S1).

**3.3.3.2.2 Microscopy and chemical composition of biominerals in fungal biomass**

**Sample preparations**

A portion of biomass of each harvested culture was prepared for Energy-dispersive X-ray spectroscopy (EDX) analysis, by technical staff in the Electron Microscopy Facility, School of Life Sciences, University of Glasgow. Samples were fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate (SC) buffer for 24 hrs, followed by three 10 min rinses with SC. Samples then underwent an ethanol dehydration series (30 % ethanol for 10 min, then 50 % for 10 min, 70 % for 10 min, 90 % for 10 min, four repetitions of absolute 100 % ethanol for 5 min, followed by four repetitions of extra dry absolute 100 % ethanol for 5mins). Finally, two 10 min drying steps with hexamethyldisilazane (HMDS: $C_6H_{19}NSi_2$) were carried out and samples allowed to dry in a desiccator until analysed.

The solid phase recovered from abiotic controls was prepared by air drying and carefully grinding into a fine powder.

**EDX analysis**

Each sample was mounted in Aluminium stubs with carbon adhesive discs and analysed on a Carl Zeiss Sigma Variable Pressure Analytical SEM with Oxford Microanalysis and Energy-dispersive X-ray spectroscopy analysis 80 mm silicon-drift detector operating at an accelerating voltage of 20kV. Analysis was performed by the author with assistance of technical staff from the Imaging Spectroscopy and Analysis Centre (ISAAC), School of Geographical & Earth Sciences, University of Glasgow.

#### 3.3.3.2.3 Identification of predominant mineral phases in biomass

**Sample preparation**

All of the remaining biomass of each sample was freeze dried at -121ºC in a vacuum for 72 hours, and ground with an agate pestle and mortar into a powder. Abiotic controls were prepared in the same manner as described for EDX analysis.

**XRD analysis**

Diffraction patterns were recorded on a Panalytical X'Pert PRO MPD (A3-26) with a PW3050/60 goniometer, a Cu anode and K-alpha radiation, from 5° to 70° 2θ, counting for 30 seconds and 0.017° 2θ per step. Mineral phases were identified by reference to all available patterns in the instrument database library. Analysis was performed by technical staff from the crystallography service, School of Chemistry, University of Glasgow; with assistance from the author.

### 3.3.4 Phenanthrene (Phen) culture experiments

#### 3.3.4.1 Experimental set up

Two Phen-spiked culture experiments were carried out with different time scales. The experimental setup was analogous to that of the Pb experiment, with harvesting times at 10, 20 and 30 days in a first experiment and 30, 50 and 70 days in a second experiment.

Modified Czapek-Dox media (MCD media) was prepared as described in section 3.3.1.3. This media was selected, rather than potato dextrose broth (PDb), as it allows a greater control on the total carbon content and yielded better recoveries for Phen with liquid-liquid extraction (method described in section 3.3.4.2.1).

From a 1.0 g $l^{-1}$ Phenanthrene (Supelco, Bellefonte, PA, USA) in Acetone (Sigma-Aldrich, St. Louis, MO. USA) stock solution, 2.0 ml was added with a glass syringe to 2.0 l of autoclaved MCD media, to give a final concentration of 1000 µg $l^{-1}$ Phen. The media was then stirred with a metallic magnetic stirrer for 15 minutes and the pH of the resulting media was measured.

As in the Pb experiment 150 ml of spiked media was added to twelve Erlenmeyer flask, of which nine were inoculated from a 10 day old MCD *P.javanicus* liquid culture, and three left as abiotic controls. Three more controls were prepared with non-spiked MCD media and inoculated.

All cultures were then left in an orbital incubator at 20°C and 150 rpm in the dark. Excess spiked media was immediately extracted as described in section 3.3.4.2.1.

To avoid the use of plastic that may adsorb Phen, cultures were harvested by filtration through a fine metallic mesh. The pH of the supernatant was measured before immediate extraction. Biomass was stored at -80°C.

### 3.3.4.2 Analytical techniques and respective sample processing

#### 3.3.4.2.1 Phen in solution in the supernatant

**Liquid-liquid extraction**

Phen was extracted from the media by liquid-liquid extraction. Precisely, three replicates of 10 ml of the supernatant of each culture were transferred with a glass pipette to 20 ml headspace glass vials. Subsequently 2 ml of dichloromethane (DCM $CH_2Cl_2$ >99.8%, Sigma-Aldrich, St Louis, MO, USA) was added with a glass syringe to each vial. Vials were then sealed with caps with PTFE/Silicone septa and hand-shaken for 5 minutes, followed by 5 minutes of sonication (Branson 5510 sonic bath) at a set temperature of 29ºC. After sonicating, vials were placed upside down, and left for 10 minutes to allow the phases to separate. The lower phase (DCM) was extracted from the vial with a glass syringe and transferred to a separate headspace glass vial. The same extraction process was repeated three times, and the three aliquots of 2 ml DCM extract were combined in the second headspace glass vial. In the last round, before extracting, vials were centrifuged at 2980 rpm for 5 minutes.

**HPLC analysis**

Extracts were analysed by the author on a Shimadzu Prominence HPLC with a SPD-M20A Photodiode Array Detector and a Purospher® STAR RP-18 Endcapped column. Column

temperature was maintained at 40°C. A mobile phase of Acetonitrile (ACN, Sigma-Aldrich, St Louis, MO, USA) and water in a gradient was run at a flow rate of 1 ml $min^{-1}$ with the following programme: 80 ACN: 20 water (min 0 to 10), 97:3 (min 10 to 13), 100:0 (min 13 to 18), 80:20 (min 18 to 25). Peak areas were measured at 248 nm and Phen concentrations were calculated using a calibration line (50, 125, 500, 1000 and 1700 µg $l^{-1}$ Phen) prepared with the same Phen stock used for the culture spike, in DCM. A blank and additional standard were included in each HPLC run.

#### 3.3.4.2.2 Phen in fungal biomass

**Accelerated solvent extraction (ASE)**

**a) Solution preparation**

Internal standards are used to improve the precision of quantitative analysis. An internal standard is a known amount of a compound that behaves similarly to the analyte and is added in every sample that is analysed, after the extraction process. A signal that can be distinguished from that of the analyte should be provided by the internal standard. Theoretically any factor that may affect the analyte signal will affect the internal standard signal to the same degree. In consequence the ratio of the two signals exhibits less variability than the analyte signal on its own, and can be used to quantify the analyte.

A surrogate is also a compound that behaves similarly to the analyte, and is added to each sample in a known amount before the extraction process, in order to assess the recovery.

A 2000 µg $ml^{-1}$ internal standard (IS) solution was prepared by weighing 20 mg of phenanthrene-d10 (Sigma-Aldrich, St. Louis, MO 63103), in a headspace vial. 10 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG) was then added and the vial sealed with a Teflon cap.

A 500 µg $ml^{-1}$ internal standard (IS) solution was prepared by weighing 10 mg of phenanthrene-d10 (Sigma-Aldrich, St. Louis, MO 63103) in a headspace vial and adding 20 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG).

A 2000 µg $ml^{-1}$ surrogate solution was prepared by weighing 20 mg of anthracene-d10 (Sigma-Aldrich, St. Louis, MO 63103) in a headspace vial and adding 10 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG).

A 500 µg $ml^{-1}$ surrogate solution was prepared by weighing 10 mg of anthracene-d10 (Sigma-Aldrich, St. Louis, MO 63103) in a headspace vial and adding 20 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG).

**b) Sample preparation**

Fungal biomass was freeze dried at -121ºC in a vacuum for 72 hours, ground with an agate pestle and mortar into a powder, and total biomass weight recorded.

For samples harvested from the first experiment, a target sample weight of 0.25 g of biomass per harvested culture was extracted. In cases were the total biomass weight was < 0.25 g, the entirety of it was extracted. For samples harvested from the second experiment, the same logic was applied, but with a target sample weight of 0.5 g.

In stainless steel extraction cells (Dionex, Thermo Fisher Scientific) with a glass fibre filter paper on the bottom end cap, the following was added (in the described order): 3.0 g of ashed (3 hrs at 450°C) and deactivated (10 % nanopure water) silica gel (Fisher Scientific, Leicestershire, LE11 5RG); a fungal biomass sample; 0.25g (for a target sample weight of 0.25 g) or 0.5 g (for a target sample weight of 0.5 g) of sodium sulphate (Fisher Scientific, Loughborough, UK); enough ashed (3 hrs at 450°C) acid purified sand (Sigma-Aldrich, St. Louis, MO 63103) to fill the remaining headspace in the cell.

For samples from experiment 1, 150 µl of the 2000 µg $ml^{-1}$ surrogate solution was added to the cell before sealing, and for samples from experiment 2, 50 µl of the 500 µg $ml^{-1}$ surrogate was added.

**c) Extraction protocol**

Extractions were performed in an ASE 350 Accelerated Solvent Extraction system (Dionex, Thermo Fisher Scientific). Two sequential extractions were performed and collected in the same vial. In the first extraction (F1) the solvent used was hexane (Fisher Scientific, Leicestershire, LE11 5RG) and in the second extraction (F2) an 8:2 hexane-toluene mixture was used. Set ASE parameters are shown in Table 3.1.

The collected mixture of F1 and F2 extracts was reduced by evaporation to 1 ml with a Buchi Syncore® Polyvap (9230 Flawil, Switzerland). Buchi settings are also shown in Table 3.1.

*Table 3.1. ASE and Buchi settings.*

| | |
|---|---|
| **Extraction solvent** | F1: toluene<br>F2: 8:2 hexane: toluene |
| **Temperature** | F1: 150°C – heat 10 min<br>F2: 150°C – heat 5 min |
| **Static time** | F1: 5 min<br>F2: 5 min |
| **Number of cycles** | F1: 1, F2: 1 |
| **Rinse volume – purge time** | F1: 50% - 60 s<br>F2: 50% - 60 s |
| **Buchi conditions** | 120 mmbar, 42°C, 200 rpm |

For samples from the first experiment, 150 µl of the 2000 µg $ml^{-1}$ IS solution was added to the final 1ml extract, giving a final solution volume of 1.15 ml with an expected concentration of 260.9 µg $ml^{-1}$ for the IS and surrogate.

For samples from the second experiment 50 µl of the 500 µg $ml^{-1}$ internal standard solution was added to the final 1ml extract, giving a final solution volume of 1.05 ml with an expected concentration of 23.8 µg $ml^{-1}$ for the IS and surrogate.

A blank cell was included in each ASE round for quality control purposes.

**GC-MS analysis**

GC-MS analysis was performed at the Environmental laboratory, in the Department of Civil & Environmental Engineering, University of Strathclyde (G1 1XQ, Glasgow), by technical staff. A Thermo Scientific Trace GC ultra with a DSQ II Mass Spectrometer and a ZB-SemiVolatile Zebron capillary column (30m length, 0.25mm I.D. and 0.25 µm film thickness) was used. The temperature programme used was as follows: 50°C (2 min hold) followed by 32°C $min^{-1}$ up to 170°C, 6°C $min^{-1}$ up to 260°C, 2.5°C $min^{-1}$ up to 295°C, 15°C $min^{-1}$ up to 325°C and a final 3 min hold. The inlet temperature was set at 280°C and detector source temperature at 230°C. Sample injection was 1 µl and the flow 1.4 ml $min^{-1}$ using helium as the carrier gas.

Recoveries were calculated with the surrogate anthracene-d10, by calculating a percentage of recovered surrogate in relation to the expected surrogate concentration (260.9 µg $ml^{-1}$ or 23.8 µg $ml^{-1}$). Phen concentration was then adjusted in each sample according to its respective recovery, as follows:

$$\text{FPhen x} = \frac{\text{ES}}{\text{Sx}} * \text{Phen x}$$

With:

- FPhen x: final Phen concentration (mg $kg^{-1}$) in a sample x.
- ES: expected surrogate (d-10 Anthracene in this case) concentration (µg $ml^{-1}$).
- Sx: obtained surrogate (d-10 Anthracene in this case) concentration (µg $ml^{-1}$) in a sample x.
- Phen x: obtained Phen concentration (mg $kg^{-1}$) in a sample x

An example of this calculation is given in Appendix CH3 A1.

As the total fungal biomass is different in each culture, increasing overtime, there is a dilution effect on Phen concentrations. For this reason, and in order to have comparable results, mg $kg^{-1}$ Phen concentrations were transformed into absolute µg Phen content, with the total biomass weight as follows:

$$\mathrm{APhen\ x} = \mathrm{FPhen\ x} * \mathrm{Wx}$$

With:

- APhen x: total Phen content (µg) in harvested fungal biomass from a culture x, represented by sample x
- FPhen x: final Phen concentration (mg $kg^{-1}$ = µg $g^{-1}$) in a sample x
- Wx: total fungal biomass dry weight (g) harvested from a culture x, represented by sample x

An example of this calculation is also included in Appendix CH3 A1.

### 3.3.5 Statistics

All statistical analysis was carried out in Microsoft Excel (2016). Errors were calculated using the t-distribution confidence interval formula, appropriate for populations under 30:

$$\mathrm{X} \pm \mathrm{t}_{n-1} \frac{\mathrm{s}}{\sqrt{\mathrm{n}}}$$

Where

X= average value for a group of measurements

n= sample size (number of measurements)

n-1= degrees of freedom

s= standard deviation

t= constant from the t Table

The two-tails probability was set at p=0.05

All remaining analysis was done using the Microsoft Excel (2016) Data Analysis package. Statistical significance was evaluated with the "t=Test: two sample assuming unequal variance" and "Anova: single factor", with significance assumed at a P-value < 0.05.

## 3.4 Results and discussion

### 3.4.1 A visual description of *P.javanicus* in plate and liquid cultures

Initial attempts at culturing *P.javanicus* in MEA plates an liquid cultures in incubators set at a temperature of 30°C resulted in no fungal growth. In consequence the incubation temperature was reduced to 20°C, with positive results for fungal growth. The strain of *P.javanicus* used in these experiments was initially isolated from mining related Pb-contaminated soils in Wanlockhead, Scotland, where the average annual temperature minimum is 2.8 ± 2.4°C and maximum 9.9 ± 3.1°C (162). It is hence coherent that lower incubation temperatures would favour its growth.

In plate cultures *P.javanicus* presented white in colour (Figure 3.1-a), acquiring local brownish and pinkish tones as it ages. From the bottom view, the base of the colony presented a brown colour (Figure 3.1-b), and white if the colony begun penetrating the agar, towards the base of the petri dish. Colony shapes varied from irregular as in Figure 3.1-a, to approximately radial/concentric, and raised to umbonate in elevation. The filamentous nature of the fungus was noticeable in its cotton-like texture. In most cases, a main larger colony grew from the inoculum and additional smaller colonies appeared scattered in the plate.

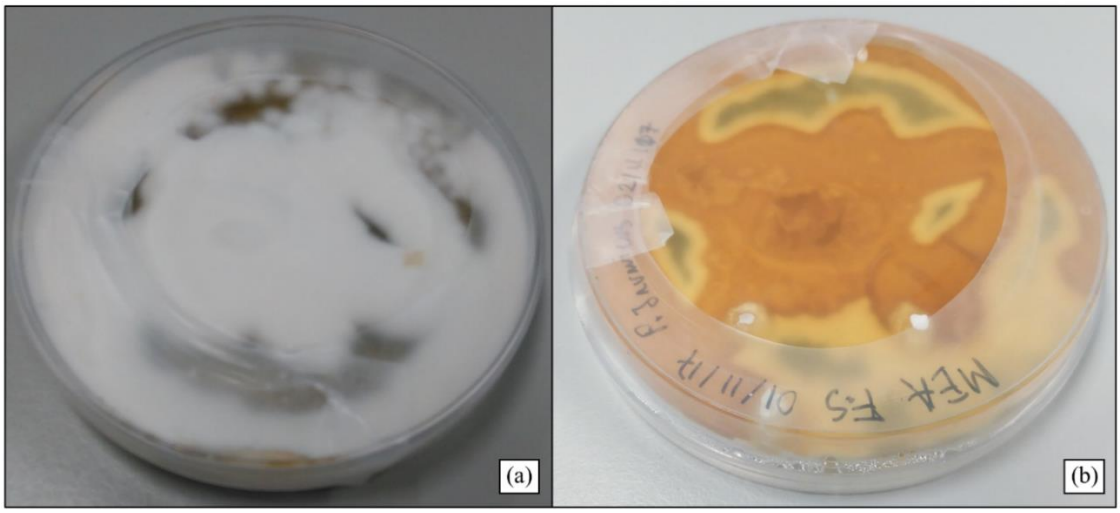


*Figure 3.1. 29 days old MEA P.javanicus plate culture: (a) top and (b) bottom.*

In liquid cultures *P.javanicus* grew in approximately spherical filamentous colonies (pompom-like) (Figure 3.2), light yellow to yellowish white in colour, of different sizes, growing in colony size overtime and/or in quantity of colonies.

In addition, a biofilm-like rim usually formed in the intersection of the surface of the media and the flask walls (Figure 3.2-b). The latter sometimes appeared with a slimy filamentous texture (Figure 3.2-b), or with a similar aspect to plate cultures, white and cotton-like on the surface, and brown on the colony base attached to the flask wall. Slimy filamentous biofilm-like growth attached to the base of the flask sometimes occurred. No specific controllable

culturing conditions were identified as causing the presence or absence of the latter, as under the same set incubation conditions and with the same media, some flasks showed growth attached to the base of the flask while some did not.

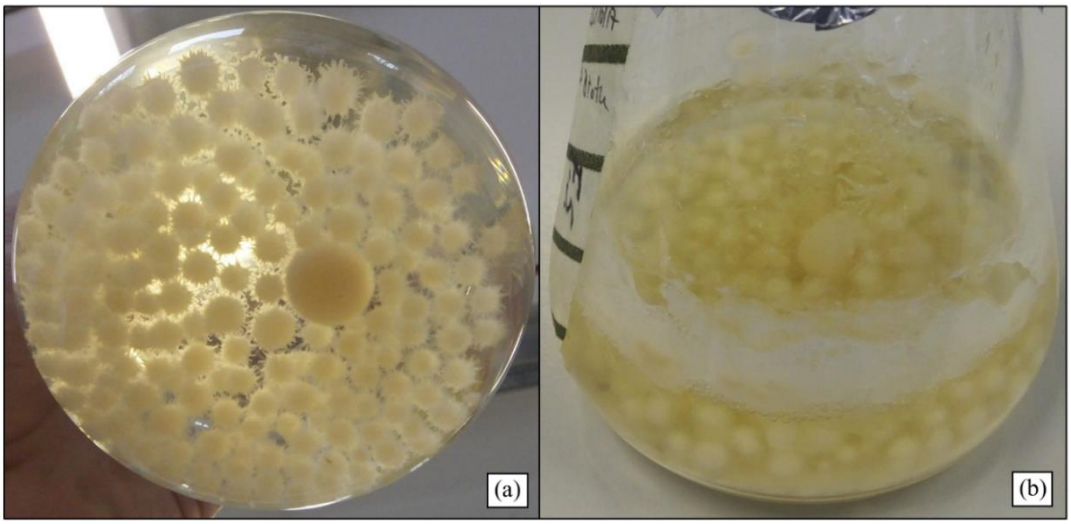



*Figure 3.2. Two examples of 13 days old liquid P.javanicus culture: (a) bottom view (b) side-top view.*

### 3.4.2 *P.javanicus* in Pb and B(a)p contaminated media: preliminary qualitative experiment

In order to study the tolerance of *P.javanicus* to Pb and PAHs, the concentrations of both Pb (400 mg $l^{-1}$) and B(a)p (10 mg $l^{-1}$) were set above reference guidelines for soil (Category 4 Screening Level for residential use with home-grown produce of 200 mg $l^{-1}$ and 5 mg $l^{-1}$ for Pb and B(a)p respectively (9)). Pb concentration was also above proposed UK Ecological Soil Guideline Value of (167.9 mg $l^{-1}$), which considers any adverse effects on wildlife such as birds, mammals, plants, soil invertebrates or on the microbial function of soils (11).

*P.javanicus* appeared to adapt to both contaminated media. After five days, growth was already significant in all cultures.

One of the Pb-spiked cultures seemed to have colonies growing in the form of sclerotia (Figure 3.3-d). Sclerotia is a mycelium morphology consistent of hard dormant bodies of compact hyphae protected by external thickened hyphae/tissue, that generally occur in adverse conditions for growth (163-165). The other Pb-spiked replicate, however, showed the usual filamentous spherical colony shape (Figure 3.3-c), suggesting a factor other than the presence of Pb was likely to have caused the sclerotia morphology.

B(a)p spiked cultures (Figure 3.3-a and b) showed a similar amount of growth to control cultures (Figure 3.3-e and f). As the solution was saturated with B(a)p (spiked concentration

of 10 mg $l^{-1}$ >> solubility 1.62*$10^{-3}$ mg $l^{-1}$ at 25 °C (159)), it would appear that B(a)p did not have toxic effects on *P. javanicus*. After 10 days the fungus had begun to grow attached to B(a)p grains in the bottom of flasks (Figure 3.4). The latter could be indication of the use of B(a)p as a carbon source, or simply a product of the physical protuberance of B(a)p grains providing a site for the fungus to attach. Regardless, this observation further suggests that B(a)p does not appear to be toxic to *P. javanicus*, and not represent an impediment for its growth.

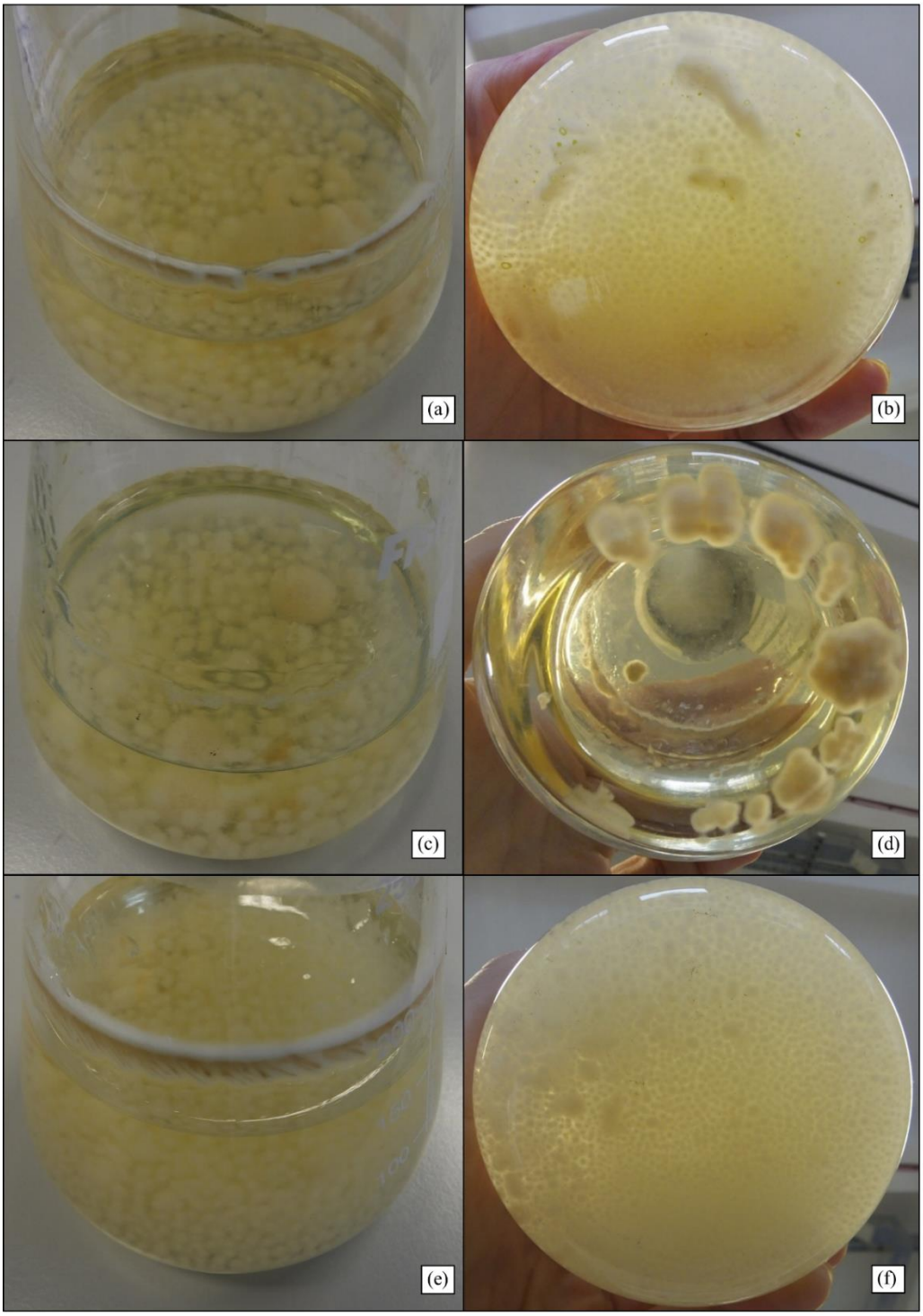



*Figure 3.3. Examples of 5 days old liquid P.javanicus cultures: (a) and (b) with B(a)p spiked media; (c) and (d) with Pb spiked media; (e) and (f) controls.*

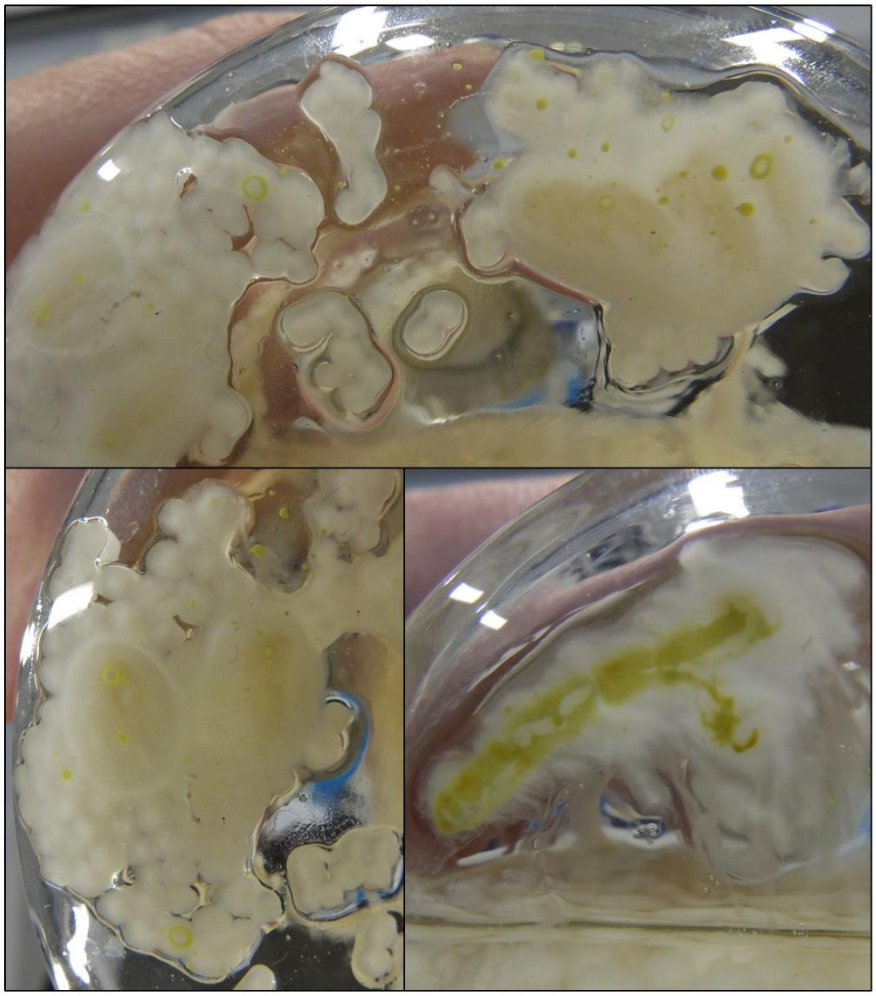


*Figure 3.4. Examples of P.javanicus growth attached to B(a)p grains, in 11 days old cultures.*

### 3.4.3 Lead (Pb) biomineralization by *P. javanicus*

An experiment with Pb-spiked liquid cultures inoculated with *P.javanicus*, harvested at intervals of 10 days over 30 days was carried out to study the biomineralization of Pb by the fungus.

After 10 days, spiked *P.javanicus* cultures showed a significant decrease of Pb in solution (Table 3.2 and Figure 3.5-a), from the initial 795.0 ± 15.63 mg $l^{-1}$, to 6.956 ± 0.762 mg $l^{-1}$. However, in the 20 day harvest, all 3 replicate supernatants showed an increase in Pb concentration to an average of 50.30 ± 6.500 mg $l^{-1}$, which remained relatively constant until the 30 day harvest, where concentrations averaged 48.92 ± 5.239 mg $l^{-1}$. Total removal rate after day 30 was ~94%. Abiotic controls also showed a significant decrease of Pb in solution, with a concentration of 202.1 ± 2.505 mg $l^{-1}$, 174.3 ± 1.354 mg $l^{-1}$ and 160.5 ± 3.847 mg $l^{-1}$ for 10, 20 and 30 day harvests respectively; indicating that even without the presence of *P.javanicus*, the set physico-chemical conditions and matix of the media, caused precipitation of Pb at the spiked concentration. Nevertheless, at 30 days Pb concentrations

in the control were 328% of those in fungal cultures. Complete data set and raw data are included in Appendix CH3 S1.

*Table 3.2. Summary table of average Pb concentrations in culture supernatants.*

| Harvest | Culture type | Average Pb (mg $kg^{-1}$) | error (mg $kg^{-1}$) |
|---|---|---|---|
| **Time 0** | *P.javanicus*-Pb | 795.0 | *15.63* |
| | Control (abiotic) | 795.0 | *15.63* |
| **10 days** | *P.javanicus*-Pb | 6.956 | *0.762* |
| | Control (abiotic) | 202.1 | *2.505* |
| **20 days** | *P.javanicus*-Pb | 50.30 | *6.500* |
| | Control (abiotic) | 174.3 | *1.354* |
| **30 days** | *P.javanicus*-Pb | 48.92 | *5.239* |
| | Control (abiotic) | 160.5 | *3.847* |

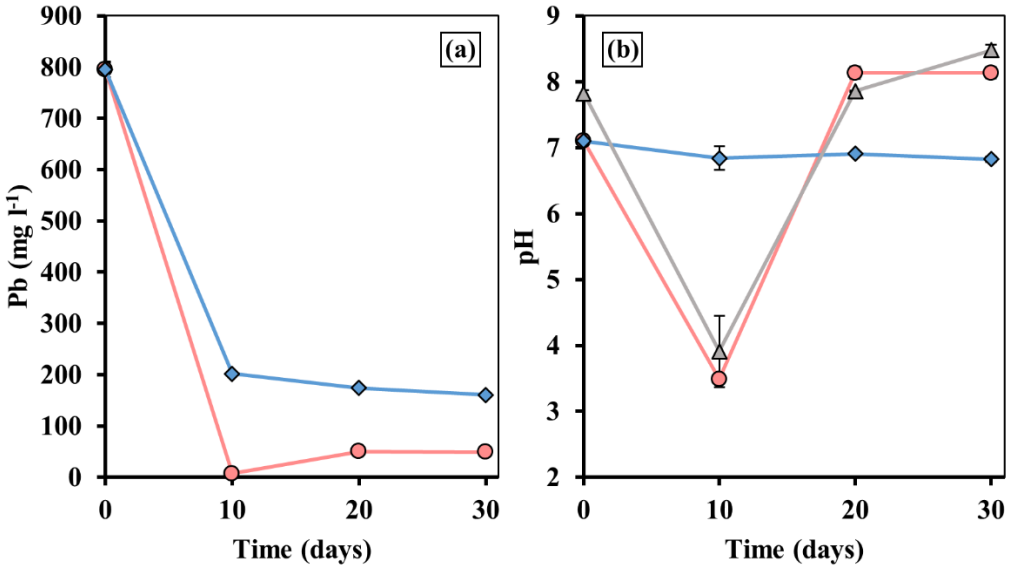



*Figure 3.5. Pb biomineralization experiment graphs: (a) Pb in solution in harvested supernatant, overtime; (b) pH of supernatant over time. In both, pink circles represent Pb-spiked P.javanicus cultures, blue diamonds the abiotic controls, and only in (b) grey triangles P. javanicus controls without Pb. Error bars (95% CI) in black are not always visible, due to the magnitude of the error in relation to Y axis scale.*

All inoculated cultures had similar pH variation over time (Table 3.3 and Figure 3.5-b), with or without Pb spike. From the initial pH of 7.1, after 10 days, spiked *P. javanicus* cultures showed a substantial drop in the pH, with an average of 3.50 ± 0.06, similar to the unspiked control which had a pH of 3.91 ± 0.50. In the 20 day harvest, pH had risen over the initial pH, to 8.14 ± 0.09 in spiked cultures and 7.86 ± 0.01 on the un-spiked control. The final harvest had a slight pH increase to 8.52 ± 0.09 and 8.48 ± 0.08 in spiked and un-spiked cultures respectively. The abiotic controls remained relatively unchanged over time, with a pH of 6.84 ± 0.18, 6.91 ± 0.01 and 6.82 ± 0.04 for 10, 20 and 30 day harvests respectively.

This differs from the pH variation reported by Liang *et al* (2016) (42) for *P.javanicus* cultures spiked with Pb and G2P, where pH gradually increased from an initial 6.00 to 6.30 ± 0.10 (10 days), 6.5 ± 0.3 (20 days) and 6.8 ± 0.4 (30 days), although the use of different culture media (MCD media in their case) and initial pH could explain the difference. Pb removal from solution in (42), also differs slightly from our results, with 53.8% (10 days) 97.2% (20 days) 99.8% (30 days) for Pb and Glycerol 2 phosphate (G2P, $C_3H_7Na_2O_6P*5H2O$) spiked cultures in their experiment, showing that the most significant difference occurs at 10 days, where pH in our experiment is sharply acidic, and Pb removal is at its highest.

*Table 3.3. Summary table of pH in culture supernatants from the Pb experiment.*

| Harvest | Culture type | Average pH | Error (pH) |
|---|---|---|---|
| **Time 0** | *P.javanicus*-Pb | 7.103 | *0.042* |
| | Control (no Pb) | 7.820 | *0.054* |
| | Control (abiotic) | 7.103 | *0.042* |
| **10 days** | *P.javanicus*-Pb | 3.487 | *0.057* |
| | Control (no Pb) | 3.910 | *0.539* |
| | Control (abiotic) | 6.840 | *0.180* |
| **20 days** | *P.javanicus*-Pb | 8.136 | *0.094* |
| | Control (no Pb) | 7.857 | *0.012* |
| | Control (abiotic) | 6.907 | *0.012* |
| **30 days** | *P.javanicus*-Pb | 8.522 | *0.090* |
| | Control (no Pb) | 8.477 | *0.082* |
| | Control (abiotic) | 6.823 | *0.042* |

Total dry weight for each sample at the different harvesting times was not obtained as each sample was subdivided for different sample preparations (EDX/SEM and XRD).

Biominerals were easily detected in the SEM/EDX analysis of harvested biomass from Pb-spiked cultures, at all harvesting times (Figure 3.6). High-sensitivity EDX detectors allow rapid determination of the chemical compositions of points or areas in a sample, in the form of high-resolution elemental maps (166). This allows to determine the composition of observed biominerals. A clear change was observed between the 10 and 20 days harvests.

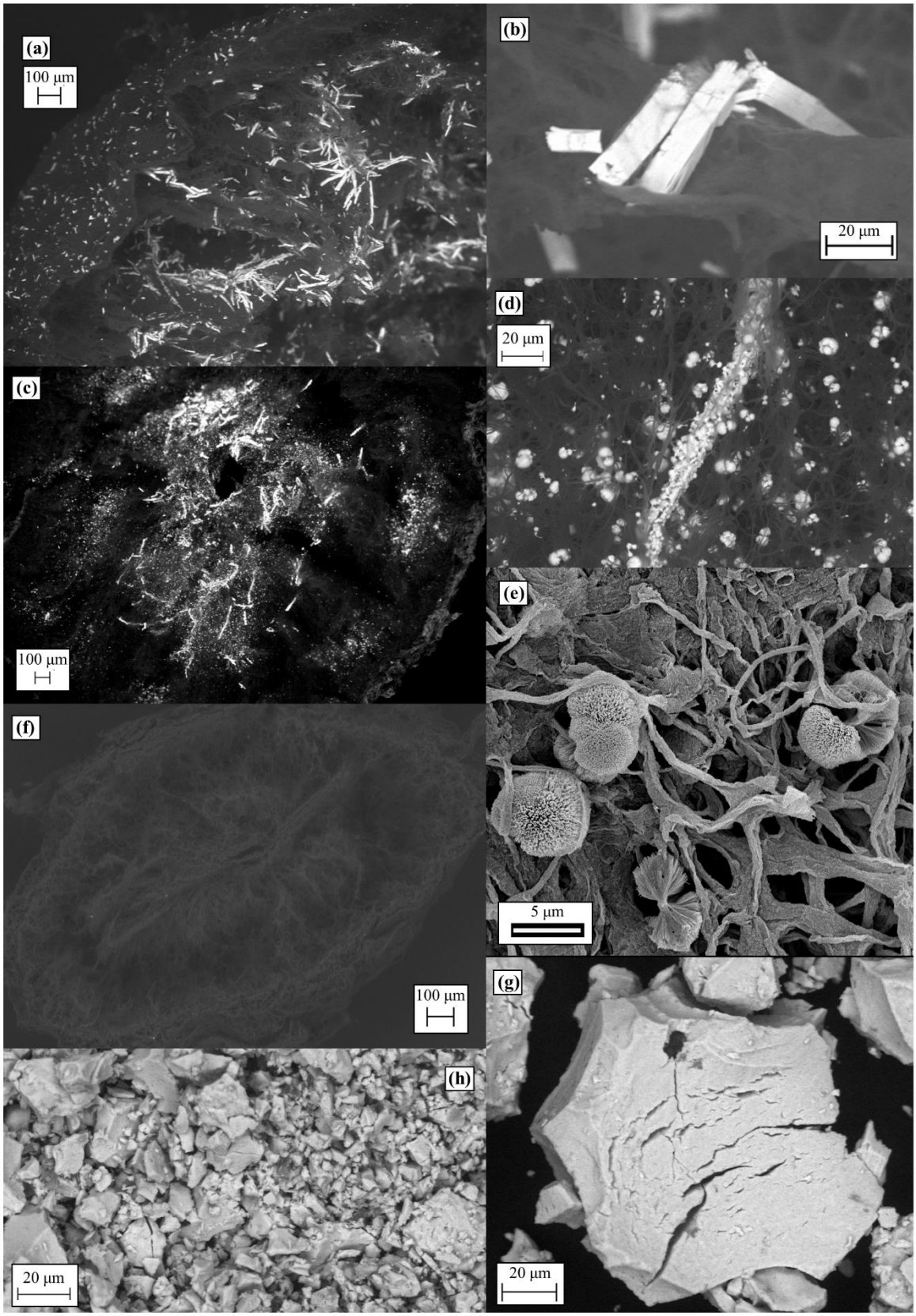



*Figure 3.6. Backscatter SEM micrographs: (a) section of a P.javanicus colony from the 10 days harvest, showing prismatic Pb biominerals (100x); (b) close up on biominerals present on the 10 days harvest (861x); (c) section of a P.javanicus colony from the 20 days harvest, showing globular and radial Pb biominerals (87x); (d) close up on globular/radial biominerals in pseudomorphism of pre-existing prismatic minerals, and disperse in the biomass (20 days harvest, 1400x); (e) close up on globular minerals with an internal radial acicular habit (20 days harvest, 3000x); (f) fungal colony harvested from a Pb-free control (10 days, 105x); (g) close up on amorphous precipitate from the abiotic control (10 days harvest, 1140x); (h) close up on amorphous precipitates from the abiotic control (10 days harvest, 1450x).*

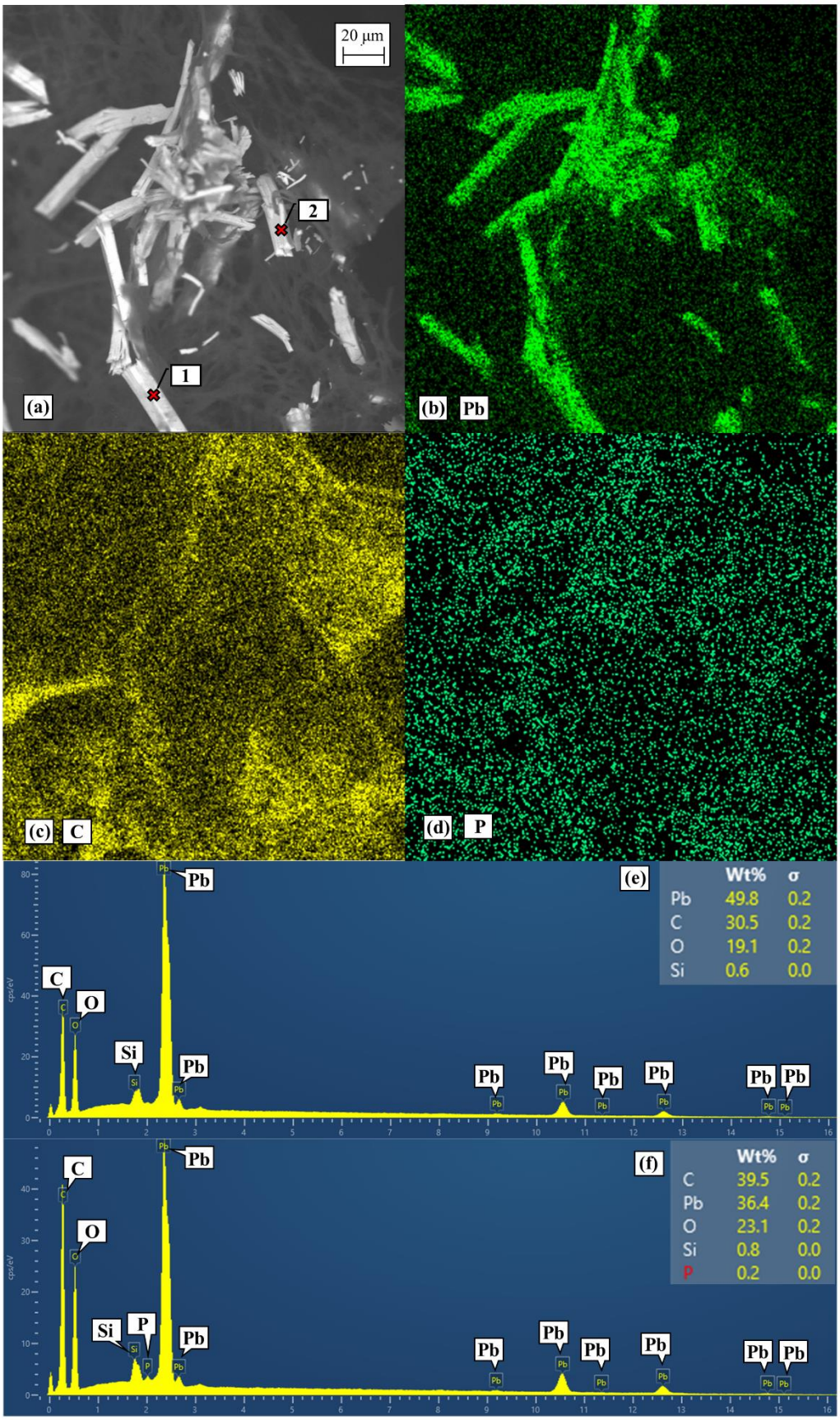



*Figure 3.7. Chemical composition of biominerals from the 10 days harvest: (a) backscatter SEM micrograph (923x) indicating EDX measurement points for spectrum 1 and 2 with a red x; (b) corresponding high-resolution elemental map for Pb; (c) corresponding high-resolution elemental map for C; (d) corresponding high-resolution elemental map for P; (e) EDX spectrum plot for point 1; (f) EDX spectrum plot for point 2.*

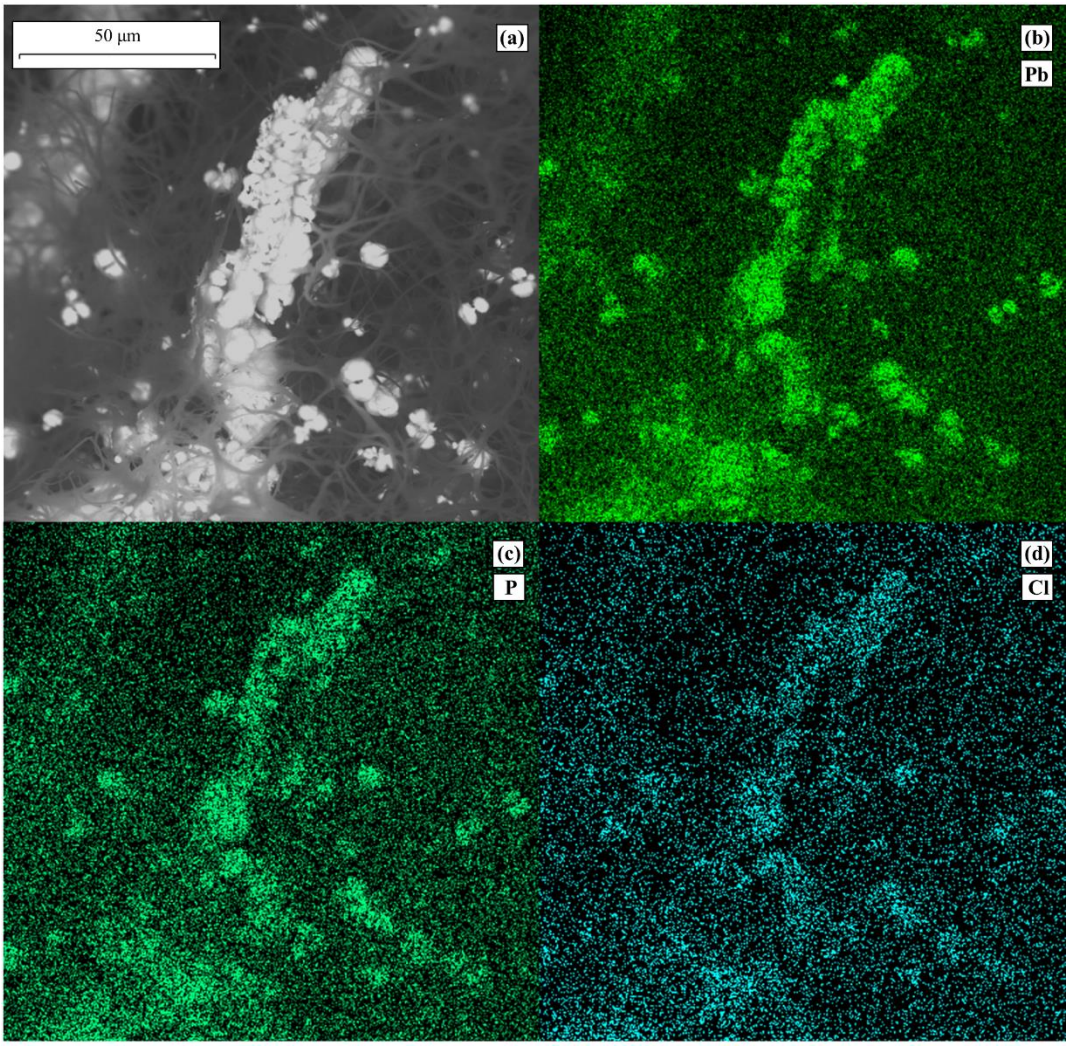



*Figure 3.8. Chemical composition of biominerals from the 30 days harvest: (a) backscatter SEM micrograph (1720x); (b) corresponding high-resolution elemental map for Pb; (c) corresponding high-resolution elemental map for P; (d) corresponding high-resolution elemental map for Cl.*

At 10 days prismatic crystals were visible (Figure 3.6-a and b), that with EDX analysis showed mainly peaks for Pb, carbon (C) and oxygen (O) (Figure 3.7). Carbon was expected in all cases, as the surrounding biomass is included in the radius of the EDX point analysis. P appears only marginally, with a very small peak (Figure 3.7-f), and the elemental map for P (Figure 3.7-d) does not clearly show the shape of the crystals, as does the map for Pb (Figure 3.7-b). In 20 and 30 days harvests, on the other hand, a globular mineral was observed both in pseudomorphism of the pre-existing prismatic minerals and disseminated amongst the fungal hyphae (Figure 3.6-c, d and e). EDX analysis indicated that Pb, C, O, and P were present as main elements (Figures 3.8 and 3.9). In the elemental map for P (Figures 3.8-c), the shape of the crystals is clearly visible, following the same pattern as Pb (Figures 3.8-b). Chlorine (Cl) appears in some spectrums (Figure 3.9-a), with a very small peak, and follows the shape of mineral crystals in elemental maps (Figures 3.8-d). Both the

composition and the globular habit of minerals present in biomass from 20 and 30 days harvests, are indicative of pyromorphite ($Pb_5[PO_4]_3X$; X= F, Cl, B or OH) (50, 167).

Abiotic controls showed no noticeable variation over time, and while also having a composition of Pb, P, C and O (Figure 3.10), no clear mineral/crystal geometry was observable, having the appearance of a fragile irregularly fractured solid (Figure 3.6-g and h). Controls with no Pb spike, as expected, had no visible biominerals (Figure 3.6-f). All obtained SEM images, EDX spectrums and elemental maps are included in Appendix CH3 S2.

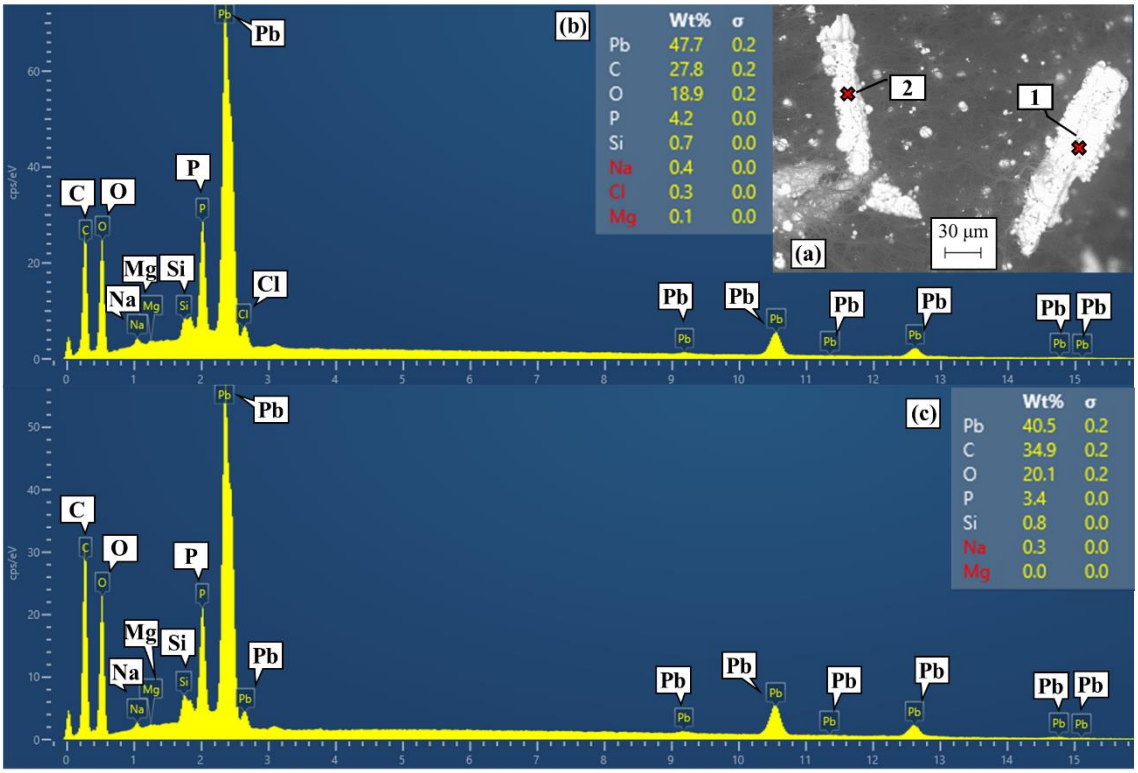



*Figure 3.9. Chemical composition of biominerals from the 30 days harvest: (a) backscatter SEM micrograph (822x) indicating EDX measurement points for spectrum 1 and 2 with a red x; (b) EDX spectrum plot for point 1; (c) EDX spectrum plot for point 2.*

X-ray powder diffraction (XRD) is an analytical technique that can be used to identify fine-grained minerals by their crystal structures and atomic spacing (168). A detector records and processes X-ray signals and converts the signal to a count rate which is then output to a device as a diffractogram (168). The latter is then compared to a library of standards available in the instrument software to identify the unknown minerals in the sample (168). Results from powder XRD analysis of fungal biomass samples were in agreement with those of the SEM/EDX analysis. Peak patterns of samples at the 10 days harvest were compatible with Pb oxalate ($PbC_2O_4$) (Figure 3.11-a); and at 20 and 30 days harvest with pyromorphite (Figure 3.11-b and c). Both abiotic (negative) controls and biotic controls grown without a Pb spike (positive) showed no peaks (Figure 3.12), indicating that the solid phase collected

from abiotic controls was indeed of an amorphous rather than crystalline nature. All obtained XRD patterns are shown in Appendix CH3 S3.

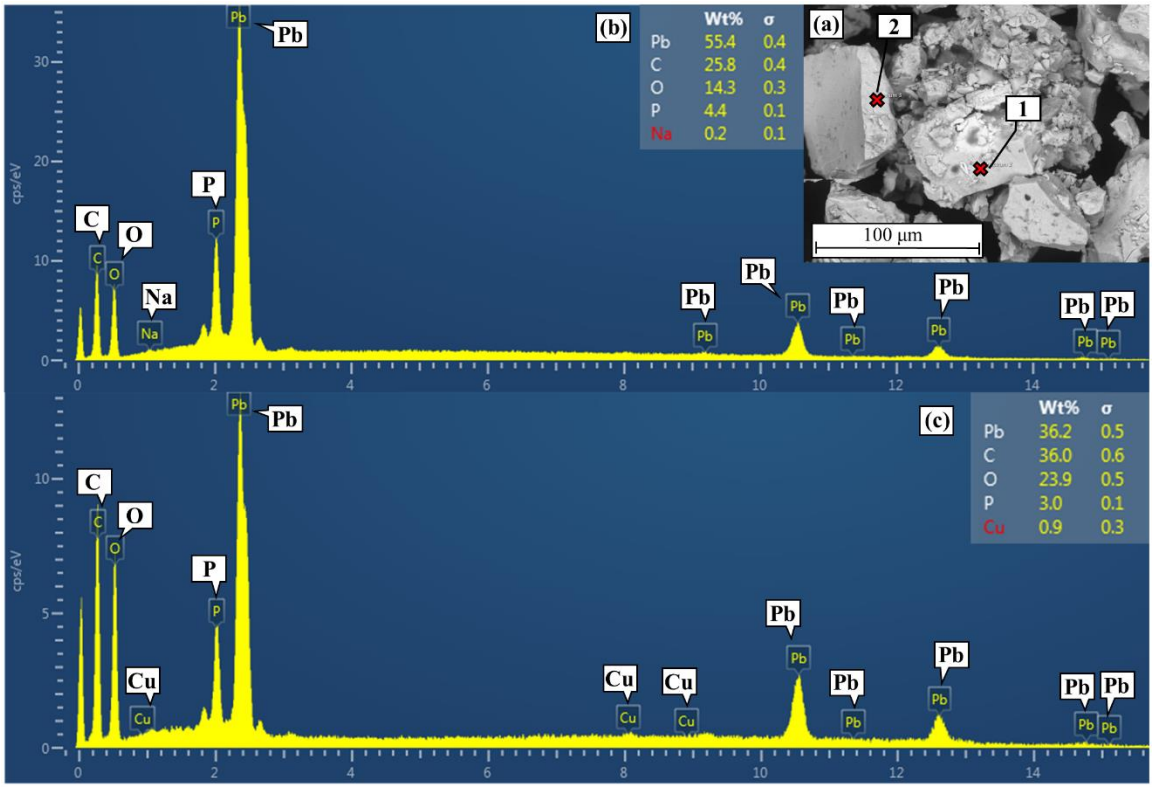



*Figure 3.10. Chemical composition of precipitated solids in the 10 days harvest abiotic control: (a) backscatter SEM micrograph (1210x) indicating EDX measurement points for spectrum 1 and 2 with a red x; (b) EDX spectrum plot for point 1; (c) EDX spectrum plot for point 2.*

These results are in agreement with what Rhee *et al.* (2012) (50) previously reported, with the biomineralization of pyromorphite by *P. javanicus* in the surface of lead shots after 30 days. In that case, other minerals (cerussite, hydrocerussite, minor litharge, and minium) were also present, both in *P.javanicus* inoculated shots and abiotic controls, which can be formed when metallic Pb is exposed to air and moisture. The researchers observed, however, that the abundance of these other minerals was reduced overtime, in favour of pyromorphite, due to the activity of the fungus.

The recrystallization of Pb oxalate to pyromorphite after 10 days is consistent with the pH variation overtime. As illustrated by Liang *et al* (2016) (42) in their Pb mineral stability diagrams (pH vs log oxalate concentration), at a determined oxalate concentration, Pb oxalate is stable at lower pH values than pyromorphite (pH 3.50 ± 0.06 at 10 days), which appears stable between 4.80-8.00, even at high oxalate concentration (pH 8.14 ± 0.09 and 8.52 ± 0.09 at 20 and 30 days respectively). The reported upper pH limit is slightly below those observed in our 20 and 30 day harvest, where pyromorphite remained stable. Mc Bride

*et al* (2019) (169) corroborated this in a laboratory study where they compare Pb-phosphate, Pb-oxalate and Pb-oxalate-phosphate aqueous systems, showing that in the latter at pH 5.00 and lower, Pb oxalate precipitation controlled Pb solubility in the system, while at pH 7.00 and above Pb phosphates did.

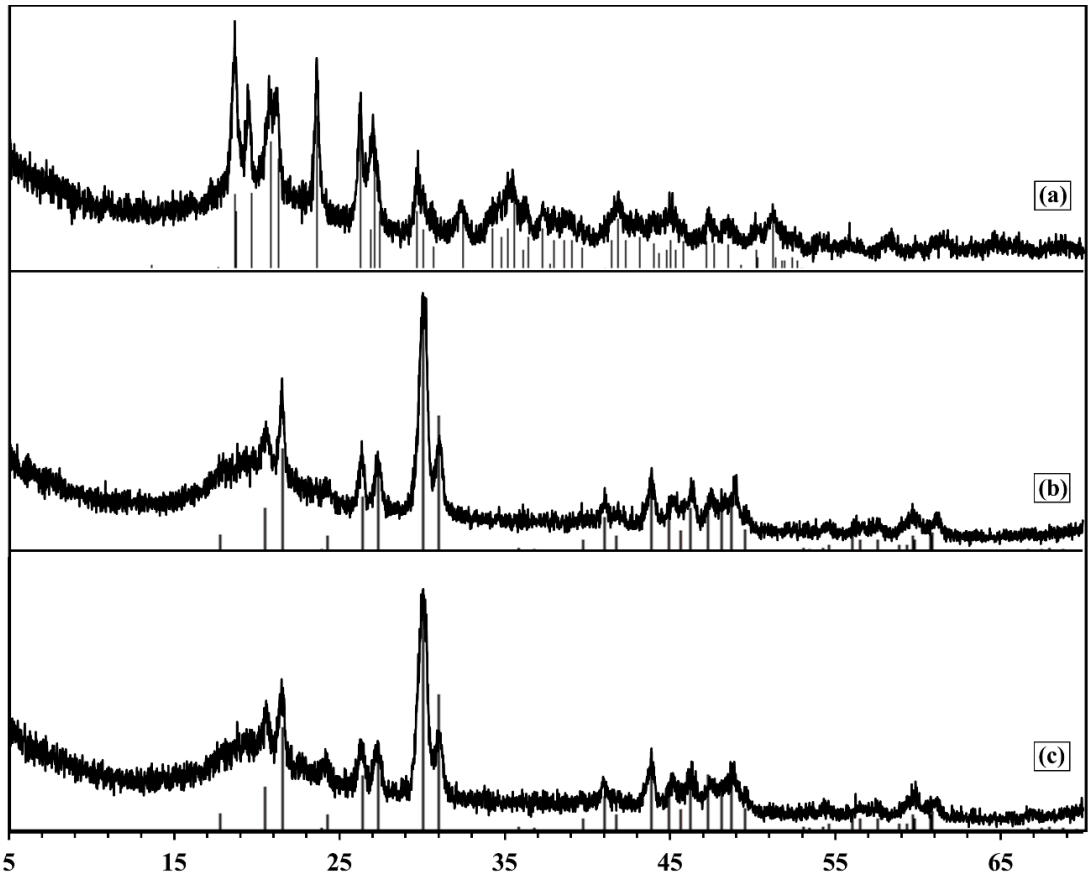



*Figure 3.11. pXRD patterns of harvested P. javanicus biomass from Pb-spiked cultures at: (a)10 days; (b) 20 days; (c) 30 days. X axis corresponds to 2θ. Vertical grey lines under sample patterns correspond to the catalogue patterns for Pb-oxalate (a) and pyromorphite (b, c).*

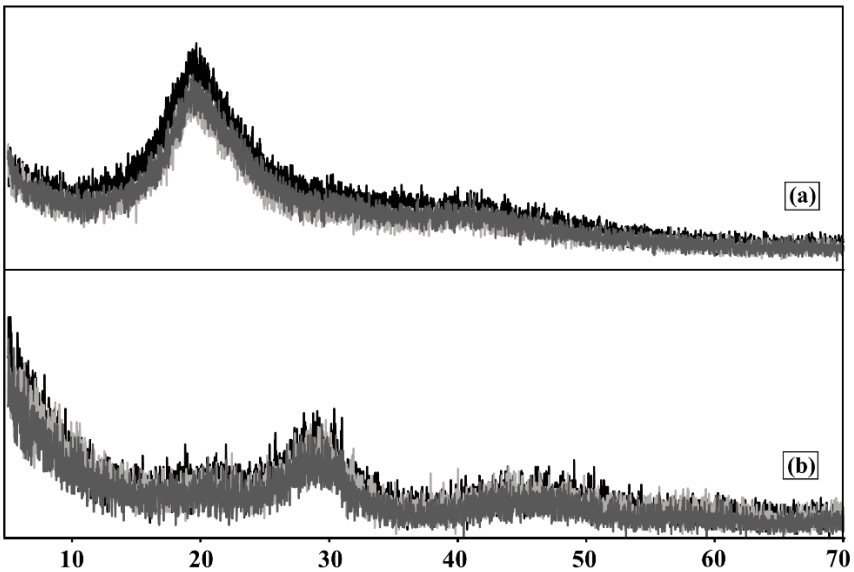



*Figure 3.12. pXRD patterns of: (a) harvested P. javanicus biomass from controls; (b) solid phase collected in abiotic controls. Patterns for 10 (black), 20 (light grey) and 30 (grey) days are superimposed. X axis corresponds to 2θ.*

Changes in the metabolism of *P.javanicus* such as production of organic acids and other exudates overtime, may have produced the change in pH and consequent recrystallization of Pb-oxalate as pyromorphite.

The increase of Pb in solution in the supernatant after 10 days, in our experiment, is thus likely an effect of the change in mineralogy. Although pyromorphite has a higher wt% of Pb (76.4%) than Pb-oxalate (70.2 %), the change in the equilibrium may have resulted in the increased dissolved Pb, possibly due to a limitation in the halogen availability ($Pb_5[PO_4]_3X$; X= F, Cl, B or OH).

The biomineralization of both Pb oxalate and pyromorphite by *P. javanicus*, also reported by Liang *et al* (2016) (169) in their study, was explained by two mechanisms: (a) Pb oxalate formation dependent on oxalate excretion and (b) organic phosphate (G2P, also used in this study) hydrolysis mediated by phosphatase, making the phosphate available for pyromorphite formation. This would explain the presence of only an amorphous solid in abiotic controls, as no oxalic acid was produced and hence no Pb oxalate could be formed, as well as no phosphatase was present to make the phosphate available for the formation of crystalline Pb phosphate minerals.

A reversed transformation of pyromorphite to Pb-oxalate has previously been reported by Fomina et al (2004), related to fungal activity (67). Solubilization of 21% pyromorphite by the entomopathogenic fungus *Beauveria caledonica* via oxalic acid production was measured (67). It was concluded that the excretion of large amounts of oxalic acid by *B. caledonica* likely contributed to Pb tolerance, and reduced pH conditions in the media as a result (67).

Furthermore, Tian *et al* (2019) (170) in their study of mechanisms of Pb tolerance in the fungal species *Aspergillus niger* and *Penicillium oxalicum,* identified the production of oxalic acid as advantageous, and the "outer defence line", or extracellular mechanism, of reducing $Pb^{2+}$ toxicity, by precipitation of Pb oxalate in a liquid medium.

Pyromorphite is considered the most stable Pb mineral in soils and the human gastrointestinal system (42, 45, 48, 56, 57, 63). However, concern that naturally occurring, biologically produced oxalate present in soil systems containing Pb and phosphate, may increase Pb solubility at mildly acidic to acidic pHs, has been raised in the literature (42, 50, 169). Observed results show that this could be overcome with fungal activity. In this culture experiment, the production of oxalate initially dominated the speciation of Pb, nevertheless allowing its immobilization and removal from solution (initial "outer defence line").

Overtime *P.javanicus* created the appropriate pH conditions in the media/environment for pyromorphite to become stable, even in the presence of oxalate.

### 3.4.4 Phenanthrene (Phen) sorption by *P. javanicus*

Two experiments with Phen-spiked liquid cultures inoculated with *P.javanicus* were carried out to study processes of biosorption and biodegradation. The first experiment was carried out with harvesting times at intervals of 10 days over 30 days, while a second experiment with harvesting times at 30, 50 and 70 days was performed to extend the timescale of the interaction between *P.javanicus* and the spiked Phen.

Considering the two Phen-spiked culture experiments, fungal growth in Phen spiked media was continuous until the 50 days harvest (t-Test p-values of biomass dry weight between harvesting times of: 10 days-20 days = 0.001; 20 days-30 days = 2.77E-4; 30 days-50 days = 1.74E-4, all $< \alpha = 0.05$), after which it plateaued (Table 3.4 and Figure 3.13-b) (t-Test p-values of biomass dry weight between 50 and 70 days harvests of $0.069 > \alpha = 0.05$). Though initially (first two harvests) the unspiked control seemed to have a faster growth, from 30 days onwards there was no notable difference with the spiked replicates. The significant cease in growth at the end of the experiment may have been due to the exhaustion of the main carbon source (glucose, at an initial 29 g $l^{-1}$) in the media.

*Table 3.4. Summary with average fungal biomass dry weights.*

| Harvest | Culture type | Average weight (g) | error (g) |
|---|---|---|---|
| **10 days** | *P.javanicus*-Phen | 0.201 | *0.078* |
| | Control (no Phen) | 0.336 | |
| **20 days** | *P.javanicus*-Phen | 0.578 | *0.115* |
| | Control (no Phen) | 0.908 | |
| **30 days (1)** | *P.javanicus*-Phen | 1.041 | *0.092* |
| | Control (no Phen) | 1.101 | |
| **30 days (2)** | *P.javanicus*-Phen | 0.985 | *0.420* |
| | Control (no Phen) | 0.916 | |
| **50 days** | *P.javanicus*-Phen | 1.469 | *0.102* |
| | Control (no Phen) | 1.493 | |
| **70 days** | *P.javanicus*-Phen | 1.374 | *0.090* |
| | Control (no Phen) | 1.480 | |

**error calculated for n = 3*

pH in spiked cultures (Table 3.5 and Figure 3.13-a) gradually reduced over the first experiment, from the initial 8.82, reaching pH 4.2 ± 0.2 at 30 days, and then remaining relatively stable at 4.43 ± 0.03 at 50 days. Unspiked controls had a similar trajectory, with an initial reduction and then staying relatively constant between pH 4 and 5 after 20 days and until 70 days. Abiotic control pH on the other hand, remained close to 8 throughout both

experiments. At 70 days spiked cultures had a marked rise to pH 8.02 ± 0.50, a value similar to the abiotic control, that was not seen in the unspiked control culture.

*Table 3.5. Summary with average pH in harvested supernatant for the two culture experiments.*

| Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|
| **Harvest** | **Culture type** | **Average pH** | **error (pH)** | **Harvest** | **Culture type** | **Average pH** | **error (pH)** |
| **Time 0** | *P.javanicus*-Phen | 8.820 | | **Time 0** | *P.javanicus*-Phen | 8.330 | |
| | Control (no Phen) | 8.700 | | | Control (no Phen) | 8.010 | |
| | Control (abiotic) | 8.820 | | | Control (abiotic) | 8.330 | |
| **10 days** | *P.javanicus*-Phen | 7.581 | *0.103* | **30 days** | *P.javanicus*-Phen | 4.760 | *0.061* |
| | Control (no Phen) | 6.287 | *0.084* | | Control (no Phen) | 4.223 | *0.062* |
| | Control (abiotic) | 7.893 | *0.062* | | Control (abiotic) | 7.847 | *0.042* |
| **20 days** | *P.javanicus*-Phen | 5.430 | *0.746* | **50 days** | *P.javanicus*-Phen | 4.431 | *0.038* |
| | Control (no Phen) | 4.043 | *0.132* | | Control (no Phen) | 4.403 | *0.124* |
| | Control (abiotic) | 7.903 | *0.280* | | Control (abiotic) | 7.820 | *0.264* |
| **30 days** | *P.javanicus*-Phen | 4.216 | *0.210* | **70 days** | *P.javanicus*-Phen | 8.020 | *0.523* |
| | Control (no Phen) | 4.673 | *0.051* | | Control (no Phen) | 4.750 | *0.286* |
| | Control (abiotic) | 8.050 | *0.088* | | Control (abiotic) | 7.733 | *0.023* |

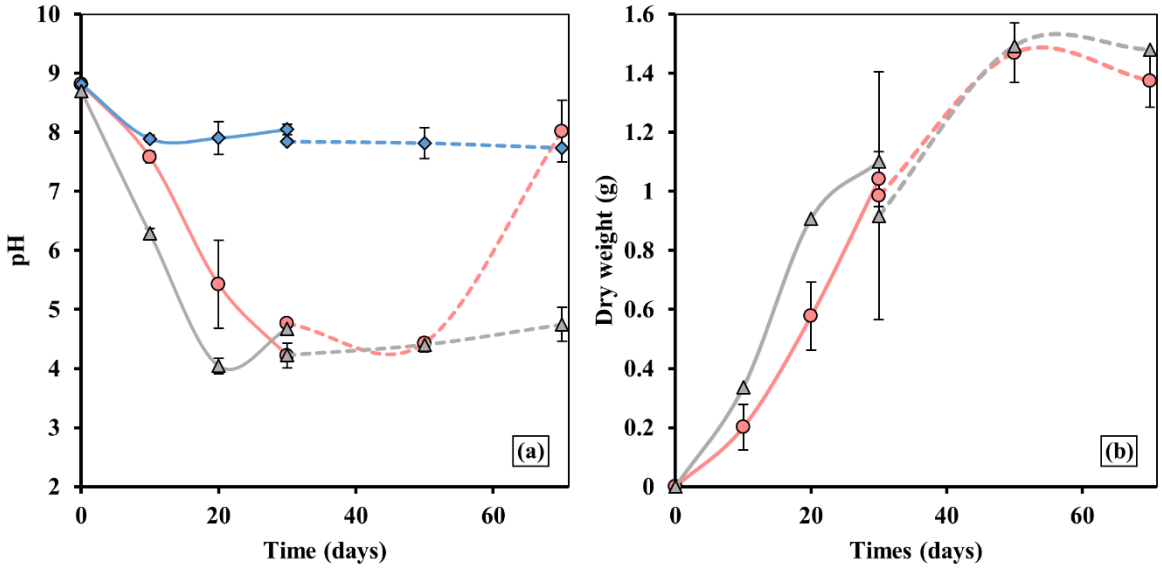



*Figure 3.13. Phen experiment graphs: (a) pH variation of culture media over time for Phen biodegradation experiment; (b) dry weight of harvested P. javanicus biomass from the Phen biodegradation experiment, over time. In both graphs continuous lines correspond to experiment 1 (10, 20 and 30 day harvests) and segmented lines to experiment 2 (30, 50 and 70 day harvests). Pink circles represent the average value in Phen-spiked P. javanicus cultures, grey triangles P. javanicus controls without Phen, and only in (a) blue diamonds the abiotic controls. Error bars in thin black lines, may not be visible when error is small in relation to the Y axis scale.*

In spiked cultures with *P. javanicus*, Phen was found in very low concentrations (16.7 ± 0.8 µg $l^{-1}$) in the supernatant only at 10 days, which translated to a ~98% reduction from the initial concentration (Table 3.6). From then on, in all harvests of both experiments, Phen was

not detectable in the supernatant (Figure 3.14, in total Phen µg instead of concentration), with no Phen-peak appearing in chromatograms. In the abiotic controls, although present in solution during both experiments, concentrations of Phen were reduced approximately by half after 10 and 20 days (502.7 ± 5.7 µg $l^{-1}$ and 489.4 ± 2.2 µg $l^{-1}$ respectively), followed by a more abrupt reduction at 30 days, to 232.5 ± 3.2 µg $l^{-1}$ in the first experiment and 180.4 ± 0.9 µg$l^{-1}$ in the second experiment, showing consistency between the first and second experiment at 30 days. Concentrations then continued to reduce more gradually to a final concentration of 96.4 ± 1.8 µg$l^{-1}$ at 70 days (Table 3.6 and Figure 3.14, in total Phen µg instead of concentration). Raw data and full data set are included in Appendix CH3 S4.

*Table 3.6. Summary with average Phen concentrations in harvested supernatant.*

| **Harvest** | **Culture type** | **Average Phen (µg $l^{-1}$)** | **error (µg $l^{-1}$)** |
|---|---|---|---|
| **Time 0 (1)** | Both | 913.8 | *82.97* |
| **Time 0 (2)** | Both | 1057 | *6.529* |
| **10 days** | *P.javanicus*-Phen | 16.74 | *0.801* |
| | Control (abiotic) | 502.8 | *18.43* |
| **20 days** | *P.javanicus*-Phen | - | |
| | Control (abiotic) | 489.4 | *7.117* |
| **30 days (1)** | *P.javanicus*-Phen | - | |
| | Control (abiotic) | 232.5 | *10.47* |
| **30 days (2)** | *P.javanicus*-Phen | - | |
| | Control (abiotic) | 180.4 | *2.970* |
| **50 days** | *P.javanicus*-Phen | - | |
| | Control (abiotic) | 113.0 | *8.927* |
| **70 days** | *P.javanicus*-Phen | - | |
| | Control (abiotic) | 96.41 | *3.640* |

Recoveries of the extraction and analysis of fungal biomass varied from 52.7 % to 103.9% (raw data and full data set in Appendix CH3 S5). Sorbed Phen (Table 3.7 and Figure 3.14), in absolute quantities (corrected with the recovery), reached an average of 68.7 ± 9.5 µg at 10 days and then had an apparent slight reduction of up to 50.2 ± 1.3 µg at 30 days (second experiment). It should be noted that both experiments again are consistent at 30 days, with 51.1 ± 3.6 µg in experiment 1 and 50.3 ± 1.3 µg in experiment 2.

The 70 days harvest had an overall average of 54.88 ± 36.99 µg, in line with the previous harvests. However, when looking at the three cultures separately (Table 3.7), one of the replicate cultures showed particularly low contents of Phen (average 6.26 µg), while the other two average 79.19 ± 15.10 µg (pink circle with a segmented red outline in Figure 3.14), higher than previous harvests. Surrogate recoveries are similar in the 3 cultures, with an

average 53.1% (SD of ± 0.37) in the low Phen replicate, and 57.1% (SD of ± 0.16) and 57.3% (SD of ± 0.74) in the remaining replicates, indicating that the Phen content difference would appear to be a sample issue, rather than an extraction/analysis issue.

*Table 3.7. Summary with average Phen content in harvested biomass.*

| | | Phen (µg) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **10 days** | **20 days** | **30 days 1** | **30 days 2** | **50 days** | **70 days** | **70 days'** |
| Replicate cultures | Average | 62.49 | 65.55 | 50.39 | 50.74 | 55.66 | 69.71 | 69.71 |
| | SD | *1.438* | *0.590* | *0.160* | *0.768* | *0.619* | *0.479* | *0.479* |
| | Average | 67.66 | 63.76 | 53.54 | 50.78 | 40.00 | 6.258* | |
| | SD | *0.082* | *0.987* | *0.552* | *0.372* | *0.322* | *0.004* | |
| | Average | 83.24 | 48.17 | 49.51 | 49.03 | 57.58 | 88.66 | 88.66 |
| | SD | - | *0.538* | *5.053* | *0.594* | *0.969* | *0.551* | *0.551* |
| Total | Average | 68.71 | 59.16 | 51.15 | 50.18 | 51.08 | 54.88 | 79.19 |
| | Error | *9.535* | *8.230* | *3.577* | *1.061* | *8.298* | *36.99* | *13.17* |

** Average for each replicate cultures calculated from two instrumental replicates (for SD, n=2). 70 days' column repeats 70 days harvest, excluding replicate * with lower Phen µg.*

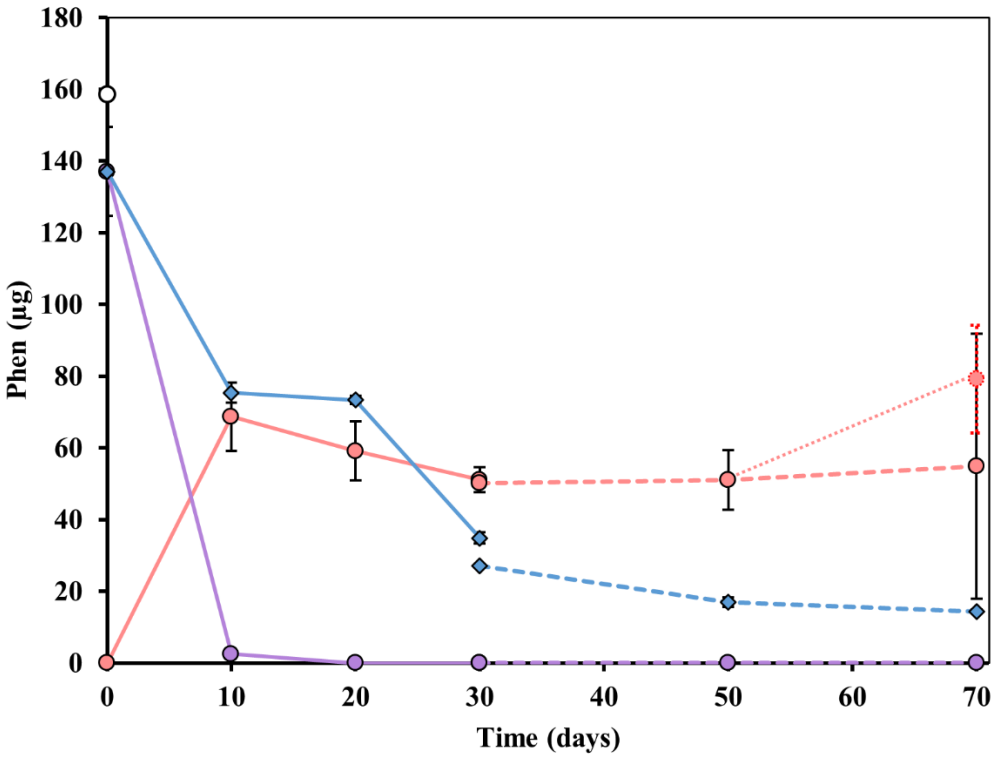



*Figure 3.14. Total Phen (µg) over time: adsorbed Phen in P. javanicus biomass (Phen concentration [µg $g^{-1}$]* biomass dry weight [g]) in pink circles. Total Phen in solution in the media (Phen concentration [µg $l^{-1}$]* total volume of media per culture [l]) of P. javanicus cultures in purple circles and abiotic control cultures in blue diamonds. Continuous lines correspond to experiment 1 (10, 20 and 30 day harvests) and segmented lines to experiment 2 (30, 50 and 70 day harvests). Error bars in thin black lines, may not be visible when error is small in relation to the Y axis scale. The white circle represents the initial Phen in solution for experiment 2. The second pink circle with a dashed red outline, and segmented red error bar at 70 days, corresponds to average Phen (µg) in fungal biomass when excluding 70 days 2 replicates, with unusually low Phen content. The circle is connected to the 50 days harvest with a thinner segmented pink line.*

This apparent difference could be explained by sample heterogeneity. Approximately 500 mg of biomass from a total 1420 mg of the low-Phen 70-day harvest replicate was extracted. As Phen was removed from the solution during the first 20 days, the fungus continued to grow after 20 days, therefore the earlier biomass is likely to have retained most of the sorbed Phen, while the later (>20-days) produced biomass (when no Phen was left in solution) of each colony would have had less sorbed Phen. In the earlier harvests, all or most of the biomass was extracted, as the total biomass weight was lower, so the likelihood of encountering heterogeneity issues was reduced. Although samples are homogenized during the preparation process, heterogeneities may have remained in larger samples, causing the unusually low Phen concentrations in one of the 70 days replicates, and high concentrations in the other two replicates.

Table 3.8 shows the p-values resulting from t-Tests and Anova analysis comparing sorbed Phen at the different harvesting time. With all 70-days replicates included, sorbed Phen in the latter is not significantly different (p value > $\alpha = 0.05$) to any of the previous harvests according to the t-Test. The null hypothesis in the Anova analysis is that the mean sorbed Phen (µg) of all harvesting times are equal. If all three culture replicates are included in the 70 days harvest, the p-value resulting from Anova analysis is > $\alpha = 0.05$ and the null hypothesis is accepted, in other words the mean sorbed Phen (µg) is equal at all harvesting times. If the low-Phen replicate is removed, however, the Anova p-value becomes < $\alpha = 0.05$, and hence the null hypothesis is rejected: the mean sorbed Phen (µg) of all harvesting times are not all equal. With the t-test, in the same case, sorbed Phen at 70 days becomes significantly (p value > $\alpha = 0.05$) higher than sorbed Phen at 20, 30 and 50 days harvests. The initial and final (10 and 70 days) sorbed Phen, nonetheless, shows no significant differences.

*Table 3.8. Summary with p-values for sorbed Phen at different harvesting times.*

| **t-Test: Two-Sample Assuming Unequal Variances and Anova p-values ($\alpha = 0.05$)** | | | | | | |
|---|---|---|---|---|---|---|
| **Harvest** | **10 days** | **20 days** | **30 days 1** | **30 days 2** | **50 days** | **70 days** |
| **20 days** | 0.0995 | | | | | |
| **30 days 1** | 0.0081 | 0.0743 | | | | |
| **30 days 2** | 0.0087 | 0.0519 | 0.5668 | | | |
| **50 days** | 0.0082 | 0.1357 | 0.9868 | 0.8113 | | |
| **70 days** | 0.4265 | 0.8013 | 0.8231 | 0.7779 | 0.8220 | |
| **70 days *** | 0.1685 | 0.0275 | 0.0160 | 0.0133 | 0.0076 | |
| **All** | 0.4834 | | | | | |
| **All*** | 3.28E-06 | | | | | |

**t-Test p-values in rows 3 to 8. Anova p-values are in the two bottom "All" rows. 70 days* and All* are with 70 days 2 replicates excluded. P-values < $\alpha = 0.05$, and hence considered significant, are highlighted in grey.*

For the rest of the harvests, when considering the results of the t-test, 30 and 50 days appear to be significantly lower than the 10 day harvest, yet all p-values between 20, 30 (from experiment 1 and 2) and 50 days indicate no significant differences. Hence, it is likely that Phen present in the fungal biomass remained constant over time, with no degradation of sorbed Phen occurring.

When compared to Phen present in solution in the abiotic control, at 10 days sorbed Phen (68.7 ± 9.5 µg) was not dissimilar to the 75.4 ± 2.7 µg in solution in the control (Figure 3.14). The remaining unaccounted Phen (relative to the initial 137.1 ± 12.4 µg) that was no longer present in solution (both in the abiotic control and *P.javanicus* cultures) or in the biomass, could be attributed to removal due to partial volatilization over time.

Each culture flask remained sealed and unopened over the course of the experiment, until (sacrificial) harvesting at each time point. While some loss may occur when opening a flask at the harvesting time, it would be expected that during incubation, after a certain amount of time in a closed system, an equilibrium would be reached between Phen in solution and in the headspace. However continuous volatilization seems to have occurred in the abiotic control instead, as indicated by the reduction of Phen concentration in solution.

After an initial removal (or volatilization) rate in the abiotic control media of ~ 6.17 µg Phen $day^{-1}$ (calculated by the difference in the total µg Phen at time 0 and 10 days, divided by the amount of days = 10), a near equilibrium seemed to be achieved between days 10 and 20 where the removal (or volatilization) rate was reduced to ~ 0.20 µg Phen $day^{-1}$. This rate however, increased again between days 30 and 50, with ~ 3.8 µg Phen $day^{-1}$, slowing down thereafter to ~ 0.51 µg Phen $day^{-1}$ between 30 and 50 days and a minimum reached of 0.12 µg Phen $day^{-1}$ between 50 days and the last harvest (70 days), indicating that the quantity of Phen in solution in the control may have been stabilising towards the end of the experiment. Photo-oxidation by reaction with oxidizing species present in the gaseous phase (e.g. NOx) may also contribute to reducing Phen concentrations (100), however cultures were cultivated in the dark and only briefly exposed to light when the incubator was opened to remove flasks for harvesting, and later during the extraction procedure.

Phen-metabolite identification in GC-MS chromatograms was planned to determine the fate of unaccounted Phen, however, prevailing circumstances (i.e. Covid-19 lockdown) made difficult the access to technical equipment and assistance, required to accomplish the analysis within the time frame of the project.

In spiked *P.javanicus* cultures, as opposed to the control, further volatilization seems to have been impeded after ~10 days, as all available Phen (µg) was sorbed by the biomass.

Biosorption of Phen and other hydrophobic compounds to biomass can occur by partitioning onto hydrophobic surfaces or due to chemical reactions (138, 140). If biomass is inactivated, sorption is more likely to be limited to the first mechanism (138-141), but with a live organism with active metabolism, chemical reactions can gain importance (138). An advantage of live biomass versus inactivated biomass, is that as it continues to grow, binding sites continue to be created, allowing increased removal of the compound (139).

Though Phen degradation by *P. javanicus* was not demonstrated here, it can be noted that in previous studies with live fungi, it has been observed that changes in nutrient conditions, in particular when nitrogen is limited, produces a shift from biosorption to biodegradation (138, 139). Degradation of biosorbed Phen by cultured fungi overtime has also been observed (139).

Ding *et al* (2013) (139) studied biosorption and biodegradation of phenanthrene and pyrene by *Phanerochaete chrysosporium*, a white-rot fungus. In 15 ml of aqueous solution with 1.0 mg $l^{-1}$ Phen and 0.1 mg $l^{-1}$ pyrene, 74.44 % of Phen and 86.24 % pyrene was removed from solution at the incubation time of 40 days (139). After 3 days of incubation, the biosorption percentages by live *P. chrysosporium* were 19.71 % and 52.21 % for Phen and pyrene respectively (139). With the increase of the incubation time to 40 days, biodegradation percentages increased from 20.40 % to 60.62 % for Phen and from 15.55 % to 49.21 % for pyrene, and the stored-PAHs in the fungal bodies decreased by 5.89 % for Phen and by 15.18 % for pyrene, which suggested that PAHs both in solution and the fungal body could be biodegraded (139). A modified Martin broth (0.5 g $l^{-1}$ $MgSO_4 \cdot 7H_2O$, 1 g $l^{-1}$ $KH_2PO_4$, 5 g $l^{-1}$ peptone) was used to test the effect of the nutritional content on biosorption and biodegradation (139). Glucose was used as the carbon source, with concentrations of 5.6 mmol $l^{-1}$ for carbon-limited groups and 56 mmol $l^{-1}$ for carbon-rich groups (139). Sodium nitrate was used as the nitrogen source, with 2.4 mmol $l^{-1}$ for nitrogen-limited groups and 24 mmol $l^{-1}$ for nitrogen-rich groups (139). 20 mL of aqueous solution with the concentrations of 1.0 mg $l^{-1}$ Phen and 0.1 mg $l^{-1}$ pyrene was added into 40-mL vials. Under the carbon-rich and nitrogen-limit nutrient conditions, the removal efficiency and biodegradation of phenanthrene and pyrene were significantly promoted, i.e. 99.55% and 92.77% for phenanthrene, and 99.47% and 83.97% for pyrene after 60 days of incubation (139). This phenomenon is ascribed to enhanced-biosorption due to the increase of fungal biomass under carbon-rich condition, and to stimulated-biodegradation under nitrogen-limit condition (139).

The marked change of pH at 70 days in our study (Figure 3.13), is showing that metabolism is changing, and that stresses on the fungi such as consumption of the main carbon source could eventually trigger Phen degradation.

### 3.4.5 Correlation between experiments and potential for remediation

The experiments show that *P.javanicus* not only tolerates but can thrive in media contaminated both with Pb (800 mg $l^{-1}$) and Phen (1000 µg $l^{-1}$). Differences in pH over time in the Pb (pH of 3.50 ± 0.06, 8.14 ± 0.09 and 8.52 ± 0.09 at 10, 20 and 30 days respectively) and Phen (pH of 7.58 ± 0.10, 5.43 ± 0.75, 4.22 ± 0.21, 4.76 ± 0.06, 4.43 ± 0.04 and 8.02 ± 0.52 at 10, 20, 30a, 30b, 50 and 70 days respectively) experiments were likely due to the use of different culture media, as the controls without contaminants behaved similarly to the spiked cultures in their corresponding experiment (for the majority of the experiment), but differently when compared to each other. The different media composition in turn may have caused different rates and types of acid production by *P.javanicus* in the different experiments. The choice to use different media was made for technical reasons, as the MCD media when spiked with Pb at the chosen concentration, caused instant precipitation, which prevented a known and homogenous Pb concentration to be maintained amongst the culture replicates. However, for the Phen experiment, MCD media yielded better recoveries than the Potato dextrose with the liquid-liquid extraction, and also allowed more control over the initial carbon content in the cultures, as it was added separately in the form of glucose. pH in the Phen experiment tended to be more acidic in fungal cultures over the majority of harvest times, which according to what was observed in Pb-experiment (section 3.4.3) would favour the formation of Pb-oxalate if Pb was present. However, the rise in pH observed in the 70 days harvest (Phen experiment) would mean that conditions favourable to pyromorphite, though delayed, would eventually be achieved, permitting the biomineralization of pyromorphite by *P.javanicus* .

With regard to Pb, the expected outcome of reducing Pb in solution via biomineralizaion of pyromorphite by *P.javanicus,* was attained. Up to ~94% of the Pb was removed from solution in the culture media, and biomineralized after 20 days, mainly as pyromorphite (Figure 3.15). This would indicate that *P.javaincus* has potential for the remediation of Pb contamination, through the reduction of its bioavailability in soil.

Phen biodegradation by *P. javanicus* was not conclusively observed, however the capacity of the latter to remove Phen from solution via biosorption (Figure 3.15), could still be advantageous. Studies have shown that PAHs can be removed from aqueous media and immobilized in soil through sorption onto different types of materials like activated carbon,

biochar and cellulose, amongst others (171). The log *K*d (where *K*d is the partition coefficient between two phases), of Phen in a soil-water system has been calculated as 1.48-2.30, whereas a range of 2.98-4.76 is observed for a variety of biomass and organic matter of different origin (138, 140), indicating that biosorption can play an important additive role in the fate of Phen in soil-water systems with added biomass (138, 140). As other PAHs such as pyrene, fluoranthene and benzo(a)pyrene have higher affinity to carbon phases than Phen (water-soot log *K*d of 6.90, 6.89, 8.21 respectively, with 6.30 for Phen (172)), it is expected that biosorption would also play an important role for these compounds.

Although biosorption, in the context of soil, may not reduce human bioavailability, it can potentially reduce Phen environmental availability to groundwater, plants and other soil organisms, whilst avoiding the formation of undesirable by-products (171). However, the long-term fate of biosorbed Phen would need to be studied, as fungal biomass decomposes. While degradation of Phen was not shown in the time-frame of the experiment, the Phen sorption capacity of *P.javanicus* could be further exploited for co-remediation of Pb and PAHs by combining the fungus with a solid material as a complementary amendment.

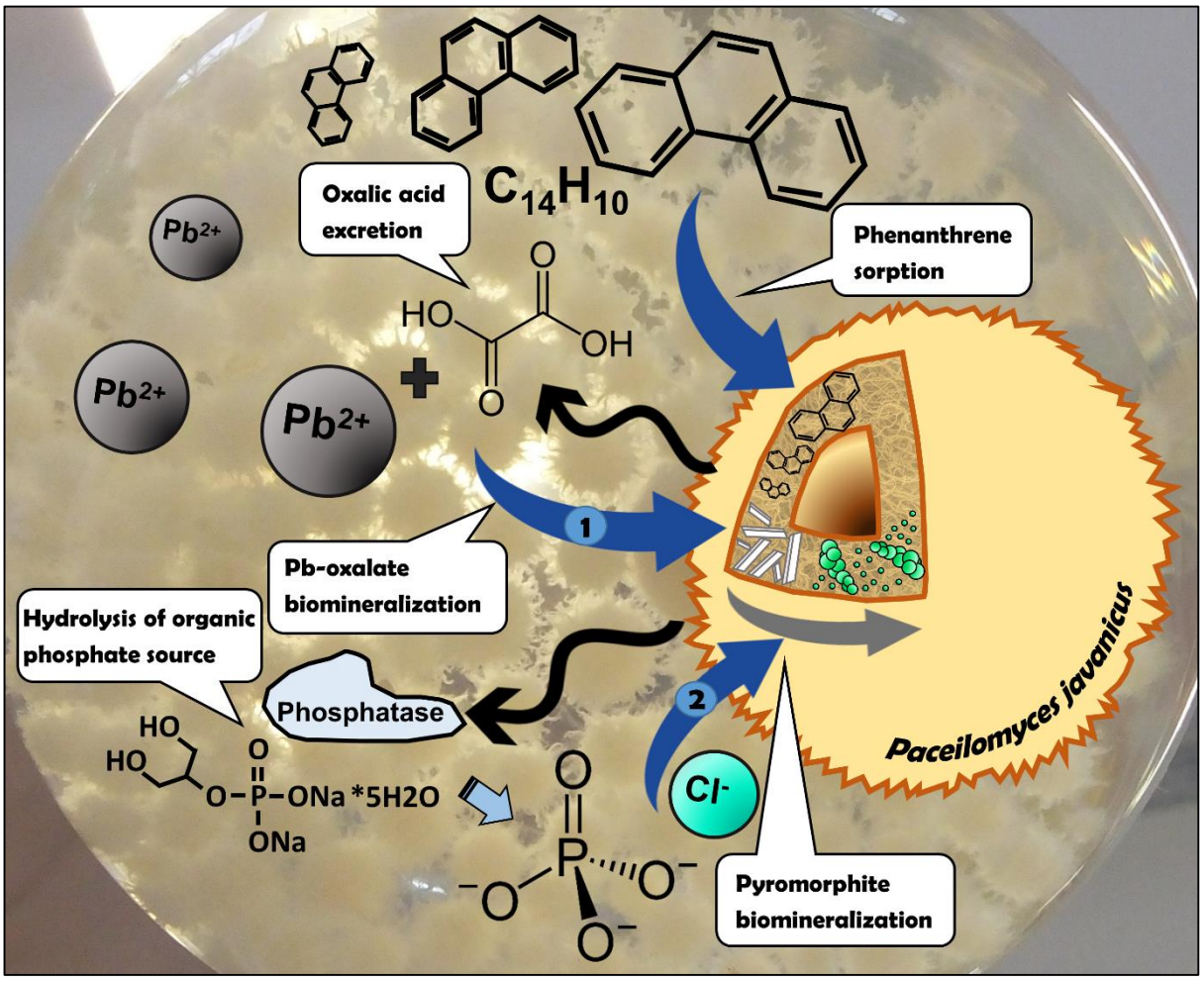



*Figure 3.15. Schematic summary of interactions of P.javanicus with Pb and Phen in the culture media.*

Furthermore, remediation applications other than *in situ* soil amendment should be investigated as the capacity of *P.javanicus* to remove Pb and Phen from solution, has shown promise for remediation in aqueous media. Contaminant sorption has been used for remediation in aqueous media such as waste water and soil washing effluent (171). Studies have shown that sorption to biomass of different origin, such as dead and live fungal biomass (138, 139), yeast, bacteria and microalgae (141, 173) can be used as treatment to remove PAHs from solution. Zhang *et al* (2018) (140) using inactivated indigenous soil bacterial biomass, achieved simultaneous enhanced biosorption of Phen and iodine from aqueous solutions, at different rates depending on the variation of physicochemical parameters, being one of the few, if not only, examples where co-remediation of Phen with an inorganic contaminant has been studied.

### 3.5 Key findings

- Growth conditions and methods for the preparations of *P.javanicus* MEA plates and MCD and PDb liquid cultures were set.
- Macroscopic visual characteristics of *P.javanicus* in plate and liquid cultures were identified.
- *P.javanicus* was tolerant to the presence of Pb in the growth medium.
- *P.javanicus* was tolerant to the presence of B(a)p in the growth medium.
- The ability of *P.javanicus* to biomineralize pyromorphite in a liquid culture spiked with Pb and an organic P source was corroborated.
- Pb-oxalate was detected as the main biomineral in *P.javanicus* biomass at 10 days of incubation.
- At 20 and 30 days of incubation, pyromorphite was detected as the main biomineral in *P.javanicus* biomass.
- A ~94 % reduction of Pb in the culture media was measured after 30 days of incubation.
- From an initial average 7.10 ± 0.04 pH in the culture media, the activity of *P.javanicus* in Pb-spiked cultures firstly produced a reduction to pH 3.49 ± 0.06 at 10 days, followed with an increase to slightly alkaline with pH 8.14 ± 0.09 and 8.52 ± 0.09 at 20 and 30 days respectively.
- *P.javanicus* showed potential for the remediation of Pb-contaminated soil, through the biomineralization of Pb as pyromorphite.
- In Phen-spiked cultures with *P.javanicus*, from 20 days of incubation until the maximum of 70 days, no Phen was detectable in the spiked supernatant.

- Total average Phen µg in *P.javanicus* biomass was not statistically different at 20, 30 and 50 days of incubation.
- Sorption by fungal biomass appeared to be the main process responsible for the removal of Phen from solution in spiked *P.javanicus* liquid cultures.
- Although PAH biosorption, in the context of soil, may not reduce human bioavailability, it can potentially reduce Phen environmental availability
- From an initial pH of 8.82 (8.33 in experiment 2) the activity of *P.javanicus* in Phen-spiked cultures produced a gradual reduction, reaching an average pH of 4.22 ± 0.21 at 30 days, remaining at acidic levels until 50 days of incubation (pH 4.43 ± 0.04), and rising to pH 8.02 ± 0.52 at 70 days.
- While pH evolutions were different in Pb-spiked and Phen-spiked experiments, the rise in pH observed in the 70 days harvest of the Phen experiment would mean that conditions favourable to pyromorphite, though delayed, would eventually be achieved, permitting the biomineralization of pyromorphite by *P.javanicus.*
- While degradation of Phen was not shown in the time-frame of the experiment, the Phen sorption capacity of *P.javanicus* could be further exploited for co-remediation of Pb and PAHs by combining the fungus with complementary amendments.
- *P.javanicus* showed potential for remediation applications other than in situ soil amendment, as its capacity to remove Pb and Phen from solution, has shown promise for remediation in aqueous media.

# CHAPTER 4 Characterization of soil samples from the Meat market site

## 4.1 Introduction

Having corroborated the biomineralization of Pb as pyromorphite by *P.javanicus*, as well as identified its ability to remove Phen from solution via biosorption in liquid cultures (Chapter 3), a progression to studies in soil matrices was sought.

Fieldwork was conducted at the Meat market site in Glasgow, described earlier (section 1.3) as an exemplary case of urban brownfield sites in Glasgow and the UK, with soil contamination issues.

Glasgow City Council commissioned ERS (Environmental Reclamation Services, environmental consultancy) to carry out fieldwork and sample collection in collaboration with the author.

## 4.2 Objectives

To obtain soil samples from the Old Glasgow Meat market site and characterize their metal and PAH content, for potential use as a real environmental matrix in soil-matrix experiments with *P.javanicus* and brewery spent grain.

## 4.3 Methodology

### 4.3.1 Sample collection

As the aim was to collect contaminated soil for use in an experimental stage, and not for a detailed characterization of the site, four sample collection points were selected using the available chemical data (section 1.3) and soil guidelines (Table 4.1 and Figure 4.1). The depth to the top and bottom of strata associated with specific chemical data was also taken into consideration (Table 4.1).

Targeted points were located with aid of a GPS and excavated using a tracked excavator with a smooth bladed bucket (Figure 4.2-b). Removed soil was piled separately according to depth. A sample of ~500g of soil was collected and analysed with a portable X-ray Fluorescence (pXRF) detector to assess the Pb content before collection of bigger samples (Figure 4.3). With the antecedent, from available chemical and descriptive data, of a correlation between the presence of visible fragments of burnt shale and high concentrations of PAHs, visual and olfactory means were utilised as a criterion for evaluating the suitability of excavated soil as a source of PAH contaminated material by the environmental consultant.

*Table 4.1. Selected locations for sample collection with description and targeted contaminant concentration.*

| Location (point name) | Depth (mbgl) | Summarised strata description | Target contaminant and concentration |
|---|---|---|---|
| CP14 | 0 – 1.3 | Made ground: dark brown sandy gravelly silt or clay. Gravel of brick, concrete, burnt shale. Strong hydrocarbon smell and staining. | Pb, 568 mg $kg^{-1}$ |
| CP08 | 0.25 – 1.5 | Made ground: black, occasionally orange-brown, slightly clayey gravelly sand. Gravel of brick, burnt shale and concrete. | Pb, 551 mg $kg^{-1}$ |
| TP03 | 0.25 – 1.5 | Made ground: dark brown occasionally orangish red gravelly silty sand. Cobbles and gravel are concrete, brick and burnt shale. | Total PAH, 238 mg $kg^{-1}$<br>B(a)p, 15.5 mg $kg^{-1}$ |
| HP01 | 0.2 – 1.4 | Made ground: dark brown slightly clayey gravelly sand. Cobbles and gravel of brick, concrete and burnt shale. | Total PAH, 155 mg $kg^{-1}$<br>B(a)p, 10.3 mg $kg^{-1}$ |

**mbgl stands for meters below ground level*

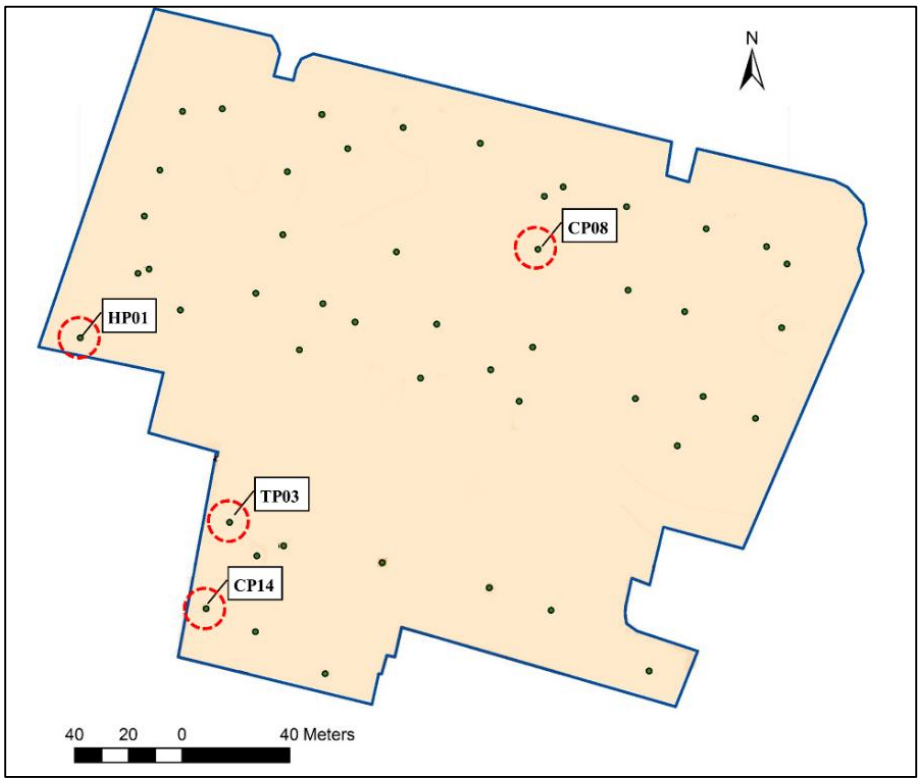



*Figure 4.1. Site plan with selected locations marked with red circles.*


*Figure 4.2 (a) Excavation pit CP08 for sample collection. (b)Tracked excavator on site.*

*Figure 4.3. pXRF analysis of soil samples on site.*

#### 4.3.1.1 On-site observations

All excavation pits correspond to made ground with varying soil characteristics (grain size distribution, colour and composition). Fragments of brick, cement, metal, plastic as well as burnt shale and coal with hydrocarbon odour were present in the four excavations.

*Table 4.2. Pb concentrations obtained by pXRF.*

| **Point** | **Depth (mbgl)** | **Pb concentration (mg kg $^{-1}$)** |
|---|---|---|
| CP14 | 0.7 | 170 |
| CP14 | 1.3 | 140 |
| CP08 | 0.6 | 225 |
| CP08 | 0.8 | 369 |

The last location excavated (point CP08, Figure 4.2-a), contained a pocket of darker soil with a strong hydrocarbon odour and higher pXRF Pb concentrations (Table 4.2). In consequence, more soil samples were collected from this point. Eleven samples of

approximately 10 to 15 kg were collected in total. The number of samples per sampling point with their respective names, are shown in Table 4.3.

*Table 4.3. Samples collected from the Meat market site.*

| Point | Number of samples | Sample names |
|---|---|---|
| CP14 | 2 | CP14(0.7m).1<br>CP14(1.2m).1 |
| CP08 | 4 | CP08(0.6m).1<br>CP08(0.6m).2<br>CP08(0.8m).1<br>CP08(0.8m).2 |
| TP03 | 3 | TP03(1.2m).1<br>TP03(1.2m).2<br>TP03(1.2m).3 |
| HP01 | 2 | HP01(1.2m).1<br>HP01(1.2m).2 |

### 4.3.2 Sample processing

#### 4.3.2.1 Subsampling

Systematic subsampling was carried out with the Japanese slabcake method (174). Samples were evenly spread on a metallic tray, handpicking big fragments (gravel and cobbles) to be discarded. Soil was then divided into 16 approximately equal portions (Figure 4.4) which were alternately collected with a flat spatula and distributed in four plastic sample bags of composite subsamples, as indicated in Figure 4.4-a. Each of the subsamples was differentiated by the addition of a number (from 1 to 4) after the original sample name (e.g. CP14(1.2m).1-1)

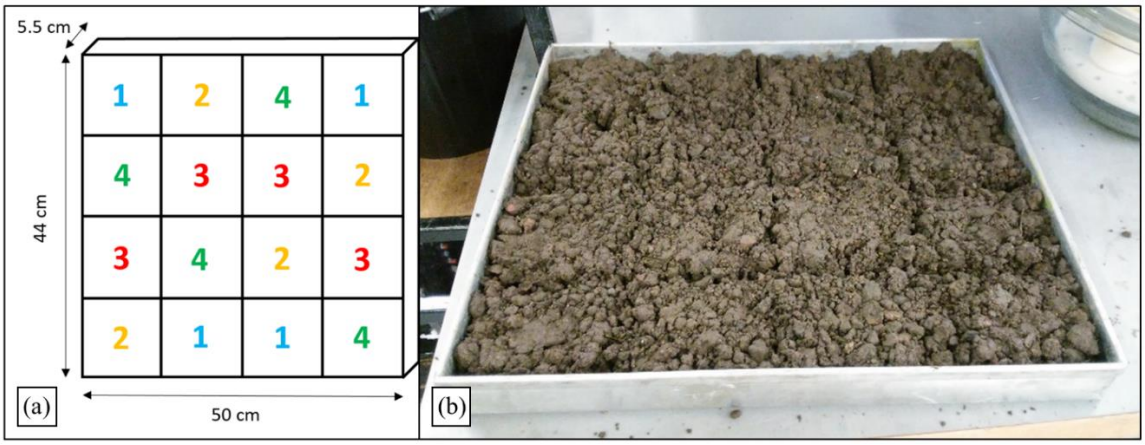



*Figure 4.4. (a) Schematic representation of the Japanese slabcake subsampling method. (b) Example of the method being applied in a real sample.*

One of the four resulting composite subsamples, from each original sample of which there were 11 in total, was subsequently divided into four smaller subsamples with the same

method. Smaller subsamples were further differentiated by the addition of a letter (from A to D) at the end of the sample name (e.g. CP14(1.2m).1-1A). One of these smaller subsamples was air dried, sieved (< 2 mm) and mechanically homogenized for further digestion/extraction and chemical analysis.

*Table 4.4. Example of sub-sampling nomenclature.*

| | |
|---|---|
| **CP14(1.2m).1** | **CP14(1.2m).1-1A** |
| | CP14(1.2m).1-1B |
| | CP14(1.2m).1-1C |
| | CP14(1.2m).1-1D |
| | CP14(1.2m).1-2 |
| | CP14(1.2m).1-3 |
| | CP14(1.2m).1-4 |

As a result, each of the 11 original samples was divided into 7 subsamples, as exemplified in Table 4.4, where one was air dried, sieved and homogenized (marked in bold letters in the example on Table 4.4), while the rest were kept stored at 4°C.

#### 4.3.2.2 General soil parameters

##### 4.3.2.2.1 pH measurement

pH was measured according to USEPA method 9045D (175). 20 g of each sample (air dried and sieved) was placed in 50 ml polypropylene centrifuge tubes. 20 ml of Milli-Q water was added, tubes capped and placed in an orbital incubator at 200 rpm for 5 minutes. Tubes were then left standing for an hour, allowing the soil to settle out from the suspension.

pH was measured with an Orion 5 star Thermo Scientific pH meter with an Orion 8156BNUWP ROSS Thermo Scientific ultra-combination pH electrode, appropriately calibrated. Three pH measurements were made per sample, and an average was calculated.

##### 4.3.2.2.2 Moisture content and loss on ignition (LOI)

Two ~5.0 g portions of each sample (air dried and sieved) were weighed into porcelain crucibles, after recording the weight of each empty crucible. Crucibles were placed in an oven at 105°C for 8 hours. The crucibles were removed and once at room temperature, their weight was recorded. Crucibles were then placed in a muffle furnace and combusted at 450°C for 8 hours. Once cool (to room temperature), the crucibles were removed and their weight recorded.

Air dried (AD), oven dried (OD) and dry ash (DA) sample weight was calculated by subtracting the previously recorded empty crucible weight from the initial empty crucible plus air dry sample weight; crucible plus sample weight after oven drying for 8 hrs at 105 °C; and crucible plus sample weight after ashing for 8 hrs at 450 °C, respectively.

Percentage moisture was calculated as follows:

$$M\% = \frac{(AD - OD) * 100}{AD}$$

With

- M%: moisture content in %
- AD: air dried sample weight in g
- OD: oven dried sample weight in g

Percentage loss on ignition (LOI) percentage was calculated as follows:

$$LOI = \frac{(OD - DA) * 100}{AD}$$

With

- LOI: loss on ignition in %
- AD: air dried sample weight in g
- OD: oven dried sample weight in g
- DA: dry ash sample weight in g

Examples for both calculations are shown in Appendix CH4 A3.

#### 4.3.2.3 Pseudo-total metal content of soil samples

##### 4.3.2.3.1 Aqua regia digestion

From each of the two ashed replicates of every sample (section 4.3.2.2.2) ~1.0 g was weighed in a DigiTUBE® (SCP Science, Quebec H9X 4B6 Canada). 10 ml of aqua regia (1:1 HCl 37%: $HNO_3$ 69%, both ARISTAR®, VWR chemicals, Leuven, Belgium) was added and the tube placed in the DigiPREP Jr.® digestion block (SCP Science, Quebec H9X 4B6 Canada) at 95°C with watch glasses on, for 4 hours. Watch glasses were then removed, and the solution allowed to reduce to ~2.0 ml. The remaining solution was transferred by filtering (Whatman Nº42 filter paper) to 100 ml volumetric flasks. To ensure transfer was complete, 5% $HNO_3$ was added repeatedly to the tubes, and filtered into the volumetric flask, until the volume was made up to a 100 ml. Solutions were then transferred into plastic bottles for storage prior to analysis.

The validation of this digestion method, by comparison to USEPA method 3050B (Acid digestion of sediments, sludges and soils (176) is shown in Appendix CH4 A1.

#### 4.3.2.3.2 ICP-OES analysis

Analysis was performed using a Perkin Elmer Optima 7300 DV ICP-OES.

A multielement calibration line (1, 5, 10, and 20 mg $l^{-1}$) was prepared from appropriate standards. Additional standards were also placed approximately every 10 samples as a quality control check for instrumental drift (3, 0.2, 15, 1 and 0 mg $l^{-1}$). At least 2 wavelengths were measured for each element, and the best value was selected according to the $R^2$ of the calibration line, consistency of the standards and RSD. Selected wavelength per element are shown in Table 4.5. The full data set can be found in the raw data included in the Appendix CH4 S1.

*Table 4.5. Selected wavelength and analytical limit of detection (LOD) per element (177, 178).*

| Element | Selected wavelength (nm) | LOD (µg $l^{-1}$) | ppm = mg $kg^{-1}$ |
|---|---|---|---|
| **Al** | Averaged 237.312 and 394.401 | | |
| **Ba** | 493.409 | 0.0049 | 0.0000049 |
| **Be** | 313.107 | | |
| **Ca** | 184.006 | 0.04 | 0.00004 |
| **Cd** | 226.502 | 0.023 | 0.000023 |
| **Co** | 228.616 | 0.083 | 0.000083 |
| **Cr** | Averaged 284.325 and 357.869 | 0.061 | 0.000061 |
| **Cu** | Averaged 324.754 and 327.396 | 0.16 | 0.00016 |
| **Fe** | Averaged 238.204 and 240.488 | | |
| **K** | Averaged 766.490 and 769.896 | 0.08 | |
| **Mg** | 285.213 | 0.002 | 0.000002 |
| **Mn** | Averaged 259.373 and 216.999 | 0.0095 | 0.0000095 |
| **Ni** | 231.604 | 0.095 | 0.000095 |
| **Pb** | Averaged 182.205 and 293.930 | 0.58 | 0.00058 |
| **Ti** | Averaged 334.941 and 338.376 | | |
| **Zn** | Averaged 206.200 and 213.856 | 0.031 | 0.000031 |

Resulting metal concentrations obtained from ICP-OES analysis were subsequently adjusted from the dry ash sample weight to a respective air dried sample weight, as shown in Appendix CH4 A2.

#### 4.3.2.4 PAH content of soil samples

#### 4.3.2.4.1 Accelerated solvent extraction (ASE)

**a) Solution preparation**

A 2000 µg $ml^{-1}$ surrogate solution was prepared by weighing 20 mg of naphthalene-d8, fluorene-d10, anthracene-d10, fluroanthene-d10 and chrysene-d10 (Sigma-Aldrich, St.

Louis, MO 63103), in a headspace vial. 10 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG) was added and the vial sealed with a Teflon cap.

A 2000 µg $ml^{-1}$ internal standard solution was prepared by weighing 20 mg of phenanthrene-d10 (Sigma-Aldrich, St. Louis, MO 63103), in a headspace vial, and adding 10 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG).

**b) Sample preparation and extraction protocol**

Air dried and sieved samples were extracted in duplicate. Two rounds of accelerated solvent extraction (ASE) were carried out. Initially using 0.25 g of soil sample, that was then increased to 1.0 g.

ASE extraction was carried out as described in section 3.3.4.2.2. For 0.25 g samples, 0.25 g of sodium sulphate and 150 µl of surrogate solution and internal standard were used. For 1.0 g samples 0.5 g of sodium sulphate and 50 µl of surrogate solution and internal standard were used.

**4.3.2.4.2 GC-MS analysis**

GC-MS analysis was performed at the Environmental laboratory, in the department of Civil & Environmental Engineering, University of Strathclyde (G1 1XQ, Glasgow), by technical staff as described in section 3.3.4.2.2.

**4.4 Results**

**4.4.1 General soil characteristics**

Table 4.6 shows a summary of general parameters measured in soil samples.

*Table 4.6. Summary of general soil sample characteristics, including soils grain size distribution (> 2mm and < 2 mm), % moisture, % loss on ignition (LOI) and pH.*

| Sample | > 2mm (%) | < 2mm (%) | Moisture (%) | SD | LOI (%) | SD | pH | error (pH) |
|---|---|---|---|---|---|---|---|---|
| **FSCP14(0.7m).1** | 37.17 | 62.83 | 2.315 | *0.005* | 5.097 | *0.174* | 9.450 | *0.215* |
| **FSCP14(1.2m).1** | 31.28 | 68.72 | 4.125 | *0.094* | 5.545 | *0.015* | 8.477 | *0.182* |
| **FSCP08(0.6m).1** | 34.27 | 65.73 | 3.996 | *0.048* | 15.99 | *2.020* | 8.203 | *0.051* |
| **FSCP08(0.6m).2** | 32.01 | 67.99 | 1.385 | *0.013* | 8.159 | *0.263* | 8.070 | *0.054* |
| **FSCP08(0.8m).1** | 34.58 | 65.42 | 2.250 | *0.009* | 13.41 | *0.592* | 8.083 | *0.042* |
| **FSCP08(0.8m).2** | 29.15 | 70.85 | 2.620 | *0.011* | 13.68 | *0.246* | 8.043 | *0.115* |
| **FSTP03(1.2m).1** | 35.92 | 64.08 | 1.278 | *0.001* | 5.891 | *0.118* | 8.223 | *0.071* |
| **FSTP03(1.2m).2** | 39.31 | 60.69 | 1.342 | *0.005* | 6.005 | *0.098* | 8.120 | *0.123* |
| **FSTP03(1.2m).3** | 35.50 | 64.50 | 1.220 | *0.006* | 6.561 | *0.129* | 8.170 | *0.073* |
| **FSHP01(1.2m).1** | 32.52 | 67.48 | 1.390 | *0.022* | 3.980 | *0.051* | 8.223 | *0.012* |
| **FSHP01(1.2m).2** | 31.92 | 68.08 | 1.645 | *0.055* | 4.131 | *0.083* | 8.257 | *0.084* |

All samples had a similar content of gravel (> 2 mm) between 30 and 40%. Moisture content ranged from 1.2% to 4.1%. LOI was the highest in CP08 samples, between 8.2% to 16.0% while in other samples ranged between 4.0% to 6.6%.

Soil pH was slightly alkaline, between 8.0 and 8.5, apart from sample FSCP14(0.7m).1 where a higher pH of 9.5 was measured.

### 4.4.2 Pseudo-total metal analysis of soil samples

Table 4.7 shows recoveries for selected elements for the Certified Reference Material (CRM) with pseudo total aqua regia digestion.

Fourteen out of 16 elements had average concentrations that fell within the certified prediction interval. The exceptions were Al and Cu that had average concentrations above and below the prediction interval, respectively. If the error is considered, however, there is overlap with the prediction interval in both cases. All elements, excepting Al, had error values that represent less than 3 % of the average concentration value. As Al was not an element of singular interest in soil samples, the greater error for this element was not considered detrimental to the continued use of this digestion method.

*Table 4.7: Average element concentrations in CRM digested for pseudo-total metal content with aqua regia, compared to certified confidence and prediction interval (CI and PI respectively).*

| CRM Sandy Clay 2010 | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Element** | **Average (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **error (%)** | **CI (mg kg$^{-1}$)** | | **PI (mg kg$^{-1}$)** | |
| **Al** | 825.2 | *170.9* | *20.71* | 537.0 | 584.0 | 394.0 | 726.0 |
| **Ba** | 12.28 | *0.190* | *1.550* | 12.40 | 13.00 | 10.60 | 14.80 |
| **Be** | 56.07 | *0.733* | *1.308* | 59.40 | 61.50 | 52.70 | 68.30 |
| **Ca** | 4261 | *31.14* | *0.731* | 4680 | 4910 | 4020 | 5570 |
| **Cd** | 72.39 | *0.626* | *0.865* | 78.90 | 81.00 | 71.50 | 88.40 |
| **Co** | 75.05 | *0.782* | *1.042* | 82.80 | 85.20 | 75.30 | 92.70 |
| **Cr** | 324.7 | *4.027* | *1.240* | 350.0 | 360.0 | 314.0 | 396.0 |
| **Cu** | 77.78 | *0.804* | *1.034* | 87.10 | 89.80 | 77.80 | 99.10 |
| **Fe** | 8176 | *46.88* | *0.573* | 8950 | 9380 | 7630 | 10700 |
| **K** | 2959 | *32.69* | *1.105* | 2960 | 3080 | 2600 | 3440 |
| **Mg** | 810.7 | *11.07* | *1.366* | 881.0 | 916.0 | 777.0 | 1020 |
| **Mn** | 579.3 | *8.768* | *1.514* | 625.0 | 646.0 | 561.0 | 710.0 |
| **Ni** | 318.2 | *3.964* | *1.246* | 339.0 | 349.0 | 304.0 | 383.0 |
| **Pb** | 112.2 | *2.390* | *2.129* | 109.0 | 112.0 | 97.50 | 124.0 |
| **Ti** | 43.55 | *0.948* | *2.176* | 46.10 | 48.00 | 41.80 | 52.40 |
| **Zn** | 488.1 | *3.523* | *0.722* | 534.0 | 549.0 | 485.0 | 599.0 |

**Concentrations that fall within the CI are highlighted with blue. Concentrations that fall within the PI are highlighted with light orange when below the CI and with bright orange when above the CI. Concentration that are lower than the PI are highlighted in grey and those higher than the PI in yellow.*

*Table 4.8: Average concentrations of analysed elements for 6 Meat market samples.*

| | FSCP14(0.7m).1-4D | | FSCP14(1.2m).1-1A | | FSCP08(0.6m).1-4D | | FSCP08(0.6m).2-1A | | FSCP08(0.8m).1-2B | | FSCP08(0.8m).2-3C | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** |
| **Al** | 13096 | *277.9* | 14370 | *200.5* | 15181 | *354.8* | 12499 | *1217* | 12896 | *17.89* | 10621 | *292.5* |
| **Ba** | 159.2 | *0.867* | 106.1 | *3.856* | 285.9 | *0.155* | 166.7 | *1.309* | 290.6 | *17.98* | 293.8 | *8.311* |
| **Be** | 1.026 | *0.031* | 1.136 | *0.005* | 3.612 | *0.265* | 1.788 | *0.039* | 3.365 | *0.062* | 3.393 | *0.075* |
| **Ca** | 27173 | *1084* | 19854 | *1378* | 16335 | *325.3* | 13777 | *516.7* | 15274 | *1300* | 14598 | *199.8* |
| **Cd** | 1.522 | *0.111* | 1.108 | *0.072* | 3.182 | *0.042* | 1.947 | *0.051* | 3.224 | *0.043* | 3.287 | *0.045* |
| **Co** | 8.608 | *0.334* | 8.214 | *0.035* | 21.11 | *0.664* | 12.64 | *0.259* | 20.93 | *1.336* | 19.86 | *0.682* |
| **Cr** | 32.58 | *0.578* | 27.24 | *0.690* | 45.80 | *6.026* | 30.84 | *0.308* | 36.49 | *0.347* | 36.45 | *0.469* |
| **Cu** | 84.82 | *9.638* | 38.50 | *0.008* | 299.4 | *31.58* | 116.8 | *4.716* | 251.6 | *6.361* | 257.2 | *5.529* |
| **Fe** | 23034 | *1850* | 16544 | *982.2* | 42414 | *175.9* | 27900 | *713.3* | 43505 | *123.4* | 43469 | *191.7* |
| **K** | 1292 | *11.13* | 1257 | *94.19* | 1388 | *16.00* | 1170 | *73.05* | 1270 | *11.28* | 1094 | *56.74* |
| **Mg** | 3099 | *59.93* | 2302 | *132.5* | 3125 | *39.06* | 3060 | *427.3* | 3058 | *298.3* | 2811 | *88.94* |
| **Mn** | 656.8 | *245.7* | 272.7 | *39.22* | 1865 | *226.9* | 1053 | *131.0* | 1762 | *235.7* | 2045 | *237.9* |
| **Ni** | 23.85 | *0.538* | 23.88 | *0.073* | 76.03 | *3.148* | 45.30 | *0.469* | 79.72 | *5.500* | 80.46 | *4.284* |
| **Pb** | 215.8 | *25.95* | 130.1 | *1.465* | 748.9 | *10.29* | 370.4 | *27.16* | 838.5 | *17.75* | 856.8 | *86.28* |
| **Ti** | 301.9 | *28.24* | 322.1 | *9.383* | 346.5 | *20.50* | 333.4 | *2.545* | 344.6 | *12.65* | 353.0 | *15.16* |
| **Zn** | 239.5 | *12.12* | 118.4 | *2.756* | 315.6 | *1.898* | 177.8 | *7.352* | 300.3 | *4.994* | 318.8 | *16.26* |

*Table 4.9: Average concentrations of analysed elements for 5 Meat market samples.*

| | FSTP03(1.2m).1-1A | | FSTP03(1.2m).2-2B | | FSTP03(1.2m).3-3C | | FSHP01(1.2m).1-2B | | FSHP01(1.2m).2-3C | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** | **Average (mg kg$^{-1}$)** | **SD** |
| **Al** | 20556 | *387.4* | 21214 | *862.1* | 23296 | *1604* | 13869 | *712.7* | 13968 | *425.6* |
| **Ba** | 190.7 | *7.024* | 190.1 | *6.728* | 169.84 | *3.515* | 312.6 | *69.69* | 213.6 | *11.00* |
| **Be** | 1.149 | *0.045* | 1.319 | *0.022* | 1.227 | *0.058* | 0.706 | *0.038* | 0.745 | *0.046* |
| **Ca** | 19747 | *46.78* | 17326 | *392.3* | 16479 | *1502* | 29803 | *211.0* | 29861 | *152.4* |
| **Cd** | 1.472 | *0.025* | 1.800 | *0.036* | 1.545 | *0.117* | 1.588 | *0.066* | 1.453 | *0.050* |
| **Co** | 10.86 | *0.142* | 12.30 | *0.272* | 11.55 | *0.183* | 9.605 | *0.410* | 9.447 | *0.096* |
| **Cr** | 26.38 | *0.614* | 28.19 | *3.453* | 26.05 | *1.583* | 21.91 | *0.436* | 23.05 | *0.603* |
| **Cu** | 47.36 | *0.217* | 77.03 | *8.118* | 42.78 | *1.898* | 36.04 | *1.385* | 47.08 | *0.582* |
| **Fe** | 23408 | *146.8* | 28313 | *512.9* | 24548 | *1580* | 22289 | *739.7* | 20399 | *650.0* |
| **K** | 1820 | *15.17* | 1701 | *28.89* | 2104 | *155.2* | 1443 | *64.31* | 1436 | *22.89* |
| **Mg** | 3691 | *41.95* | 3694 | *158.1* | 3808 | *174.2* | 4271 | *97.42* | 3798 | *129.5* |
| **Mn** | 485.6 | *4.472* | 546.5 | *55.75* | 519.1 | *18.90* | 482.0 | *8.624* | 425.3 | *5.712* |
| **Ni** | 28.60 | *0.947* | 36.85 | *6.191* | 31.27 | *1.430* | 22.37 | *0.463* | 24.70 | *0.600* |
| **Pb** | 178.9 | *8.149* | 302.6 | *133.2* | 142.3 | *14.36* | 263.8 | *0.916* | 295.0 | *7.867* |
| **Ti** | 326.7 | *2.458* | 292.5 | *2.187* | 184.8 | *4.767* | 518.9 | *10.35* | 472.3 | *2.279* |
| **Zn** | 161.0 | *7.274* | 166.7 | *8.054* | 147.1 | *6.192* | 174.2 | *12.76* | 175.7 | *7.452* |

*Table 4.10: Compilation of generic assessment criteria and background values, with the respective number of Meat market samples (from the total of 11) which surpass the given value.*

| | C4SL Res. whgp[1] (mg kg $^{-1}$) | n | C4SL Allot.[2] (mg kg $^{-1}$) | n | C4SL POS[3] (mg kg $^{-1}$) | n | CLEA SGV (2011) Res.[4] (mg kg $^{-1}$) | n | Dutch Soil Intervention Value[5] (mg kg $^{-1}$) | n | Proposed UK Ecological SGV[6] (mg kg $^{-1}$) | n | Glasgow Background BGS mean[7] (mg kg $^{-1}$) | n | SH UK (USM)[8] (mg kg $^{-1}$) | n |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cd** | 26.00 | 0 | 4.900 | 0 | 220.0 | 0 | 10.00 | 0 | 13.00 | 0 | 1.150 | 10 | 0.380 | 11 | 0.280 | 11 |
| **Cr** | | | | | | | | | 180.0 | 0 | 21.10 | 11 | 125.0 | 0 | 38.30 | 1 |
| **Cu** | | | | | | | | | 190.0 | 3 | 88.40 | 4 | 76.20 | 6 | 34.80 | 11 |
| **Ni** | | | | | | | 130.0 | 0 | 100.0 | 0 | 25.10 | 7 | 54.90 | 3 | 26.20 | 7 |
| **Pb** | 200.0 | 8 | 80.00 | 11 | 630.0 | 3 | | | 530.0 | 3 | 167.9 | 9 | 180.1 | 8 | 118.0 | 11 |
| **Zn** | | | | | | | | | 720.0 | 0 | 90.10 | 11 | 201.0 | 4 | | |

*Table 4.11: Elements with concentrations above generic assessment criteria and background values, for each sample.*

| Sample | C4SL Res. whgp[1] | C4SL Allot.[2] | C4SL POS[3] | Dutch Soil Intervention Value[4] | Proposed UK Ecological SGV[5] | Glasgow Background BGS mean[6] | SH UK (Urban Scotland mean)[7] |
|---|---|---|---|---|---|---|---|
| **FSCP14(0.7m).1** | Pb | Pb | | | Pb, Cd, Cr, Zn | Pb, Cd, Cu, Zn | Pb, Cd, Cu |
| **FSCP14(1.2m).1** | | Pb | | | Cr, Zn | Cd | Pb, Cd, Cu |
| **FSCP08(0.6m).1** | Pb | Pb | Pb | Pb, Cu | Pb, Cd, Cr, Cu, Ni, Zn | Pb, Cd, Cu, Ni, Zn | Pb, Cd, Cr, Cu, Ni |
| **FSCP08(0.6m).2** | Pb | Pb | | | Pb, Cd, Cr, Cu, Ni, Zn | Pb, Cd, Cu | Pb, Cd, Cu, Ni |
| **FSCP08(0.8m).1** | Pb | Pb | Pb | Pb, Cu | Pb, Cd, Cr, Cu, Ni, Zn | Pb, Cd, Cu, Ni, Zn | Pb, Cd, Cu, Ni |
| **FSCP08(0.8m).2** | Pb | Pb | Pb | Pb, Cu | Pb, Cd, Cr, Cu, Ni, Zn | Pb, Cd, Cu, Ni, Zn | Pb, Cd, Cu, Ni |
| **FSTP03(1.2m).1** | | Pb | | | Pb, Cd, Cr, Ni, Zn | Cd | Pb, Cd, Cu, Ni |
| **FSTP03(1.2m).2** | Pb | Pb | | | Pb, Cd, Cr, Ni, Zn | Pb, Cd, Cu | Pb, Cd, Cu, Ni |
| **FSTP03(1.2m).3** | | Pb | | | Cd, Cr, Ni, Zn | Cd | Pb, Cd, Cu, Ni |
| **FSHP01(1.2m).1** | Pb | Pb | | | Pb, Cd, Cr, Zn | Pb, Cd | Pb, Cd, Cu |
| **FSHP01(1.2m).2** | Pb | Pb | | | Pb, Cd, Cr, Zn | Pb, Cd | Pb, Cd, Cu |

**In both tables: [1] Category 4 Screening Level for residential use with home-grown produce (DEFRA, 2014 (9)); [2] Category 4 Screening Level for allotment use (DEFRA, 2014 (9)); [3] Category 4 Screening Level for public open space use (DEFRA, 2014 (9)) [4] CLEA 2011 soil guideline value for residential use (Fordyce et al., 2014 (11)); Dutch Soil Intervention Value for soils (VROM, 2009 (13)) [6] Proposed UK Ecological Soil Guideline Value (considering any adverse effects on wildlife such as birds, mammals, plants, soil invertebrates or on the microbial function of soils) (Fordyce et al., 2014 (11)); [7] (Fordyce et al., 2014 (11)); [8] Soil and Herbage UK mean background value for urban areas in Scotland (EA, 2007 (14)).*

Table 4.8 and Table 4.9 show the average concentrations of analysed elements for the 11 Meat market samples. The same generic assessment criteria (guideline values) used to study the pre-existing chemical data of the Meat market site (section 1.3), were used in this case, to identify element of interest or concern.

Table 4.10 compiles guideline values according to different assessment criteria and background concentrations for Cd, Cr, Cu, Ni, Pb and Zn, and the respective number of samples (n) that surpass each value. This information is complemented by Table 4.11, that shows which elements surpass guideline and/or background values in each specific sample.

Pb and Cu are the only two elements whose concentrations surpass guideline values, other than ecological guideline values, in some of the samples.

All samples have Pb concentrations higher than the C4SL for allotments (80 mg $kg^{-1}$ (9)), however this value is already lower than published backgrounds for the city of Glasgow (11) and general urban areas in Scotland (14). The C4SL for residential use with home grown-produce (200 mg $kg^{-1}$ (9)), is surpassed in 8 out of 11 samples, the exceptions being samples FSCP14(1.2m).1, FSTP03(1.2m).1 and FSTP03(1.2m).3. Three samples (FSCP08(0.6m).1, FSCP08(0.8m).1 and FSCP08(0.8m).2) have Pb concentrations above both the Dutch soil intervention value (530 mg $kg^{-1}$ (13)) and C4SL for public open spaces (630 mg $kg^{-1}$ (9)), all collected from the same location on site (CP08), where a pocket of dark soil with a strong hydrocarbon odour and higher pXRF Pb concentrations was observed. These same three samples have concentrations of Cu that surpass the Dutch soil intervention value (190 mg $kg^{-1}$ (13)).

All elements have concentrations above the ecological guideline value (11) in some or all samples, however in most cases, except for Cd and Cu, this guideline value is lower than the average background for Glasgow (11). The latter is surpassed in a minimum of three samples for all elements, with the exception of Cr that is lower than the average Glasgow background (125 mg $kg^{-1}$ (11)) in all samples.

These results agree with conclusions drawn from the pre-existing chemical data (section 1.3). Pb was the main element of concern and distribution of this element was shown not to be widespread on site but localized in hotspots such as the CP-08 sample collection point.

### 4.4.3 PAHs in Meat market soil samples

After the first round of ASE extractions, with 0.25g soil samples, technical staff (Environmental laboratory, department of Civil & Environmental Engineering, University

of Strathclyde G1 1XQ, Glasgow) reported that no PAH identification or quantification had been possible from soil extracts.

For this reason, samples were re-extracted, increasing the sample weight to 1.0 g and reducing the surrogate solution and internal standard from 150 µl to 50 µl, adapting the method to lower soil PAH concentrations. However, despite these changes, PAH identification and quantification was still not possible.

It was hence concluded that although there was a record of PAH contamination in the Meat market site, and certain indications of potential contamination (e.g. presence of burnt shale and coal with hydrocarbon odour) were observed during soil excavation on site, collected soils were not representative of soil PAH contamination. PAHs concentrations in soil samples were below the limit of detection of the extraction/analysis method.

### 4.4.4 CP08 soil sample homogenization

The four samples collected from point CP08 (FSCP08(0.6m).1, FSCP08(0.6m).2, FSCP08(0.8m).1, FSCP08(0.8m).2), with the highest Pb concentrations amongst all 11 collected samples, were selected as a potentially suitable matrix for future bioremediation experiments with *P. javanicus.*

In consequence all CP08 subsamples were combined and homogenized as follows.

Subsamples, previously stored at 4°C, were spread in open 42 l plastic storage boxes, under fume hoods for a week. Once dried, all the soil was sieved (< 2 mm) and collected in a separate 42 l plastic storage box. In order to homogenise the soil, mechanical mixing with a trowel was performed every time a new portion of soil was sieved and collected in the box.

From the final composite soil (~ 25 l), six ~1.0 g samples were taken from six different random points (at different surface position and depth in the box) and digested with aqua regia for pseudo-total metal content, as described in section 4.3.2.3.1. ICP-OES analysis was performed externally by a commercial laboratory (Concept Life Science laboratory, East Kilbride, G75 0YF).

Resulting concentrations are summarized in Table 4.12.

*Table 4.12. Summary with average element concentrations in homogenized CP08 samples.*

| | CP08 | | CRM | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Average (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **Average (mg kg$^{-1}$)** | **SD (mg kg$^{-1}$)** | **CI (mg kg$^{-1}$)** | | **PI (mg kg$^{-1}$)** | |
| **Al** | > LOD | | 799.2 | *0.449* | 537.0 | 584.0 | 394.0 | 726.0 |
| **Ba** | 271.8 | *28.09* | 13.06 | *0.013* | 12.40 | 13.00 | 10.60 | 14.80 |
| **Be** | 3.063 | *0.123* | 57.18 | *0.292* | 59.40 | 61.50 | 52.70 | 68.30 |
| **Ca** | > LOD | | 5047 | *20.64* | 4680 | 4910 | 4020 | 5570 |
| **Cd** | 0.770 | *0.095* | 83.26 | *0.515* | 78.90 | 81.00 | 71.50 | 88.40 |
| **Co** | 21.86 | *0.542* | 85.70 | *0.463* | 82.80 | 85.20 | 75.30 | 92.70 |
| **Cr** | 49.68 | *8.291* | 374.9 | *2.368* | 350.0 | 360.0 | 314.0 | 396.0 |
| **Cu** | 247.7 | *6.260* | 87.94 | *0.710* | 87.10 | 89.80 | 77.80 | 99.10 |
| **Fe** | > LOD | | > LOD | | 8950 | 9380 | 7630 | 10700 |
| **K** | 1298 | *47.23* | 3152 | *29.21* | 2960 | 3080 | 2600 | 3440 |
| **Mg** | 3525 | *436.4* | 843.0 | *3.383* | 881.0 | 916.0 | 777.0 | 1020.0 |
| **Mn** | 1604 | *334.7* | 684.5 | *2.010* | 625.0 | 646.0 | 561.0 | 710.0 |
| **Ni** | 74.29 | *1.636* | 362.3 | *1.384* | 339.0 | 349.0 | 304.0 | 383.0 |
| **P** | 645.8 | *39.56* | 11.82 | *0.636* | | | | |
| **Pb** | 669.7 | *84.18* | 115.7 | *0.084* | 109.0 | 112.0 | 97.50 | 124.0 |
| **Ti** | 478.3 | *21.85* | 47.36 | *0.352* | 46.10 | 48.00 | 41.80 | 52.40 |
| **Zn** | 310.0 | *8.809* | 584.2 | *0.664* | 534.0 | 549.0 | 485.0 | 599.0 |

**Element concentrations in the CRM digested alongside samples are also included with the respective certified confidence and prediction intervals (CI and PI respectively) Concentrations that fall within the CI are highlighted with blue. Concentrations that fall within the PI are highlighted with light orange when below the CI and with bright orange when above the CI. Concentration that are higher than the PI are highlighted in yellow. SD is shown in replacement of error as there were only two replicates.*

## 4.5 Key findings

- Three out of eleven soil samples from the Old Glasgow Meat market site, collected from the same sampling point (CP08), had Pb concentrations above the selected reference guideline value for public open space use.
- The four samples with the higher Pb concentrations (CP08 samples) were homogenised in a composite sample, for potential use in experimental studies and as a reference environmental matrix.
- With GC-MS analysis, no detectable PAH concentrations were identified in soil samples.
- No other contaminants of concern were identified.

## CHAPTER 5. Brewery spent grain as a matrix for soil bioaugmentation

### 5.1 Introduction

A common contamination profile found in urban soil is that of Pb and PAHs (1, 4, 6-8). The Old Glasgow Meat market is an example of this type of contamination, with Pb, total PAHs (16 priority contaminants USEPA) and B(a)p surpassing selecting soil guideline values, in data provided by Glasgow City Council (section 1.3).

In Chapter 3, the ability of *P.javanicus* to biomineralize pyromorphite in liquid cultures spiked with Pb and an organic P source was shown. Changes in the pH of the culture media, from an initial average 7.10 ± 0.04, to pH 3.49 ± 0.06 at 10 days accompanied the capture of Pb in the fungal biomass as Pb-oxalate. Pb-oxalate was then replaced in pseudomorphism by pyromorphite, detected as the main mineral phase at 20 and 30 days of incubation. This change in mineralogy was accompanied with changes in the pH of the media, to pH 8.14 ± 0.09 and 8.52 ± 0.09 at 20 and 30 days respectively.

Pyromorphite ($Pb_5[PO_4]_3X$; X= F, Cl, B or OH) is the most stable form of Pb in soils, under a wide range of physicochemical conditions (e.g. pH and Pb concentration), as well as in the human gastrointestinal system (42, 45, 48, 52). While phosphate amendments have been used for the *in situ* remediation of Pb-contaminated soil, through immobilization of Pb as pyromorphite (45, 56, 57, 63), limitations in the efficiency of this remediation strategy have been shown in soil with neutral to alkaline pHs, as more acidic pHs are required for the pre-existing Pb and P phases to be solubilized and react to form pyromorphite (45, 46, 57, 68, 70-72).

*P.javanicus* showed potential for the remediation of Pb-contaminated soil, through the biomineralization of Pb as pyromorphite, and its ability to change the pH in its microenvironment. The latter could promote the formation of pyromorphite in soils with neutral pH, typical of urban environments, where the use of phosphate amendments alone have limited effects on Pb immobilization as pyromorphite.

Chapter 3 also showed that *P.javanicus* was tolerant to the presence of B(a)p and Phen in culture media. No detectable Phen remained dissolved in liquid culture media after 20 days of incubation in *P.javanicus* cultures. Sorption by fungal biomass appeared to be the main process responsible for the removal of Phen from solution in spiked *P.javanicus* liquid cultures, as total Phen content in biomass was statistically equal at 20, 30 and 50 days of cultivation.

Although biosorption, in the context of soil, may not reduce human bioavailability, it can potentially reduce Phen environmental availability to groundwater, plants and other soil organisms, whilst avoiding the formation of undesirable by-products (171). Degradation of biosorbed Phen by cultured fungi overtime may also occur (139).

The Phen sorption capacity of *P.javanicus* could be further exploited for co-remediation of Pb and PAHs by combining the fungus with complementary amendments that can promote biodegradation through biostimulation of the native microbiota.

Brewery spent grain is an agro-industrial waste product available in abundance and at no cost in Scotland (142, 145). Its rich nutritional content (143) (detailed in section 2.3) led to the selection of brewery spent grain as a potentially suitable matrix for the cultivation/growth of *P.javanicus*, to create a spent grain + *P.javanicus* inoculum for the bioaugmentation of soils with the fungus. As a lignocellulosic material (143), the use of spent grain as a soil amendment may also have potential in stimulating the activity of native lignin degrading microorganisms, known to play a role in the degradation of PAHs (section 1.3.2).

The efficiency of bioaugmentation is determined by many biotic and abiotic factors (102). The selected microorganisms must not only be able to degrade or biomineralize contaminants but also successfully compete with indigenous microflora (102) and adapt to soil conditions (75). Poor soil conditions, common in contaminated soil and made ground, can often prevent successful inoculation of additional microorganisms and sustain their growth. Addition of available nutrients (biostimulation) is sometimes necessary to achieve a successful inoculation (75).

Experiments were carried out to determine the suitability of brewery spent grain as "growth media" for the preparation of *P.javanicus* inoculums.

Subsequently soil microcosms were used to study the feasibility of *P.javanicus* inoculation, propagation and growth in soils, with the use spent grain + *P.javanicus* inocula, and additional amendment of soil with sterile and non-sterile brewery spent grain.

In Chapter 4 it was shown that three out of eleven collected soil samples from the Old Glasgow Meat market site, had Pb concentrations above the selected reference guideline value for public open space use. With GC-MS analysis, however, no detectable PAH concentrations were identified in these soil samples.

A preliminary soil experiment using real environmental soil samples from the Meat market site was carried out to evaluate the bioaugmentation approach using spent grain + *P.javanicus*. Due to the exploratory nature of this initial stage, the experiment was conceived

as a qualitative observation-focused study, hence a simplified experimental set up was devised. As the aim was to study the transference of fungal growth into a soil matrix, no initial chemical characterization (e.g. Pb speciation) of the soil was sought. Further quantitative chemical data was later obtained (shown in Chapter 6).

The chemical composition of spent grain used as an amendment in the preliminary soil experiment, compared to fresh spent grain (spent grain not used as an amendment in soil), was studied to investigate the interaction of the spent grain and soil matrix, and identify any input or sorption processes occurring.

Finally, Pb, Phen and B(a)p spiked soils treated with different amendment combinations, including *P.javanicus* and spent grain, were studied in microcosms to assess the bioaugmentation and biostimulation approaches, and effects on Pb, Phen and B(a)p contamination (detailed in Chapter 6 and 7 respectively).

## 5.2 Objectives

- Determine the suitability of brewery spent grain for the preparation of *P.javanicus* inoculums.
- Study the feasibility of *P.javanicus* inoculation, propagation and growth in real urban soils and in Pb, Phen and B(a)p spiked soils with the selected inoculum preparation and additional spent grain additions.
- Study the composition of brewery spent grain before and after use as a soil amendment in microcosms and determine its contribution in terms of elements released and sorbed from the soil.
- Study pH changes in Pb, Phen and B(a)p spiked soils caused by the different treatments, for correlation with treatment effect on the contamination.

## 5.3 Methodology

### 5.3.1 Spent grain

Brewers spent grain was obtained from a local craft brewery (Jaw Brew, 67 Montrose Avenue, Glasgow G52 4LA) with a strong focus on promoting sustainability and "circularity" in their waste management practices. Their current disposal of spent grain is in the form of cattle feed in a local farm and production of high fibre bread and snack bars by a local bakery.

Collected spent grain consisted of brown malted barley used in the production of Porter beer, retrieved from the mashing-filtration stage of the brewing process the day before collection.

Spent grain was then portioned into glass jars and stored at -80°C for preservation until further use.

### 5.3.2 Inoculum preparation: experimental set up

An experiment was carried out in two rounds to study the suitability of spent grain as a matrix for the preparation of *P.javanicus* inocula. The first round consisted of three replicates of sterile spent grain inoculated with *P.javanicus* (SSG-Pj1, 2 and 3), a control with non-sterile spent grain inoculated with *P.javanicus* (SG-Pj), and a control with only sterile spent grain (SSG). The second round consisted of another four replicates of sterile spent grain inoculated with *P.javanicus* (SSG-Pj4, 5, 6 and 7) and a control with only non-sterile grain (SG).

In both rounds, portions of 10g of spent grain were weighed and placed in 250ml Erlenmeyer flasks. Replicates SG-Pj and SG were prepared in previously sterilised flasks and 10 ml of sterile Milli-Q water were added with a sterile pipette to the spent grain (Figure 5.1). All other replicates were prepared in non-sterile flasks, 10 ml of Milli-Q water were added and then the flasks were autoclaved at 121ºC for 15 minutes.

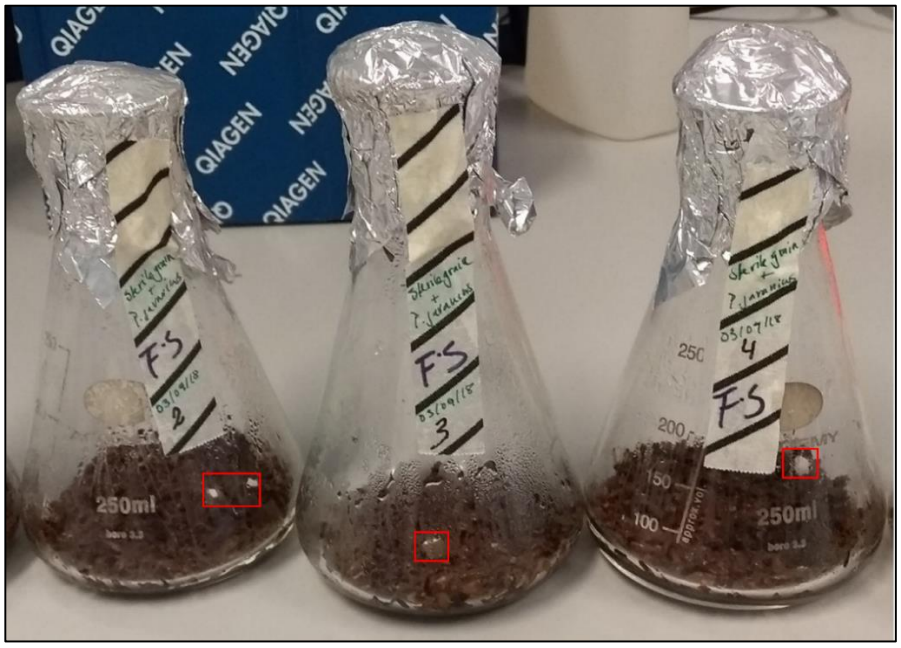



*Figure 5.1: Example of spent grain-P.javanicus inoculum preparation experiment set up at time zero, with MEA inoculation plugs marked by the red squares.*

*P.javanicus* was inoculated into SG-Pj and SSG-Pj replicates from 10 days old MEA culture plate prepared from the glycerol stock (section 3.3.1.4), using sterile glass Pasteur pipettes to cut out a plug from the edge of a growing colony and to drop it in the flask with the aid

of sterile syringe needles (Figure 5.1). All flasks were incubated at 20ºC and 150 rpm in the dark. Round 1 ran for 25 days and round 2 for 32 days.

Growth was monitored by qualitative visual observation and photographs were taken regularly to record the growth pattern.

### 5.3.3 Preliminary soil experiment: experimental set up

#### 5.3.3.1 Soil preparation

The soil matrix was prepared from the soil samples collected from the Meat Market site, previously processed during subsampling (section 4.3.2.1) by air drying and sieving (< 2 mm). A portion (20g) of each Meat Market sample (11 in total) was combined and homogenized, mechanically mixing by hand (homogenized mixture = MMS).

#### 5.3.3.2 Microcosm preparation

Brewery spent grain was added to the homogenized soil in a 1:4 mass ratio (55g grain [moist weight]: 220g soil) and thoroughly mixed. A quarter of the soil grain mixture was separated, by coning and quartering, into a different glass container and autoclaved at 121ºC for 15 minutes, while the rest was kept un-sterilized.

An equal amount of the soil-grain mixture (68.75g) was then added to 25 days old spent grain-*P.javanicus* inoculums, prepared in 250ml Erlenmeyer flasks (as described in section 5.3.2: 10g of spent grain with *P.javanicus*), creating the following four microcosm systems: a control with non-sterile soil (MMS) and spent grain (SG) mixture and no *P.javanicus* inoculum (Control = MMS+SG); two flasks with non-sterile soil (MMS) and spent grain (SG) mixture added over the *P.javanicus* inoculum (Pj), named P.jav1 and P.jav3 (= MMS+SG+Pj ) and one flask with sterile soil (SMMS) and sterile grain (SSG) mixture added over the *P.javanicus* inoculum (Pj) named P.jav2 (= SMMS+SSG+Pj). 25 ml of sterile Milli-Q water was added to each flask with sterile disposable pipettes. All flasks (Figure 5.2) were then placed in an orbital incubator at 20ºC and 150 rpm. Incubation took place over 70 days, during which time growth was monitored by qualitative visual observation, and photographs were taken regularly to record the fungal growth.

Special emphasis was put on qualitative observations of microcosms over time to monitor fungal growth. Changes observed in the different microcosm treatments, contrasted to the control, were noted and later compared to available literature on indicators of microbial growth in sediments.

At 70 days, flasks were removed from the incubator, stored at -80°C until freeze drying was carried out at -121ºC in a vacuum for 48 hours. Dry contents of the flasks where then gently disaggregated with a ceramic pestle inside the same flasks, and transferred to glass jars for storage at -20°C.


*Figure 5.2. All microcosm flasks at time zero before incubation.*

#### 5.3.3.3 Spent grain digestion

##### 5.3.3.3.1 Sample preparation

A portion of spent grain from each preliminary soil experiment microcosm, separated from the soil by sieving (> 2 mm), was thoroughly washed with Milli-Q water three times, to remove soil particles. After washing the harvested spent grain was again freeze dried for 24 hours. A portion of fresh spent grain was freeze dried for 48 hours for comparison.

##### 5.3.3.3.2 Digestion method

Approximately 0.5 g of dry spent grain was weighed in duplicate for each microcosm system (P.jav1, 2, 3 and Control), as well as for the fresh spent grain, and digested according to USEPA method 3050B (Acid digestion of sediments, sludges and soil) (176) as follows.

Samples were placed in Teflon beakers and 5.0 ml of Milli-Q water + 5.0 ml of $HNO_3$ (ARISTAR®, VWR chemicals, Leuven, Belgium) was added. Beakers were placed on a hot plate covered with watch glasses and heated for 15 minutes without boiling. After cooling to room temperature, 5.0 ml of $HNO_3$ was added and beakers returned to the hot plate with watch glasses for 30 minutes. This procedure was repeated until no brown fumes were given off by the samples. The solution in beakers was allowed to reduce to approximately 5.0 ml without boiling. After cooling to room temperature, 2.0 mL of Milli-Q water + 3.0 ml of

30% $H_2O_2$ was added. Beakers were covered with watch glasses, returned to the hot plate and heated until effervescence subsided. A further 3.0 ml of $H_2O_2$ was added in 1.0 ml portions. Again the solution was allowed to reduce to approximately 5.0 ml without boiling. The remaining solution was transferred by filtering (Whatman Nº42 filter paper) to 100 ml volumetric flasks. To make sure transference was complete, 5% $HNO_3$ was added repeatedly to the tubes, and filtered into the volumetric flask, until the volume was made up to a 100 ml. Solutions were then transferred into plastic bottles for storage.

The digestion method although intended for a different kind of matrix, fully dissolved the spent grain.

#### 5.3.3.3.3 ICP-OES analysis

Analysis was carried out in a Perkin Elmer Optima 7300 DV ICP-OES.

A multielement calibration line (1, 5, 10, 15, 20 and 25 mg $l^{-1}$) was prepared from appropriate standards (PlasmaCAL – Quality Control Standard 1, catalogue number 140-102-01X. Lot: S1908150004; PlasmaCAL ICP/ICPMS Standard – Phosphorus 1000 µg $ml^{-1}$, catalogue number 140-050-15x. Lot: S190819014, both SCP Science, Quebec H9X 4B6 Canada). 1 mg $l^{-1}$ standards were also placed every 10 samples as a quality control check for instrumental drift and 5% $HNO_3$ blanks approximately every 15 samples. At least 2 wavelengths were measured for each element, and the best value was selected according to the $R^2$ of the calibration line, consistency of 1 mg $l^{-1}$ standards and RSD. Selected wavelength per element are shown in Table 5.1. Raw data is included in Appendix CH5-6 S1.

*Table 5.1. Selected wavelength and analytical limit of detection (LOD) per element (177, 178).*

| Element | Selected wavelength (nm) | LOD (µg $l^{-1}$) | ppm = mg $kg^{-1}$ |
|---|---|---|---|
| **Cd** | 228.802 | 0.023 | 0.000023 |
| **Co** | 228.616 | 0.083 | 0.000083 |
| **Cr** | Averaged 267.716 and 357.869 | 0.061 | 0.000061 |
| **Cu** | Averaged 324.754 and 327.396 | 0.16 | 0.00016 |
| **Fe** | Averaged 238.204 and 240.488 | | |
| **Mg** | 285.213 | 0.002 | 0.000002 |
| **Mn** | Averaged 259.373 and 293.930 | 0.0095 | 0.0000095 |
| **Ni** | 221.647 | 0.095 | 0.000095 |
| **P** | 177.495 | 0.04 | 0.00004 |
| **Pb** | 220.353 | 0.58 | 0.00058 |
| **Ti** | 334.941 | | |
| **Zn** | Averaged 206.200 and 213.856 | 0.031 | 0.000031 |

#### 5.3.3.3.4 Calculations

In order to understand the effect of each specific soil system on the final composition of the amendment spent grain, obtained average element concentrations for the fresh grain (spent grain not used as soil amendment) were considered as a background and subtracted from spent grain used as soil amendment. The subtraction of the background fresh grain from the amendment spent grain resulted in delta values that were denominated "sorbed elements" from the soil to the spent grain (increased concentrations in respect to the background of fresh grain) and elements "released" from the spent grain into the soil (decreased concentrations in respect to the background of fresh grain).

As the total mass of spent grain used as a soil amendment in each microcosm (13.75 g) is different to the total mass of soil in the same respective microcosms (55 g), if a certain (fixed) quantity of an element (mg) moves from the soil (0.055 kg) to the amendment spent grain (0.01375 kg) via sorption, it becomes concentrated (mg $kg^{-1}$).

For this reason, in order to compare sorbed/released element concentrations in spent grain with element concentrations in the respective soil, concentration in spent grain (mg sorbed/ released element per kg spent grain) were recalculated according to the respective soil mass (mg sorbed/ released element per kg soil), according to the following equation:

$$\mathrm{SR} = \frac{(\mathrm{ASGxn} - \mathrm{FSGx}) * 0.01375}{0.055}$$

With:

- ASGxn: average concentration (mg $kg^{-1}$) of an element x in the amendment spent grain of microcosm n (n= P.jav1, P.jav2, P.jav3 and Control).
- FSGx: average concentration (mg $kg^{-1}$) of an element x in the fresh spent grain.
- 0.01375: total weight in kg of amendment spent grain in each microcosm (P.jav1, P.jav2, P.jav3 and Control).
- 0.055: total weight in kg of soil in each microcosm (P.jav1, P.jav2, P.jav3 and Control).

The resulting value (SR) in mg of an element in the amendment spent grain per kg of soil (mg $kg^{-1}$ soil), was named "sorbed" when positive (+) or "released" when negative (-).

A percentage of sorbed/released element x in relation to the total (sorbed/released + currently present in soil) concentration was then calculated as follows (% of SG+S).

For sorbed:

$$\%\ \mathrm{of\ S + SG} = \frac{\mathrm{Sbxn} * 100}{\mathrm{Sbxn + Xn}}$$

For released:

$$\% \text{ of } S + SG = \frac{Rlxn * 100}{Xn}$$

With:

- Sbxn: concentration of an element x sorbed in amendment spent grain from microcosm n (n= P.jav1, P.jav2, P.jav3 and Control).
- Rlxn: concentration of an element x released by amendment spent grain from microcosm n (n= P.jav1, P.jav2, P.jav3 and Control).
- Xn: average concentration of element x in the soil of microcosm n (n= P.jav1, P.jav2, P.jav3 and Control).

That is, the sorbed percentage of an element, was in respect to a theoretical initial total concentration of the element in the soil, consisting of the amount of this element present in excess in the spent grain and the amount present currently in the soil (Appendix CH5 A2). While the released percentage of an element will be in respect to the current concentration in the soil, and hence presuming the latter is a product of an initial content of this element in the soil, plus the quantity of it that was released into the soil by the spent grain. As an example, if in a microcosm 454.5 mg of Pb per $kg^{-1}$ soil were sorbed by the grain and 454.5 mg $kg^{-1}$ Pb remained in the soil:

- Xn = 454.5 mg $kg^{-1}$
- Sbxn = 454.5 mg $kg^{-1}$

$$\% \text{ of } S + SG = \frac{454.5 \text{ mg kg}^{-1} * 100}{454.5 \text{ mg kg}^{-1} + 454.5 \text{ mg kg}^{-1}} = 50 \% \text{ of Pb was sorbed}$$

An example for these calculations with real data is shown in Appendix CH5 A1.

### 5.3.4 Amendment of Pb, Phen and B(a)p spiked soil with spent grain and *P.javanicus*

#### 5.3.4.1 Experimental set up

##### 5.3.4.1.1 Soil Preparation

Non- sterile commercial garden soil was dried at 50ºC for 48 hours and sieved (<2 mm). 30g of soil were then placed in 26 glass 250ml beakers. 1920 mg of $Pb(NO_3)_2$ (Sigma-Aldrich, St. Louis, MO. USA) was dissolved in 200 ml of Milli-Q water. To achieve a concentration of 1000 mg $kg^{-1}$ Pb, 5 ml of this solution was transferred with a pipette into each 30g soil portion, making sure to distribute it evenly. Beakers were then mechanically shaken by hand and placed to dry at 50 ºC for 12 hours, after which they were allowed to return to room

temperature. 22.5 mg of Phen (Supelco, Bellefonte, PA, US) and B(a)p (Supelco, Bellefonte, PA, US) were dissolved in 60 ml of Acetone (Sigma-Aldrich, St. Louis, MO. USA). To achieve a concentration of 25 mg $kg^{-1}$ Phen and B(a)p, 2 ml of this solution was transferred with a glass syringe into each 30g soil portion, making sure to distribute it evenly. Beakers were then mechanically shaken by hand and left uncovered in a fume hood, allowing the acetone to evaporate. Soil was aged for five weeks in the fume hood, and mechanically mixed once a week with a glass stirrer for further homogenization.

#### 5.3.4.1.2 Measurement of general soil properties

**a) Moisture and LOI**

Moisture content and loss on ignition (LOI) were calculated on prepared soil without contaminant spike. 3 ~5g samples of soil were placed in an oven for 8 hrs at 105ºC to calculate moisture content, followed by 8 hrs at 450 ºC for the determination of organic matter content by LOI. Calculations were performed as in section 4.3.2.2.2

**b) pH**

pH was measured before and after spiking with an Orion 5 star Thermo Scientific pH meter with an Orion 8156BNUWP ROSS Thermo Scientific ultra-combination pH electrode. A modified version of USEPA method 9045D was used (175), where the sample weight was changed from 20g to 4g due to the reduced total sample volume, and the 1g:1ml soil: water ratio was changed to 1g: 2ml due to the high water holding capacity of the soil. pH before spiking was of 6.195 ± 0.221 and 6.364 ± 0.080 after.

#### 5.3.4.1.3 Selection of amendment combinations

As a follow up from the preliminary experiment, bioaugmentation with *P.javanicus* and biostimulation with spent grain amendment were included in this experiment. Although it was to be expected that commercial garden soil would contain sufficient P, the exact concentration of P in soil was unknown. For this reason, a treatment with a complementary addition of a P amendment, in the form of superphosphate (Westland, Cambridgeshire PE28 4HY, United Kingdom) was included.

In consequence, seven different treatment combinations and a control, with three replicates each, were planned: (a) Pb-Phen-B(a)p spiked soil on its own as a control (S1, 2 and 3); (b) soil + superphosphate (S+SP4, 5 and 6); (c) soil + spent grain (S+SG7, 8 and 9); (d) soil + inoculum (S+Pj10, 11 and 12); (e) soil + spent grain + inoculum (S+SG+Pj13, 14 and 15); (f) soil + sterile spent grain + inoculum (S+SSG+Pj16, 17 and 18); (g) soil + superphosphate

+ inoculum (S+SP+Pj19, 20 and 21); (h) soil + spent grain + superphosphate + inoculum (S+SG+SP+Pj22, 23 and 24).

Superphosphate (SP) was added to soil at a P/Pb ratio of 4, recommended in literature for P amendment of Pb contaminated soil (46, 57, 68), considering a content of 17.5% phosphorus pentoxide ($P_2O_5$). NaCl (Merck KGaA, Darmstadt, Germany) was also added in all treated soils to ensure the availability of Cl, required to form chloropyromorphite ($Pb_5(PO_4)_3Cl$), at an excess Pb/Cl molar ratio of 5/3.5.

The ratio of soil/spent grain was adapted from that proposed in Crosier (2014) (153) for agricultural utilization of raw (not composted) brewers spent grain, where 30 (American) gallons of spent grain (equivalent to 113.56 l) were added to a volume of soil described by the top 8 cm of a 4.5$m^2$ plot area, giving a volume ratio of soil/spent grain of 3.17. The average density of our soil, calculated by weighing in triplicate, a beaker filled with 50 ml of soil, without compressing, was approximately 0.6g $ml^{-1}$, and that of the spent grain (with the same method) was 0.5g $ml^{-1}$. At these given densities and with the literature volume ratio, a total addition of 6.572g of spent grain, including both the inoculum and additional amendment, was calculated for 25g of spiked soil.

#### 5.3.4.1.4 Microcosm preparation

Fifteen spent grain-*P.javanicus* inoculums were prepared as described in section 5.2.2, but using only 5g of spent grain and 5ml of Milli-Q water in 125ml Erlenmeyer flasks (Figure 5.3). Grain was in turn inoculated from two weeks old MEA plates, prepared from the glycerol stock. *P.javanicus* was incubated at 20ºC and 150 rpm in the dark for 30 days (Figure 5.3).

*Figure 5.3. Examples of 30 days old P.javanicus-spent grain inoculums before addition of soil.*

At 30 days nine additional 125 Erlenmeyer flasks were sterilised (121ºC for 15 minutes). From each 30g spiked soil portions, 25g were added into each Erlenmeyer flask (15 with and 9 without inoculum). Additional amendments were then added according to the different

treatments, as shown in Table 5.2. Soil and added amendments were mixed using sterile glass stirrers, and 30 ml of sterile Milli-Q water was added to each replicate. Flasks were then placed in the incubator at 20ºC and 150 rpm in the dark (Figure 5.4).

*Table 5.2. Weights of different components of each microcosm treatment combination.*

| Treatment | Soil (g) | Inoculum (spent grain g) | NaCl (g) | Superphosphate (g) | Spent grain (g) |
|---|---|---|---|---|---|
| S (control) | 25 | | | | |
| S+SP | 25 | | 0.005 | 0.196 | |
| S+SG | 25 | | 0.005 | | 6.572 |
| S+Pj | 25 | 5 | 0.005 | | |
| S+SG+Pj | 25 | 5 | 0.005 | | 1.572 |
| S+SSG+Pj | 25 | 5 | 0.005 | | 1.572 (sterile) |
| S+SP+Pj | 25 | 5 | 0.005 | 0.196 | |
| S+SP+SG+Pj | 25 | 5 | 0.005 | 0.196 | 1.572 |

**The g of Inoculum refer to the amount of spent grain utilized for the preparation of the inoculum as described in section 5.3.2 and shown in Figure 5.3.*

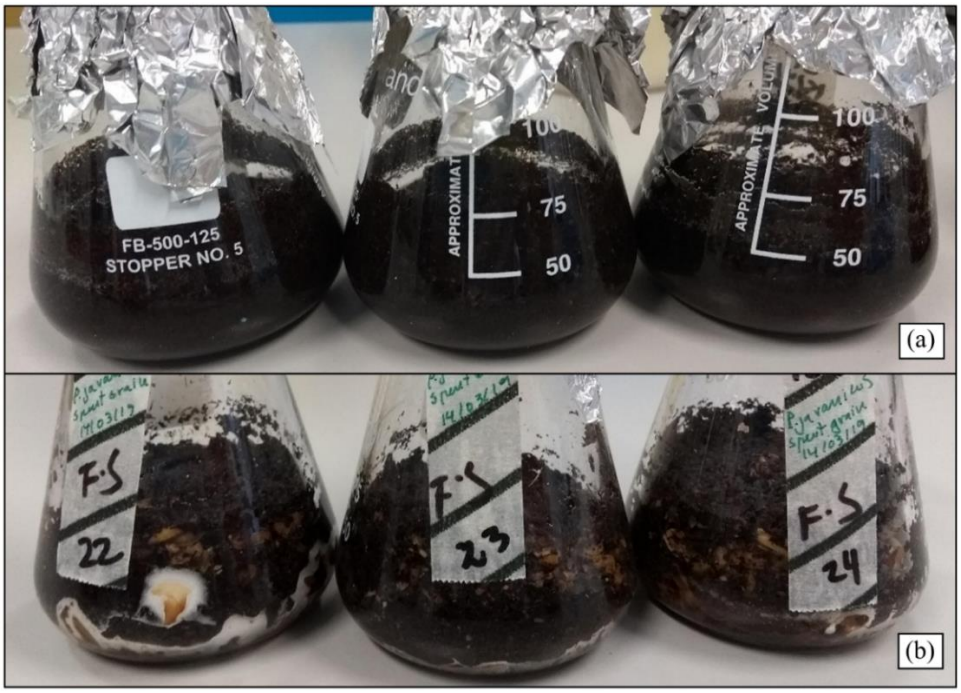



*Figure 5.4. Examples microcosms before incubation: (a) all 3 replicates from the S+SP treatment; (b) all 3 replicates of S+SP+SG+Pj treatment.*

Growth was monitored by qualitative visual observation, and photographs were taken regularly to record the fungal growth.

#### 5.3.4.1.5 Interventions during the experiment

Responding to *in situ* observations, the following interventions were carried out overtime, all in a sterile environment (the justification and effect of such interventions is discussed further in section 5.4.3.1):

Day 15: 5ml of sterile Milli-Q water was added with sterile pipettes to treatments S+SG, S+SG+Pj, S+SSG+Pj, and S+SP+SG+Pj, that is all those with grain amendment additional to the inoculum grain. All remaining treatments received an addition of 2.5ml of sterile Milli-Q water.

Day 44: treatments S+Pj, S+SG+Pj, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj were re-inoculated with two Pasteur pipettes cut outs (as described in section 5.3.2) from two weeks old MEA plates, prepared from the same glycerol stock used for the original inoculation.

Day 60: 3ml of sterile Milli-Q water was added to treatments S+SG, S+Pj, S+SG+Pj, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj. Soil was then gently agitated with sterile pipettes to allow oxygenation.

Day 70: treatments S+Pj, S+SG+Pj, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj were re-inoculated from a 10-days liquid (PDb) culture, prepared from the same glycerol stock used for the original inoculation. The excess supernatant was carefully poured out from the liquid culture flask and with a sterile spatula a small portion of the fungal biomass was transferred into each soil flask.

Day 91: 3 ml of sterile Milli-Q water were added into all treatments.

Day 129: 3 ml of sterile Milli-Q water were added into all treatments.

#### 5.3.4.1.6 Harvest

After 150 days all flasks were removed from the incubator and immediately stored at -80°C. Freeze drying was carried out, six flasks at a time, at -121ºC in a vacuum for 72 hrs. Flask mouths were covered with a fine metallic mesh to avoid sample loss. After drying, the content of flasks (soil, grain and fungi) were disaggregated with a porcelain pestle, sealed and returned to -80°C storage. Once all flasks were dried, approximately 2/3 of the content was separated into a sample bag and stored at -4°C for further processing for the analysis of Pb (Chapter 6) and pH measurement (as described in section 5.3.4.1.2). The remaining sample was kept in the flasks at -80°C, for further processing for the analysis of Phen and B(a)p (Chapter 7).

### 5.3.5 Statistics

All statistical analysis was carried out in Microsoft Excel (2016). Errors were calculated using the t-distribution confidence interval formula, appropriate for populations under 30:

$$X \pm t_{n-1} \frac{s}{\sqrt{n}}$$

Where

X= average value for a group of measurements

n= sample size (number of measurements)

n-1= degrees of freedom

s= standard deviation

t= constant from the t Table

The two-tails probability was set at p=0.05

All remaining analysis was done using the Data Analysis package. Statistical significance was evaluated with the "t=Test: two sample assuming unequal variance" and "Anova: single factor", with significance assumed at a P-value < 0.05.

## 5.4 Results and discussion

### 5.4.1 Fungal growth on inoculated spent grain

Experiments were carried out to determine the suitability of brewery spent grain as "growth media" for the preparation of *P.javanicus* inocula. *P.javanicus* was successfully inoculated into sterilized spent grain, indicating the latter is a suitable matrix for the growth of the fungus, as well as sufficient as the sole source of nutrients for the preparation of *P.javanicus* inoculums. The growth rate however differed between the two rounds.

In the first round, after 5 days, fungal growth was mostly limited to the inoculation MEA plug, while the beginning of some obvious transference to the surrounding grain was observable in one of the replicates. At 10 days fungal growth had a clear increase, being well spread around the surface of the spent grain layer, with the main growth area occurring around the inoculation MEA plug, but also with separate smaller colonies beginning to grow (Figure 5.5-c). By day 12, as the main and smaller colonies grew, they began to merge and by day 17 the surface of the spent grain layer was almost entirely covered with fungus in convex shaped colonies (Figure 5.6-a). At this time, areas of the colonies (initially white) acquired a slightly pinkish brown colour, and exudate droplets appeared on their surface. Penetration of the mycelium into the spent grain layer also began to be obvious, with a few growth spots observable from the base of the flask. From then on, the surface layer of fungi thickened slightly (Figure 5.7-a) while the penetration towards the base of the flask increased, being quite evident by day 24 (Figure 5.7-c). As successful colonization of the spent grain matrix had been achieved, flasks were removed from the incubator after 25 days.

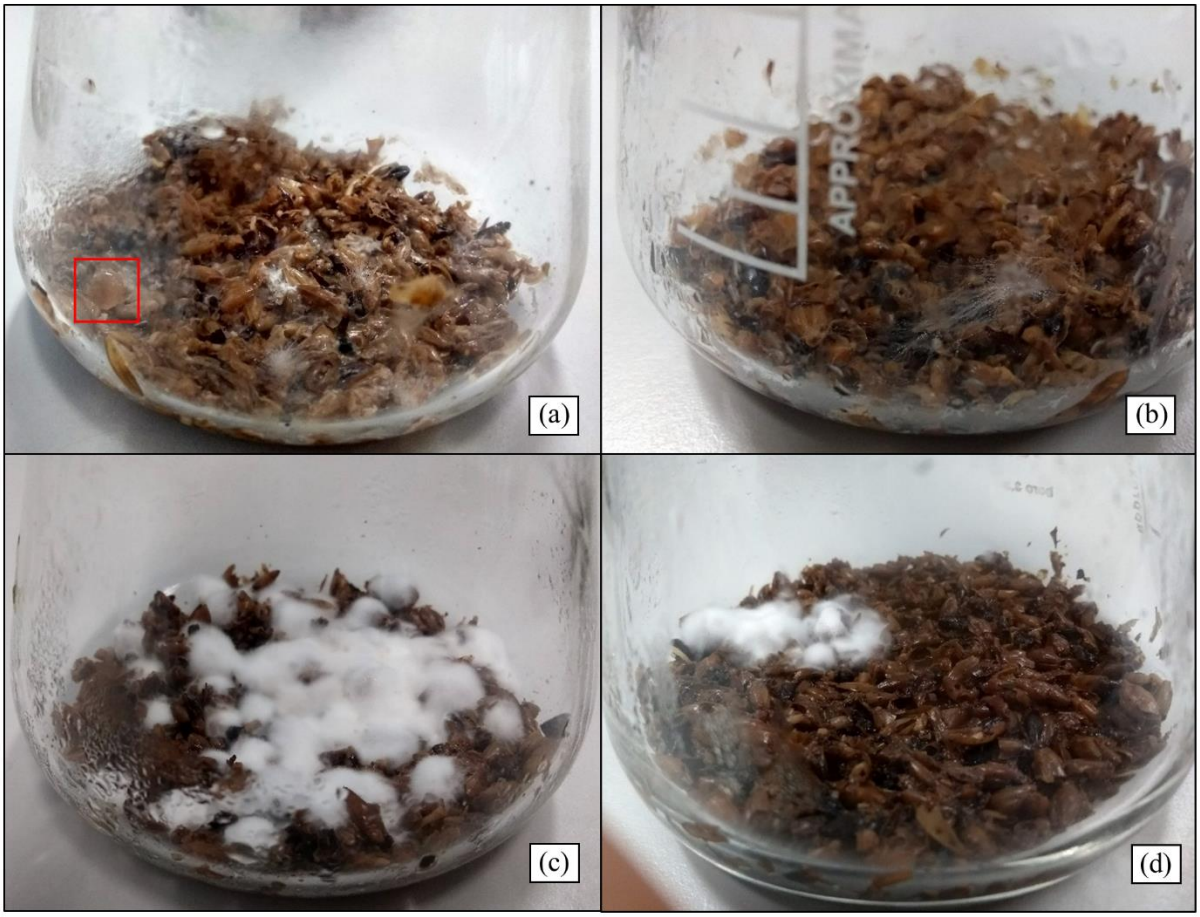



*Figure 5.5. Examples of growth on spent grain inoculums: (a) SG-Pj (round 1) at 10 days with the MEA inoculation plug marked with a red square; (b) SG control (round 2) at 11 days for comparison with SG-Pj; (c) SSG-Pj1 (round 1) at 10 days; SSG-Pj5 (round 2) at 11 days.*

The SSG control remained unchanged over the incubation time, with no obvious decomposition occurring, and no observable growth (Figure 5.7-d).

The SG-Pj flask, with non-sterile spent grain, showed no growth spreading from the inoculation MEA plug, as can be observed in Figure 5.5-a, at 10 days of incubation. Over time the spent grain began to show signs of decomposition, acquiring a lighter yellowish-brown colour and a slimy aspect. Growth of a white filamentous ("hairy" looking) fungus was also observed and increased over time (Figure 5.7-e at 24 days). This indicates that, while the sterilized spent grain proved appropriate for the growth of *P.javanicus*, the microorganisms already present on non-sterile spent grain had a competitive advantage over the inoculated population of *P.javanicus*, preventing its growth.

In the second round SSG-Pj replicates displayed a similar behaviour in the first few days, with fungal growth initially limited to the inoculation MEA plug, and transference onto the surrounding grain being observed after the first 5 days.

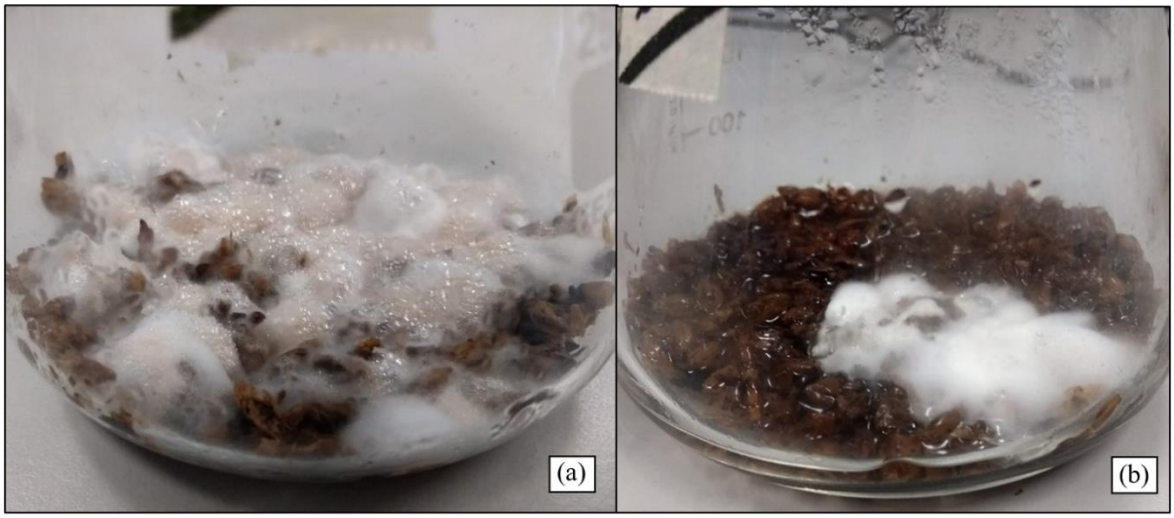



*Figure 5.6. Examples of growth on spent grain inoculums at 17 days: (a) SSG-Pj1 (round 1); (b) SSG-Pj5 (round 2) after addition of extra 10 ml of sterile Milli-Q water.*

At day 11 however, growth was considerably reduced in respect to the first round. With the exception of one replicate (SSG+Pj4), growth was limited to the immediate surrounding of the inoculation MEA plug, and no additional/separate smaller colonies had appeared (Figure 5.5-d). Growth continued to appear much slower than in the first round. Although the same amount of water was added as in the first round, before sterilization, more of it may have been lost in the form of vapour when opening the culture flasks for inoculation. For this reason, on day 17, a further 10 ml of sterilized Milli-Q water was added into the flasks with sterile pipettes (Figure 5.6-b).

After this addition of water, fungal growth rate did increase, with *P.javanicus* covering a significant portion of the surface of the spent grain layer by day 23 (Figure 5.7-b, next to an example from the first round for comparison Figure 5.7-a). Besides replicate SSG+Pj4 which had developed separate smaller colonies earlier on, growth of the other replicates after the water addition occurred in a different way than the first round. Rather than various convex shaped colonies merging with one another, as was observed in the first experiment, growth seemed to continue to stem from the main colony, in the form of a thin layer covering the surface of the spent grain, that thickened as the days passed. By day 32 the fungal layer was well developed over the spent grain (Figure 5.8-c), and flasks were removed from the incubator. Penetration into the spent grain layer, observable from the base of the flask (Figure 5.8-d), was considerably reduced compared with the first round.

The SG control, on the other hand, had a similar behaviour to that of the SG-Pj control in the first round. Spent grain began showing signs of decomposition after a few days, and by day 11 growth of a white filamentous (“hairy” looking) fungus could also be observed (Figure 5.5-b).

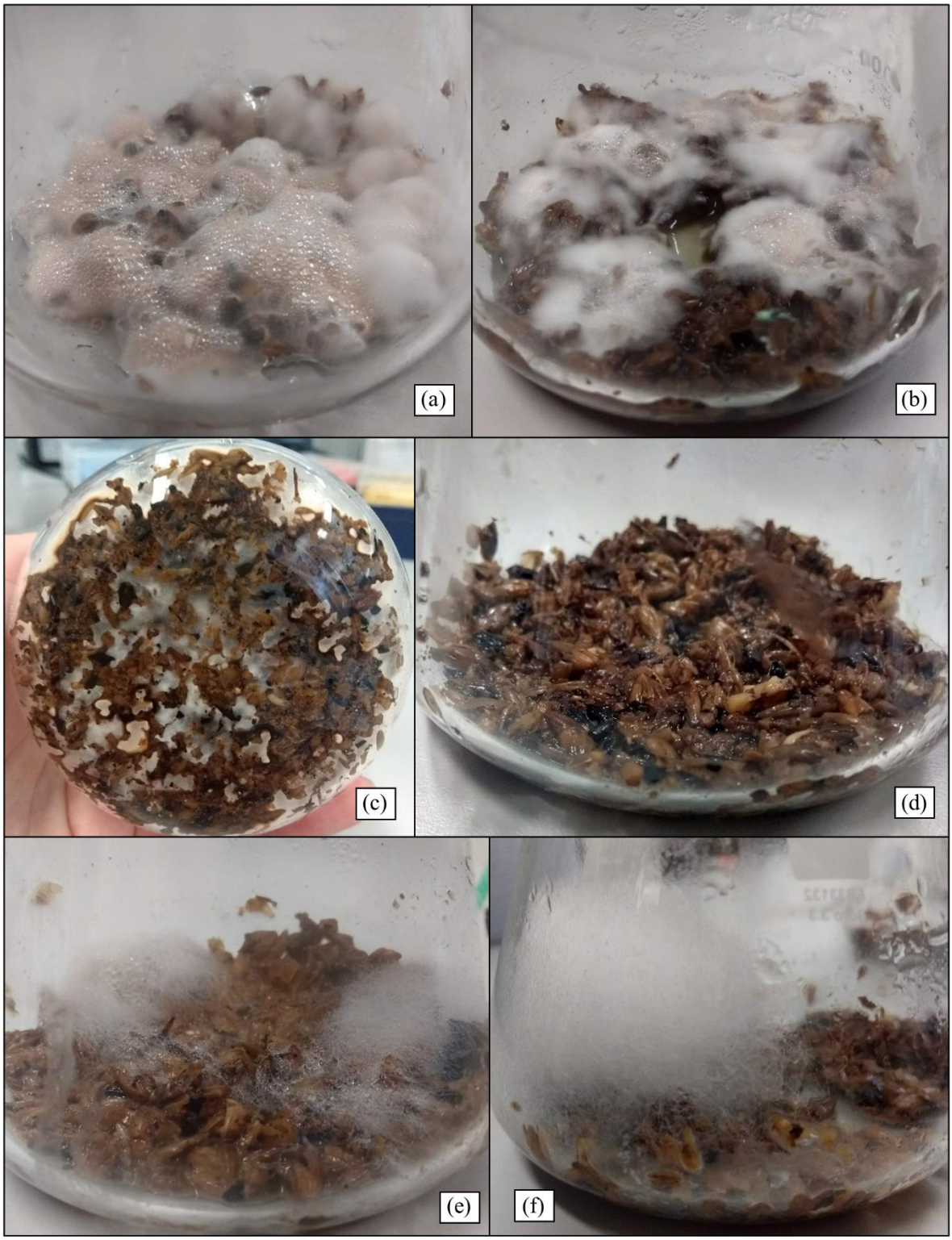



*Figure 5.7. Examples of growth on spent grain inoculums: (a) SSG-Pj2 (round 1) at 24 days; (b) SSG-Pj6 (round 2) at 23 days; (c) SSG-Pj1 (round 1) at 24 days, growth observed from the base of the flask; (d) SSG control (round 1) at 24 days; (e) SG-Pj (round 1) at 24 days; (f) SG control (round 2) at 23 days.*

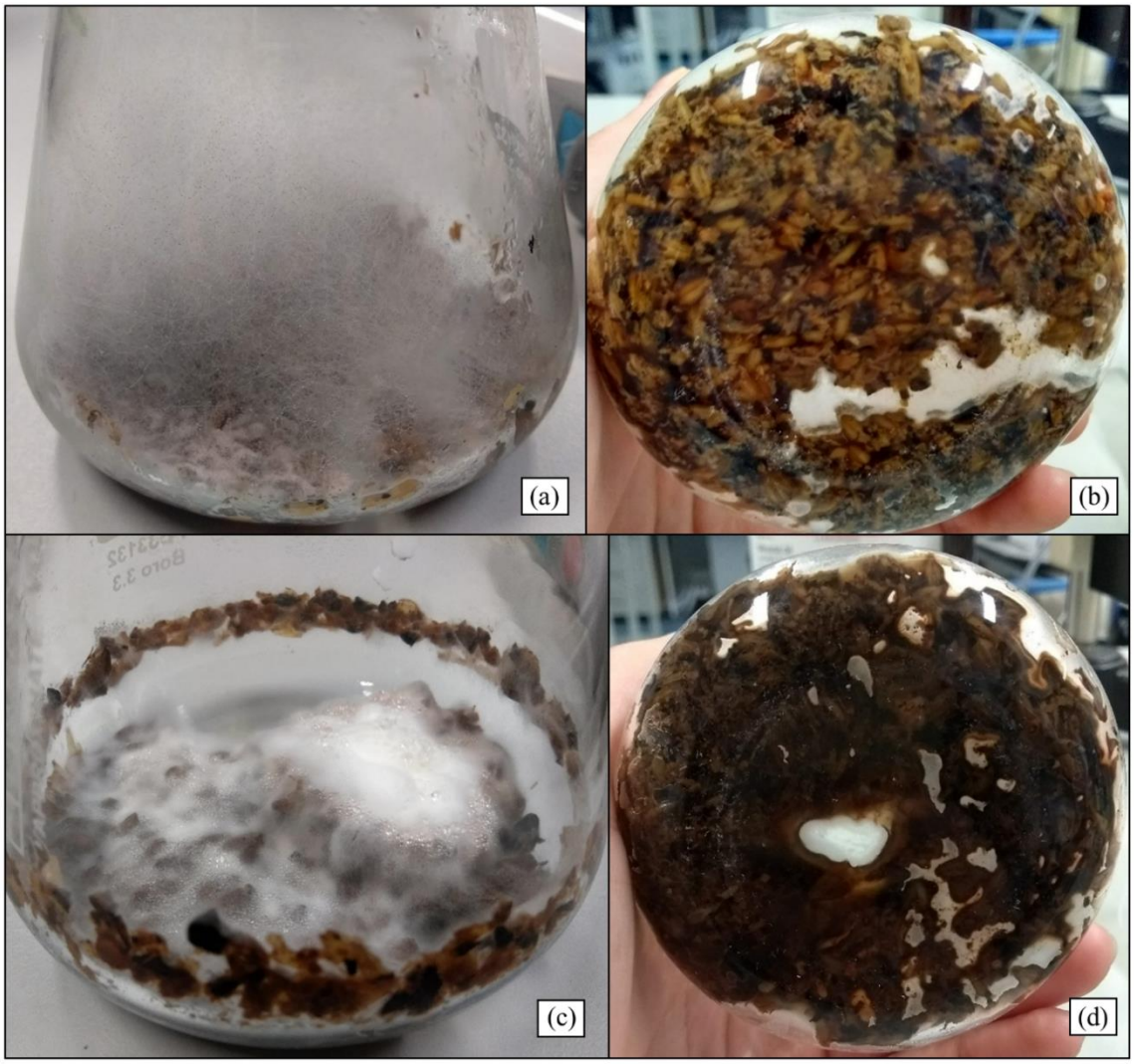



*Figure 5.8. Examples of growth on spent grain inoculums from round 2 at 32 days: (a) SG control; (b) SG control, growth observed from the base of the flask; (c) SSG-Pj6; (d) SSG-Pj4, growth observed from the base of the flask.*

Growth of this unidentified fungus in non-sterile grain was faster in the second round, as can be observed in Figure 5.7-f (next to SG-Pj for comparison Figure 5.7-e). By day 28, growth of a pinkish organism (or consortium of organisms) was also observed on the surface of sections of spent grain. At the harvesting time (day 32, Figure 5.8-a and b) the filamentous fungus had grown beyond the 200ml flask mark, in height, and the pinkish layer covering the grains had also spread further.

The differences in the growth rate and pattern of *P.javanicus* between the two rounds, despite the set incubation conditions being the same, shows a level of sensitivity in the growth rate of the fungus to subtle variations in such conditions. Nevertheless, spent grain was proven as a suitable matrix for inoculum preparation when sterilised.

### 5.4.2 Preliminary soil experiment

The efficiency of bioaugmentation is determined by many biotic and abiotic factors (102). Poor soil conditions, common in contaminated soil and made ground, can often prevent successful inoculation of additional microorganisms and sustain their growth. Addition of available nutrients (biostimulation) is sometimes necessary to achieve a successful inoculation (75).

Brewery spent grain, in section 5.4.1, was proven as a suitable matrix for growing the fungus *P.javanicus*, when sterilised.

A preliminary soil experiment using real environmental soil samples from the Meat market site was carried out to evaluate the feasibility of inoculating *P.javanicus* in soil using the spent grain + *P.javanicus* preparation as an inoculum. Further addition of sterile and non-sterile spent grain a soil amendment to assist a successful inoculation was also tested.

### 5.4.2.1 Fungal growth on inoculated soil

After placing flasks in the orbital incubator, the soil quickly liquefied (179). The Meat market soil which was poor quality, made-ground with limited organic matter content or soil structure to influence soil water holding capacity, led to saturation, which in addition to the 150rpm mechanical shaking in the incubator, caused the soil to liquefy. In consequence the added mixture appeared as an aqueous sludge, which in flasks other than the Control, overlaid the inoculum spent grain that adhered to the bottom of the flask.

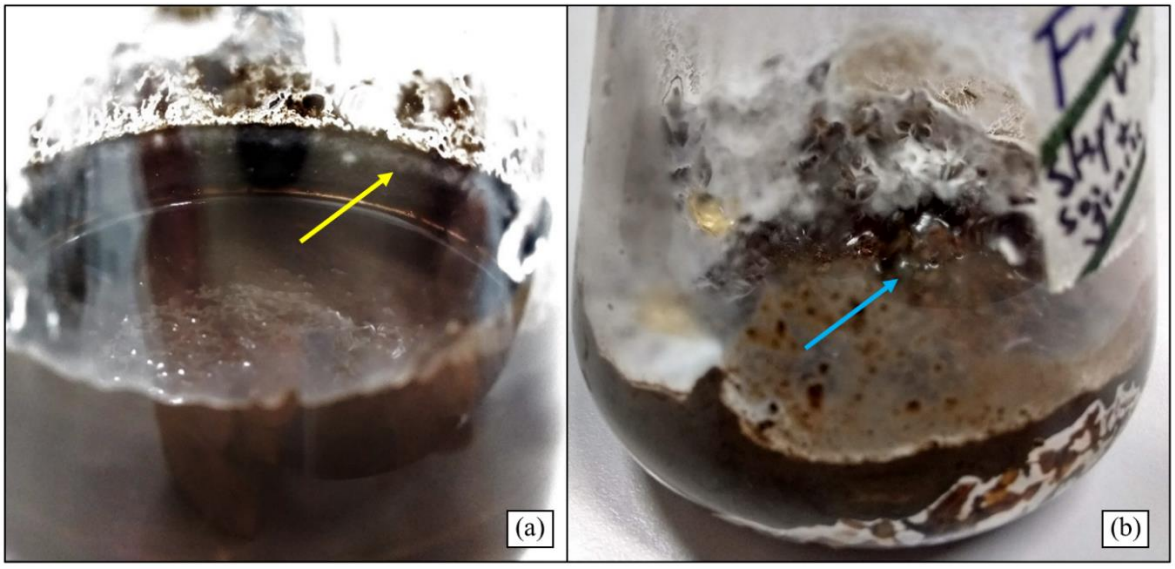



*Figure 5.9. Examples of growth after 6 days: (a) P.jav1 (MMS+SG+Pj), yellow arrow indicates fungal growth as a rim in the intersection between the sludge surface (when shaking in the incubator) and the flask wall; (b) P.jav2 (SMMS+SSG+Pj), blue arrow indicates sedimented soil with clear water over it.*

*Table 5.3. Summary of observations made over the incubation time of microcosms of the preliminary soil experiment (part 1).*

| Day of experiment | Observations |
|---|---|
| 6 | P.jav2 (SMMS+SSG+Pj) already showed signs of significant growth of *P.javanicus*, with a sizeable reduction of water content, and sedimentation of the soil (Figure 5.9-b and Figure 5.10-a). An apparent selective accumulation of spent grain was observed on the surface, associated to the main fungal growth body, surrounding a central area of sedimented sandy soil covered with a layer of clear water. P.jav1 and 3 (MMS+SG+Pj), in contrast, had only developed fungal growth as a rim in the intersection between the rotating sludge surface and the flask wall (Figure 5.9-a). |
| 8 | The Control (MMS+SG) flask began showing growth of a similar organism after 8 days, suggesting that the organism observed in P.jav1 and P.jav3 (MMS+SG+Pj) had most likely originated from the added non-sterile spent grain or soil, and not from the inoculum. |
| 13 | The consistency of the sludge in P.jav1 and P.jav3 (MMS+SG+Pj) seemed to have thickened in comparison to Control (MMS+SG), and in P.jav 2 (SMMS+SSG+Pj) there was a reduction in the quantity of the water over the soil. |
| 17 | The growth of fungus on the rim of Control (MMS+SG) had continued, further populating a circumference around the flask wall, but with no increase in wideness (Figure 5.10-b), unlike P.jav1 (Figure 5.10-c) and P.jav3 (MMS+SG+Pj). In the latter, the continuous increase in the viscosity of the sludge lowered the intersection of its surface with the flasks walls, allowing the rim of fungus to widen. In P.jav2 (SMMS+SSG+Pj) the clear water covering the soil had now disappeared, and instead a very thin layer of fungus covered the soil. This layer thickened slightly after a few days, becoming more clearly visible (Figure 5.10-d). |
| 23 | The sludge in P.jav1 and P.jav3 (MMS+SG+Pj) appeared to have fully solidified, unlike the control that continued to be aqueous (Figure 5.11). It can also be observed in Figure 5.11, how the growth of fungi in the flask walls had continued to expand in P.jav1 (MMS+SG+Pj), while the control showed no further growth since day 17. P.jav2 (SMMS+SSG+Pj) continued showing the same trend, with active growth of *P.javanicus* visible in the surface of the soil, but also at the base of the flask, in the inoculum spent grain, unlike P.jav1 and 3 (MMS+SG+Pj) where growth in the inoculum grain was no longer visible. |
| 27 | The fungus growing on the flask walls of P.jav1 and P.jav3 (MMS+SG+Pj) had begun to spread onto the sludge surface. The appearance was similar to that in the flask walls, white and filamentous in a thin layer, but also disseminated on this layer, white granules had appeared, visible in Figure 5.12-a and b. |
| 38 | Quite suddenly a crater appeared in the centre of P.jav3 (MMS+SG+Pj), with solid vertical walls and aqueous sludge inside (Figure 5.12-a). Material (sludge with spent grain) from the crater was expelled to one side over the solid sludge surface. |
| 55 | The water in the crater (P.jav3-MMS+SG+Pj) was clear, having the mud particles been removed, leaving the bottom visible with light coloured sandy sediment (Figure 5.12-b). The walls of the crater appeared to have a grainy texture, much like the fungus growing on the surface of the solidified sludge (Figure 5.12-b). Small elongated filaments could also be observed underwater, stemming from the crater walls. Lastly, the grain layer at the bottom of the flask had slightly detached from the glass. The latter was more evident in P.jav1 (MMS+SG+Pj), where the contraction and detachment of the grain and soil mixture from the bottom of the flask, had left a clear air pocket (Figure 5.12-c). P.jav2 (SMMS+SSG+Pj) was beginning to show growth within the soil profile, visible through the flask walls (Figure 5.12-d). |

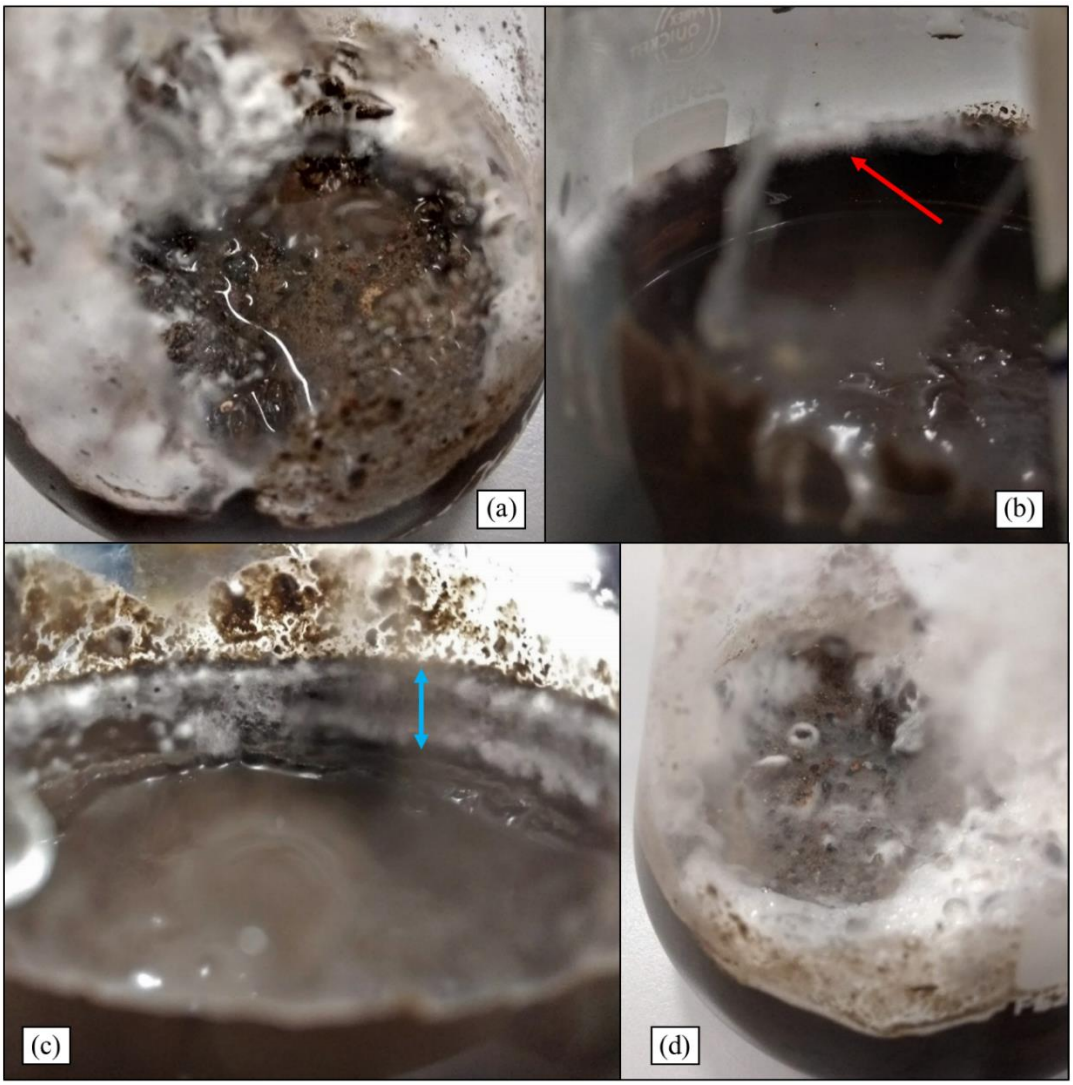



*Figure 5.10. Examples of changes in microcosms overtime: (a) P.jav2 (SMMS+SSG+Pj) at day 8, showing P.javanicus growing around a central area with sand and clear water; (b) Control (MMS+SG) at day 17, red arrow indicates fungus growing as a rim in the intersection between the sludge surface and the flask wall; (c) P.jav1 (MMS+SG+Pj) at day 17, with blue arrow indicating widening of rim with fungal growth; (d) P.jav2 (SMMS+SSG+Pj) at day 20, showing P.javanicus growing over the central sandy soil area.*


*Figure 5.11. Comparison of P.jav1 (MMS+SG+Pj) (left) and Control (MMS+SSG) (right) at day 23. P.jav 1 is noticeably dryer and has wider ring of fungal growth on the flask wall (red arrow) than the control (blue arrow).*

*Table 5.4. Summary of observations made over the incubation time of microcosms of the preliminary soil experiment (part 2).*

| Day of experiment | Observations |
|---|---|
| 64 | Abruptly, the contracting mud and grain mixture in P.jav 1 (MMS+SG+Pj) collapsed to the bottom of the flask, having detached from the flask wall. The inoculum grain on the base acquired a blackened colour. |
| 70 | Flasks where removed from the incubator for harvesting. P.jav 2 (SMMS+SSG+Pj) continued to show actively growing colonies of *P.javanicus*, with abundant biomass visible in the surface, base and profile of the soil (Figure 5.13-a, b and c). P.jav 1, after the collapsing of the mud towards the bottom of the flask, showed no noticeable changes (MMS+SG+Pj). After the initial spread towards the sludge surface, no major fungal growth was noticeable, and unlike P.jav2 no growth was clearly visible on the basal grain or on the soil profile (Figure 5.14-a, b and c). In P.jav 3 (MMS+SG+Pj), the central crater no longer contained water and walls had receded, showing an approximately layered structure (Figure 5.14-d). The fungus growing on the surface of the mud acquired a yellowish tone (Figure 5.14-d). As in P.jav1, no visible growth continued on the basal grain (Figure 5.14-e). The control remained mostly unchanged overtime, only a slight increase in viscosity was noticeable, and the small amount of fungal growth on the flask wall, which appeared earlier during the first 15 days, didn't increase like in other flasks (Figure 5.13-d). |

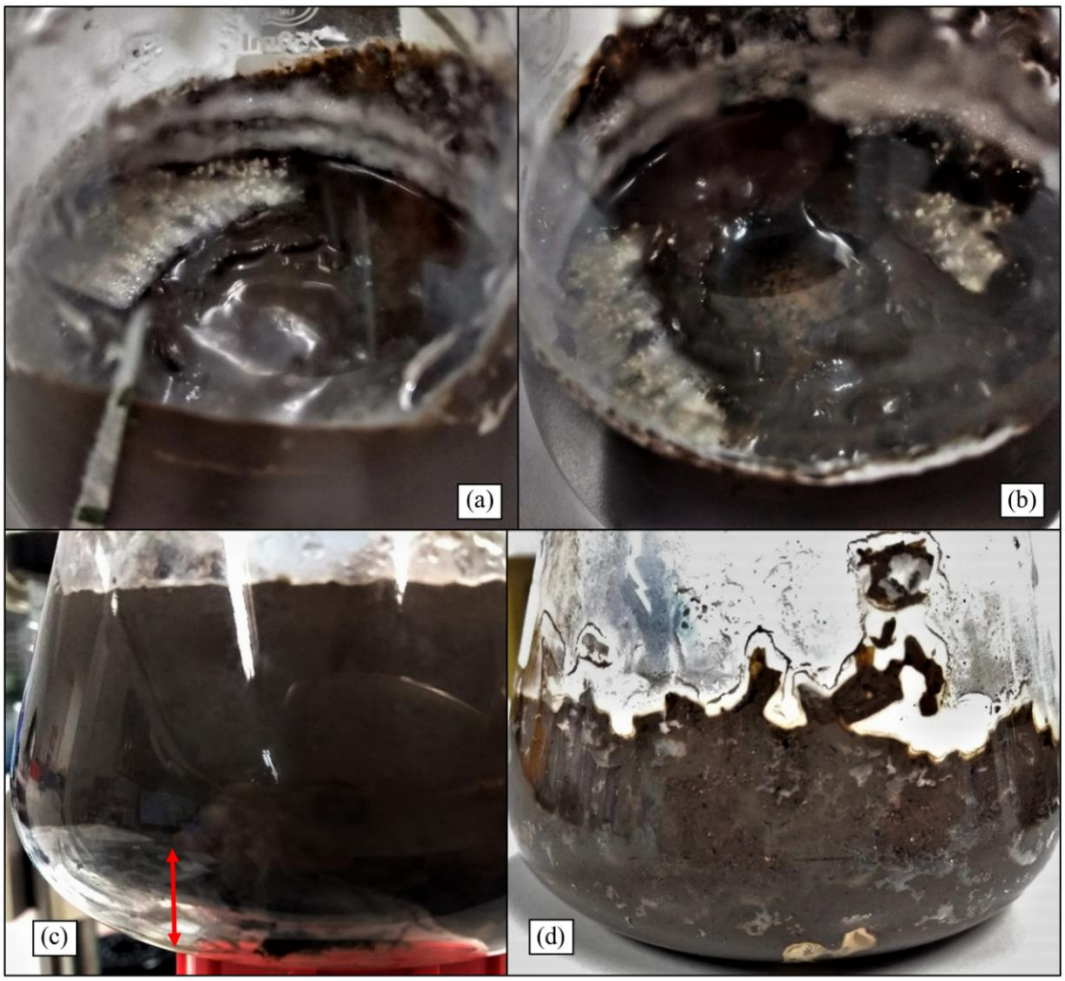



*Figure 5.12. Examples of changes in microcosms overtime: (a) P.jav3 (MMS+SG+Pj) at day 44 with central crater filled with muddy water; (b) P.jav3 (MMS+SG+Pj) at day 55, with clear water filling the central crater and sand at the bottom; (c) P.jav1 (MMS+SG+Pj) at 55 days, red arrow indicates detachment of the spent grain and soil from the base of the flask, leaving an air pocket (d) P.jav2 (SMMS+SSG+Pj) at day 55 with fungal growth appearing on the soil profile.*

Although simple, this experiment resulted in useful observations that aided the decision making for consecutive experimental steps. P.jav2 (SMMS+SSG+Pj) acted as a control with idealised sterile conditions (in both soil and grain), removing entirely the microbial competition with native microorganisms present in the soil and spent grain. This provided more confidence that any visible fungal growth corresponded to *P.javanicus*. As a result, it was concluded that when excluding any effects of the native microbial population, it was indeed possible to introduce *P.javanicus* in soil using a spent grain inoculum, and additional spent grain amendment as a nutrient source.

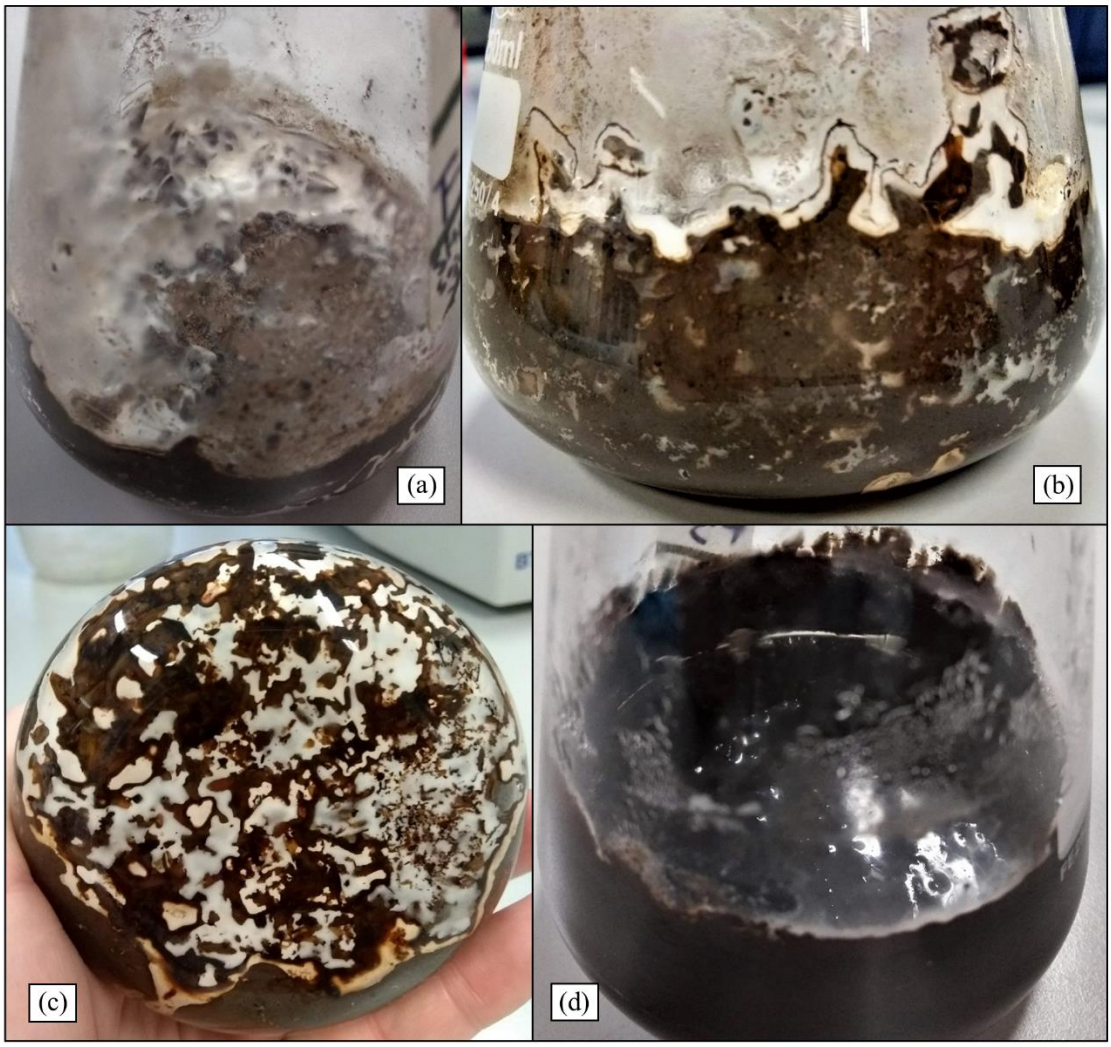



*Figure 5.13. Microcosms before harvesting: (a) P.jav2 (SMMS+SSG+Pj) at 69 days view from above; (b) P.jav2 (SMMS+SSG+Pj) at 69 days, fungal growth visible in soil profile; (c) P.jav2 (SMMS+SSG+Pj) at 70 days, abundant fungal growth visible from the flask base; (d) Control (MMS+SSG) at 70 days.*

In the case of P.jav1 and P.jav3 (MMS+SG+Pj), although the successful introduction of *P.javanicus* in the soil matrix cannot be asserted, some conclusions can be drawn from noted observations.

In sedimentary systems physical dynamics as well as chemical processes translate in specific patterns and structures of erosion and deposition of sediment. However, microorganisms also have an active response to the physical and chemical conditions of their muddy or sandy substrate. This response leaves traces behind, which in sandy deposits have been termed microbially induced sedimentary structures (MISS) (180). These structures arise from the interaction of biofilms and microbial mats with the physical sediment dynamics in siliciclastic aquatic environments (180).

Biofilms are considered to be a layered consortia of microbes, in which materials needed to sustain life can be recycled (181). Extracellular polymeric substances (EPS) are usually an important component of biofilms, and consist of mechanically stable mucilages composed of complex polysaccharides, enzymes, proteins, lipids, extracellular DNA, as well as detritus from the environment (180). EPS are electrostatically charged and in consequence clays and other minerals can be trapped. They also provide chemical bonding sites due to their content in pyrovate ketals, uronic acids, sulfate and phosphate groups (180). Microbial mats are defined as macroscopically visible layered biofilms (180). Biofilms and microbial mats can hence engineer their sedimentary habitat to promote their survival by buffering erosion and deposition. They do so through processes called biostabilization, which counteracts erosion, and by baffling and trapping, which facilitates deposition (180).

When it comes to MISS and biofilms, attention has mostly been focused on photoautotrophic organisms in marine/coastal environments such as stromatolite forming cyanobacteria. However, various types of microorganisms, including fungi, can form and be found in biofilms in various environments, such as caves, forests and woodland soils, toxic and acid waste sites, anoxic lakes and ponds (181).

Although the chemical-physical dynamics and microbial population in the artificial systems of this experiment are different from those developed in well studied marine-coastal environments, parallels can be made between structures observed in the experiment, particularly in P.jav 1 and P.jav3 (MMS+SG+Pj) microcosm systems, and defined/described MISS.

Physical/hydraulic conditions (shaking at 150 rpm) remained unchanged overtime during the entire experiment. Flasks also remained sealed (they were not opened at any time point during the experiment) meaning the system remained closed to the escape of gases or water vapour. In addition, they were kept at a constant temperature, therefore an equilibrium between liquid and gaseous water should have been achieved, preventing further evaporation of water from occurring. However, the sludge in P.jav1 and P.jav3 (MMS+SG+Pj) became

increasingly viscous, until complete stagnation (Figure 5.11-a). If no changes in the hydraulic conditions or continuous evaporation of water was occurring, the consumption of water and deposition of sediment could only be attributed to the baffling, trapping and biostabilization abilities of a growing microbial population. Microbial filaments create micro-zones of lower current velocity that allow silt-sized grains to settle (180).

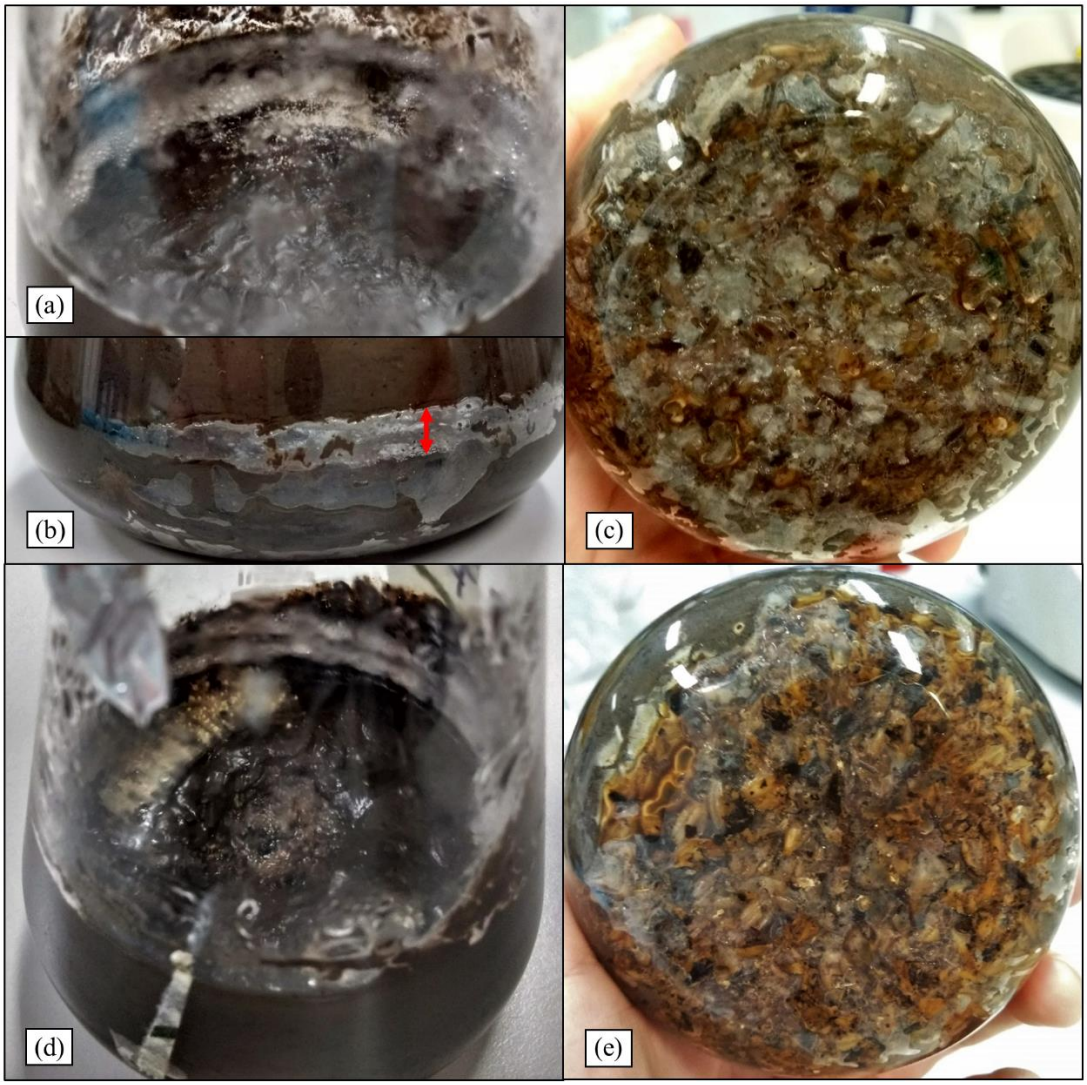



*Figure 5.14. Microcosms before harvesting: (a) P.jav1 (MMS+SG+Pj) at 70 days, view of surface; (b) P.jav1 at day 70, profile view, with red arrow indicating detachment of flask wall after collapse towards the bottom of the flask; (C) P.jav1 at 69 days, basal view with darkened spent grain; (d) P.jav3 (SMMS+SSG+Pj)at 70 days view of mud surface with central crater; (e) P.jav3 at 70 days basal view of inoculum spent grain.*

Gas domes, typically described for tidal settings with a high water line, form when mucilages of microbial mats block the quick escape of gases produced by the decay of underlying organic matter (180). These gasses become trapped underneath mats, exerting localized pressure that forms convex domes. Eventually these domes rupture, allowing the gases to escape, and collapsing in consequence (180). A similar process may have caused the sudden appearance of a crater in P.jav3 (MMS+SG+Pj, Figure 5.12-a). A biofilm formed within the

stagnated sludge may have blocked the escape of gases formed by the decay of spent grain towards the headspace of the flask. The consequent pressure build-up would have produced the rupture of the biofilm and expulsion of material over the sludge surface. The observed texture in the crater walls (granular and with filaments) support the idea of microorganisms having grown within the sludge forming the biofilm. The muddy water inside the crater, in contrast with the solidified structured crater walls around it, may have emanated from the internal structure of the ruptured biofilm.

The water in the crater became clear overtime, revealing a sandy bottom layer (Figure 5.12-b). At a constant 150 rpm, the removal of fine particles from the water could have only been a product of the microbial activity. Baffling refers to sedimentary particles suspended in supernatant seawater being 'combed out' by vertically oriented filaments (180). In this case, observed filaments stemming from the crater walls may have combed out the fine particles, causing the bottom of the crater to become sandy instead of muddy, once the water was clear. A parallel with P.jav2 (SMMS+SSG+Pj) can also be made. Although fungal growth and sedimentation occurred too quickly for different stages in the process to be observed, in P.jav 2 the main fungal colony also surrounded a sandy sedimented area covered with clear water (Figure 5.10-a). This could be indication that *P.javanicus* combed out fine particles from the surficial water, leading to the formation of this sandy sediment cover.

Towards the end of the experiment the clear water in the crater of P.jav3 (MMS+SG+Pj) disappeared. The walls of the crater receded revealing an approximately laminated-levelled structure (Figure 5.14-d), typical of layered microbial mats (180). It has been described (180) that when a microbial mat is locally disturbed, by the deposition of a mollusc shell for example, a local shadow is formed and microorganism react by migrating away. In consequence, the mat texture around the local shadow becomes less coherent. A similar effect may have been caused by the crater, leading to a migration of the surrounding microorganisms and the consequent recession of the crater walls.

Lastly, the contraction of the sludge-grain mixture, most apparent in P.jav1 (MMS+SG+Pj, Figure 5.12-c), was most likely a consequence of the growing biofilms inside the sludge, reacting to a limited supply of water. Structures arisen from the shrinkage of microbial mats have been described in literature (180), and although mud can contract (abiotically) due to drying, as flasks were sealed this is unlikely to be the cause.

All the parallels drawn with MISS strongly suggest that an active microbial population was driving the changes in P.jav1 and P.jav3 (MMS+SG+Pj) overtime. Although there is no concrete evidence that *P.javanicus* was responsible or actively participating in this process,

the clear difference with the Control (MMS+SG) microcosm, that remained largely unchanged, suggests that the inoculation of *P.javanicus* did play an important role in the P.jav1 and P.jav3 (MMS+SG+Pj) microcosm systems. In consortium with other organisms present in the soil and spent grain, *P.javanicus* may have generated the described structures. As observed previously in liquid cultures (section 3.4.1), *P.javanicus* can form biofilm-like assemblages attached to flask walls, as well as elongated filaments stemming from floating and attached colonies.

Biofilm formation on hydrocarbon contaminated matrixes has been said to be especially well suited for the treatment of recalcitrant or slow-degrading compounds (75). Furthermore, the mucilaginous sheath (mainly polysaccharide) which can surround the surface of hyphae to provide protection, can also act as a matrix for fungus-metal-mineral interactions, harbouring mineral-weathering and metal-chelating agents which can result in crystal growth and deposition of mycogenic biominerals (78).

The formation of biofilms and/or hyphal networks within the sludge may hence have provided the necessary medium for interaction with potential contaminants such as Pb and PAHs, posing a positive precedent for the study of biomineralization of pyromorphite and effect on PAH contents by inoculation of *P.javanicus* in contaminated soils.

The use of sterile spent grain-*P.javanicus* inoculum and suitability of non-sterile spent grain for biostimulation, as a soil amendment to encourage the growth of *P.javanicus* in soil, showed some promising results, allowing for further study.

#### 5.4.2.2 Spent Grain and soil interaction

The digestion and ICP-OES analysis of spent grain, had both the purpose of studying any possible inputs into the soil system that may result from the addition of grain as an amendment, as well as how its composition changed after interacting with the components of each soil system (P.jav1, 2 and 3 and Control). For this reason, the grain used in microcosm was analysed alongside samples of "fresh spent grain", not used as soil amendment. As the sample preparation describes (section 5.3.3.3.1), grain was thoroughly washed to remove attached soil particles, so the resulting chemical concentrations would represent only the contents of the spent grain matrix itself.

Table 5.5 shows a summary with the average concentrations (2 replicates) per sample for the analysed elements, including standard deviation (SD), and Figure 5.15 shows box plots for each sample per element. Compared to registered composition in literature (143) (discussed in section 2.3), obtained element concentrations were lower, yet similar in magnitude in most

cases (Cr 5.9 mg $kg^{-1}$, Cu 18 mg $kg^{-1}$, Fe 193.4 mg $kg^{-1}$, Mg 1958 mg $kg^{-1}$, Mn 51.4 mg $kg^{-1}$, P 5186 mg $kg^{-1}$, Zn 178 mg $kg^{-1}$ (143)). Additional analysed elements (Cd, Co, Ni, Ti) had low concentrations <5 mg $kg^{-1}$. Pb with 21.6 ± 4.0 mg $kg^{-1}$, was slightly higher than some of the other metals, yet not significantly high in relation to soil concentrations.

Apart from Cd and P, all elements had higher concentrations in spent grain used as soil amendment. Cd had similar concentrations to fresh grain, while P had significantly lower concentrations in amendment spent grain.

*Table 5.5 Average concentration of elements in fresh spent grain and amendment spent grain samples.*

| | Fresh SG | | SG P.jav1 | | SG P.jav3 | | SG P.jav2 | | SG Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Average | SD | Average | SD | Average | SD | Average | SD | Average | SD |
| **Cd (mg $kg^{-1}$)** | 1.202 | *0.887* | 0.996 | *0.383* | 0.884 | *0.639* | 1.189 | *1.039* | 1.601 | *0.057* |
| **Co (mg $kg^{-1}$)** | 0.114 | *0.114* | 4.177 | *1.073* | 3.475 | *0.294* | 2.069 | *1.133* | 5.661 | *0.607* |
| **Cr (mg $kg^{-1}$)** | 0.465 | *0.465* | 11.48 | *0.487* | 12.31 | *0.150* | 13.14 | *1.424* | 14.46 | *0.015* |
| **Cu (mg $kg^{-1}$)** | 6.985 | *0.065* | 74.67 | *0.850* | 73.94 | *3.597* | 104.9 | *3.077* | 112.9 | *1.266* |
| **Fe (mg $kg^{-1}$)** | 78.89 | *1.255* | 11494 | *581.8* | 10818 | *803.9* | 8045 | *624.9* | 9123 | *534.6* |
| **Mg (mg $kg^{-1}$)** | 1654 | *8.176* | 1804 | *83.78* | 1708 | *213.1* | 1809 | *152.0* | 1945 | *103.9* |
| **Mn (mg $kg^{-1}$)** | 30.17 | *1.430* | 435.4 | *29.53* | 568.6 | *75.48* | 323.6 | *10.94* | 395.4 | *10.06* |
| **Ni (mg $kg^{-1}$)** | 4.504 | *1.326* | 27.47 | *0.115* | 27.71 | *2.231* | 23.53 | *2.222* | 25.25 | *0.460* |
| **P (mg $kg^{-1}$)** | 4551 | *66.93* | 2444 | *157.4* | 2283 | *355.8* | 2755 | *150.7* | 1688 | *94.53* |
| **Pb (mg $kg^{-1}$)** | 21.58 | *3.997* | 243.5 | *1.503* | 249.7 | *1.749* | 160.5 | *4.890* | 616.7 | *7.591* |
| **Ti (mg $kg^{-1}$)** | 0.745 | *0.119* | 73.99 | *9.701* | 82.51 | *9.166* | 33.66 | *5.327* | 120.1 | *26.49* |
| **Zn (mg $kg^{-1}$)** | 86.04 | *3.246* | 189.7 | *8.779* | 206.5 | *4.829* | 170.2 | *3.300* | 218.5 | *1.089* |

* *Control = MMS+SG; P.jav1 and P.jav3 = MMS+SG+Pj; P.jav2 = SMMS+SSG+Pj.*

In order to understand the effect of each specific soil system on the final composition of the amendment spent grain, a comparison with soil concentrations was made (Appendix CH5 A3 and Figures 5.16 and 5.17). Figure 5.16 shows calculated sorbed/released concentrations (mg $kg^{-1}$ soil, Appendix CH5 A3) compared to the respective soil concentration. Figure 5.17 shows percentages (% of SG+S, Appendix CH5 A3) of sorbed/released elements per microcosm (calculated as shown in section 5.3.3.3.4). Due to the nature and original goal of this experiment (section 5.1), the exact weight of soil and spent grain per flask was not registered. The total quantity of soil (220 g) and spent grain (55 g) was weighed and homogenized, to create the soil-spent grain mixture that was then divided in the four portions used in each microcosm, with a final theoretical soil and spent grain weight of 55 g and 13.75 g respectively each. Furthermore, the soil mixture was used completely in the experiment so no chemical analysis of soil without grain amendment is available. For these reasons the following analysis is considered semi-quantitative rather than quantitative. In consequence evaluation of statistical significances with t-Test and Anova was not carried out.

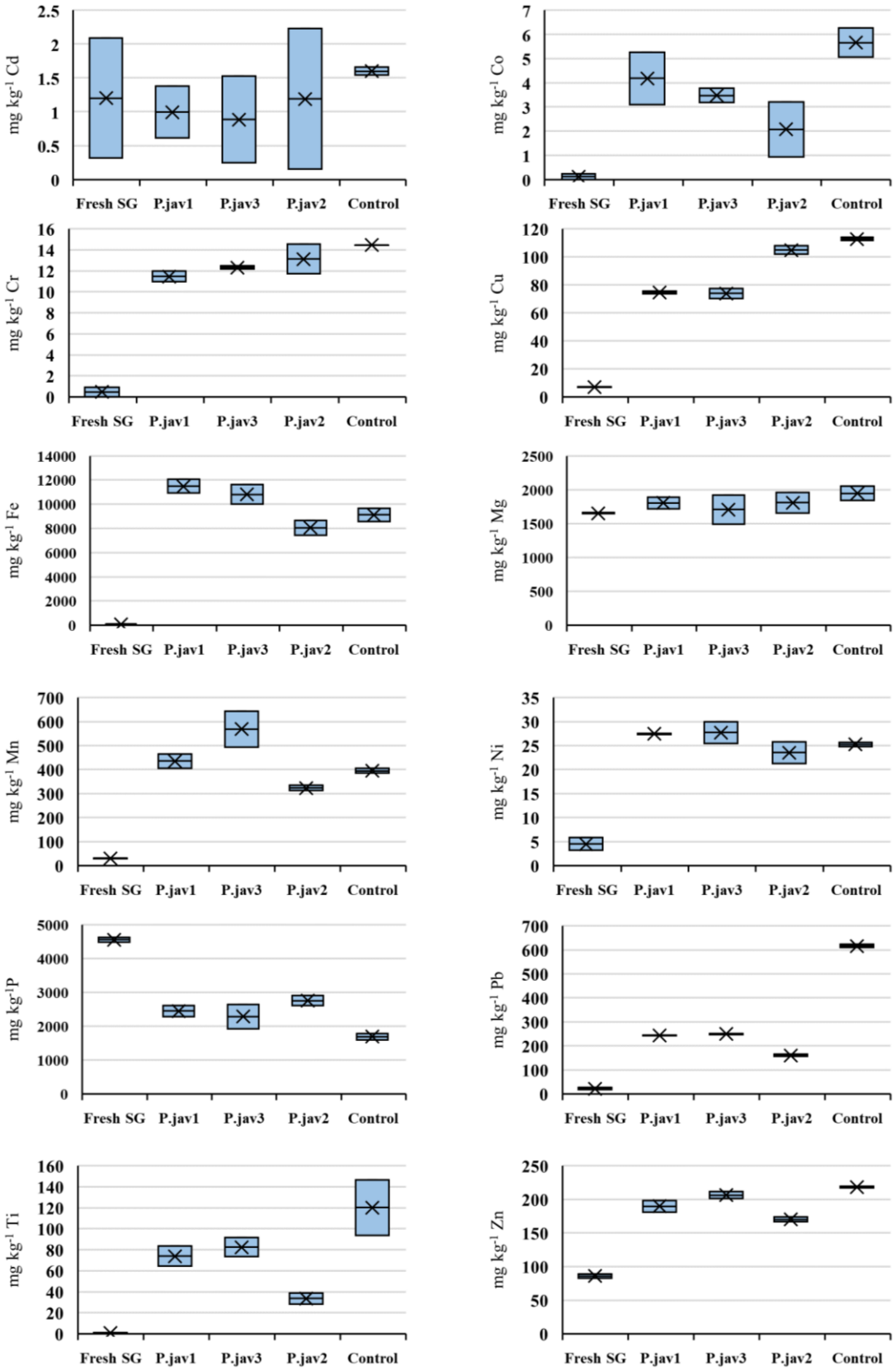



*Figure 5.15. Box plots with average concentration of elements in fresh spent grain and amendment spent grain samples. Control = MMS+SG; P.jav1 and P.jav3 = MMS+SG+Pj; P.jav2 = SMMS+SSG+Pj.*

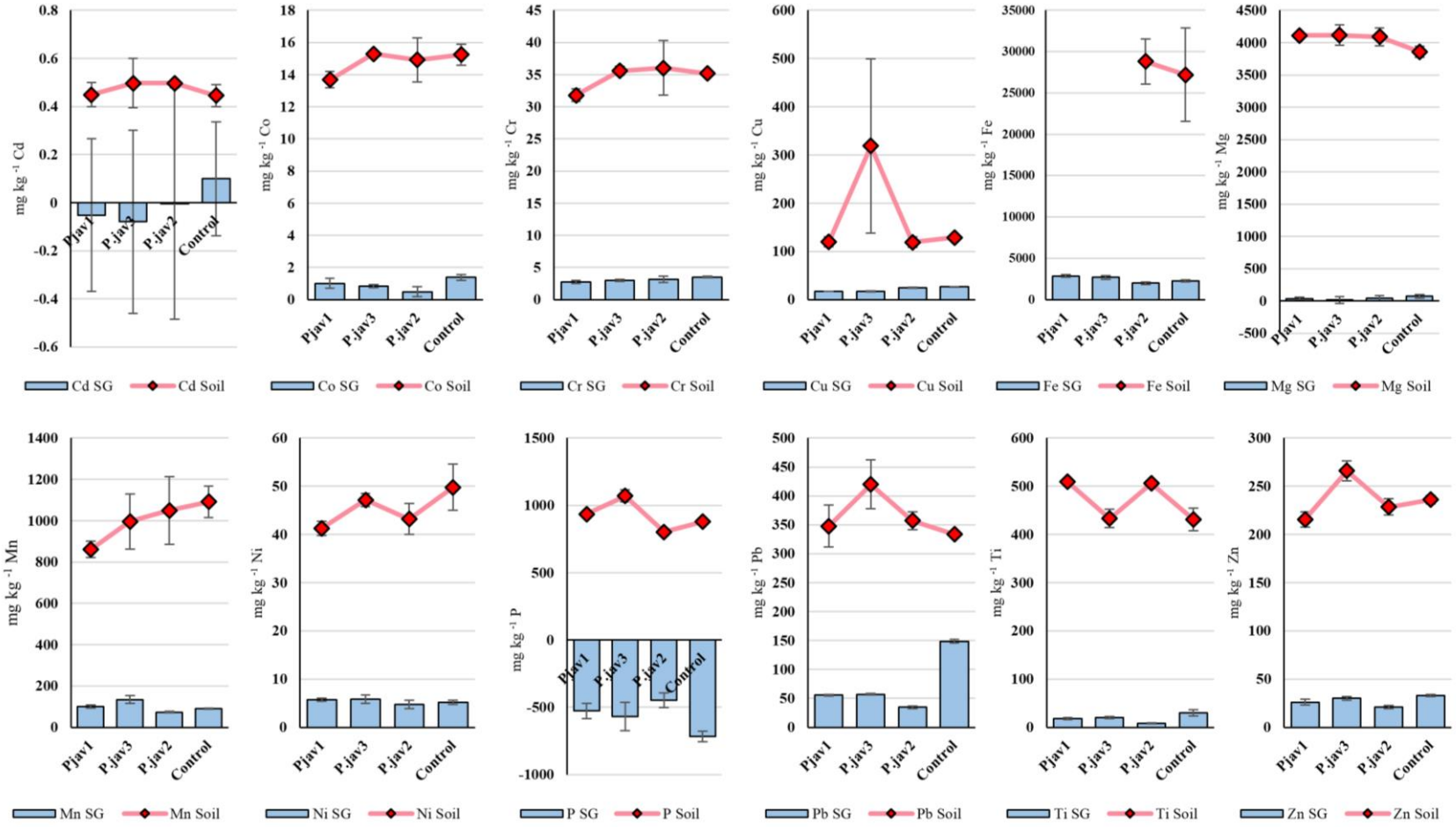



*Figure 5.16. Graphs with sorbed/released concentrations per element in light blue bars, compared to soil concentrations in red diamonds. Note that the scale of the Y-axis is different in every graph, as the magnitude of the concentrations are very different in each element.*

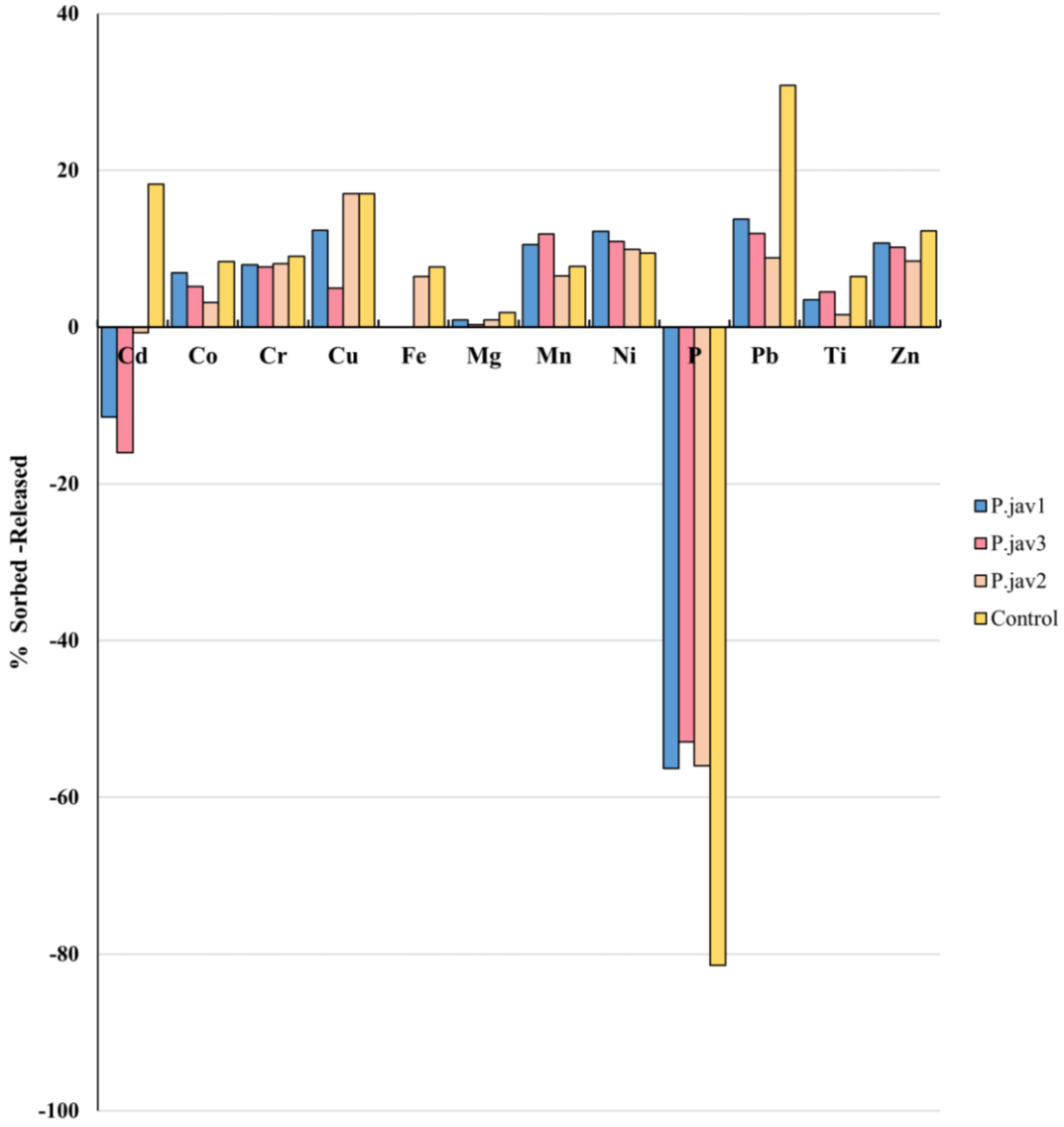



*Figure 5.17. Sorbed/released percentage per element.*

#### 5.4.2.2.1 Pb and P

From all the described calculations and comparisons with soil concentrations, the following observations can be made for P and Pb:

P is the only element that appears as markedly "released" into the soil by the grain, as amendment spent grain have considerably lower concentrations of P than fresh spent grain. Control (MMS+SG) has the highest released P, by a substantial amount. This could indicate that more release of P into the system occurs when the grain has less fungal/microbial growth on its surface. If this is the case, the activity of *P.javanicus* would not seem to produce, or at least enhance, the release of P from the spent grain into the soil. The calculated high P release from Control microcosm spent grain, however, is not reflected by higher P concentrations in the Control soil in comparison to the other microcosm soils.

In the opposite direction to P, Pb sorption onto the grain is also substantially higher in the Control (MMS+SG: 30.8% versus < 14% in the rest). No related trends appear to exist between sorbed Pb and the respective soil Pb concentrations, although Control soils do have a slightly lower average Pb concentration than the rest, which would be coherent with more Pb being removed from the soil via sorption to the grain. The numerical difference in soil concentration does not equate to that in the sorbed Pb, but, as these are only semi-quantitate, they may still be reflection of a real tendency. Again, as there is no fungal growth on the surface of the spent grain in Control, this could be leading to increased sorption. As *P.javanicus* has an affinity with Pb, when "coating" the grain it may have retained part of Pb from sorbing into the spent grain. In relation to the subsequent sequential extraction of soils from these microcosms (results detailed ahead in Chapter 6), Control (MMS+SG) has a higher % of Pb in the BCR1 (target phases: soil solution, carbonates, exchangeable metals) stage and relatively equal in the BCR2 stage (target phases: Iron/manganese oxyhydroxides) in relation to P.jav2 (SMMS+SSG+Pj). This suggests there is no apparent reduction of the more mobile fraction of Pb as a consequence of an increased sorption onto spent grain. BCR3 (target phases: organic matter, sulfides), however, is lower in Control than in P.jav2, and an affinity between organically complexed Pb with the organic matrix of the spent grain could be partly responsible for this.

#### 5.4.2.2.2 Other elements

From the described calculations and comparisons with soil concentrations, the following observations can be made for other element:

Cd concentrations are very low in general, both in the fresh and spent grain, and in the soil. Concentrations in the amendment grain, when the SD is considered, is not different from that in the fresh spent grain, and for this reason, besides having included Cd in graphs and tables, no interpretation was made from such results.

Co has relatively low concentrations in soil, and all sorbed concentrations are < 2 mg $kg^{-1}$. There is a subtle variation in the grain with Control > P.jav1 > P.jav3 > P.jav2 (both in mg $kg^{-1}$ and %), but it does not seem to relate with concentrations in soils. This could be suggesting that sorption of Co is slightly reduced in grain where fungus/microorganisms are growing, and hence coating the grain surface.

There is a certain amount of sorbed Cr that doesn't vary notably between samples (7.7 to 9%). A similar trend between soil and respective sorbed Cr concentrations, may indicate that slightly lower concentrations in soil translated in slightly less sorption in the grain.

In terms of mg $kg^{-1}$ P.jav2 (SMMS+SSG+Pj) and the Control (MMS+SG) have similar and slightly higher concentrations of sorbed Cu than P.jav1 and 3 (MMS+SG+Pj). As P.jav3 has a particularly high soil average concentration, but with an equally high SD, the % of sorbed Cu becomes lower in that sample. If this unusual soil concentration is omitted, there is no clear connexion between soil and sorbed Cu concentrations, that allows to make distinctions between treatments.

As Fe has high concentration, over the upper most limit detection range of ICP-OES when total digestion of soils was carried out, a respective total concentration was only added for P.jav2 and Control, obtained from the ∑BCR (Appendix CH5 A3) P.jav1 and 3 (MMS+SG+Pj) have higher sorbed Fe than P.jav2 and Control, however as no soil concentration for them is available, no comparison with the soil can be done.

Besides there being high concentrations of Mg in soils, sorbed Mg in % is quite low in all samples. This may be related to the fact that Mg concentrations are already high in the fresh spent grain (1654.0 ± 8.2). SD considered, sorbed Mg in P.jav1, 2 and 3 are relatively similar, while Control (MMS+SG) is higher. Control (MMS+SG) also has the lowest soil Mg concentrations. This could be interpreted, as for Co, as increased sorption occurring when there is no fungal growth coating on the grain, hence producing a reduced Mg concentration in soil where more sorption to the grain has occurred (Control).

Sorbed Mn is higher in P.jav1 and 3 (MMS+SG+Pj) samples, both in mg $kg^{-1}$ and % and lowest in P.jav2. This however does not relate to soil concentrations, neither higher or lower at increased sorption.

Ni concentrations are relatively low, in soil and more so sorbed in spent grain. P.jav1 and 3 have subtly higher sorbed concentrations, but as seen for Mn, this shows no apparent connexion with the concentration variation in soils.

Sorbed Ti is highest in Control (6.5%), followed by P.jav3 > P.jav1 > P.jav2. Spent grain with higher sorbed Ti, in this case seem to correspond with soils with lower remaining Ti concentrations.

Sorbed Zn percentages decrease in the order Control > P.jav1-3 > P.jav2. This again concurs with an increased sorption when there's no fugal coating, and least sorption with the most fungal growth. A clear connexion with soil concentrations is not apparent.

#### 5.4.2.2.3 Overall observations

Although the numerical values of sorbed/released elements should not be taken as exact, the general trends observed from this analysis may be truthful. It appears there is a substantial interaction between spent grain used as amendment and the soil system it has been added into. Sorption of metals into the grain matrix and release of P into the system, if not in the exact magnitudes shown through approximate calculation, is most likely occurring. It would appear that such interaction may be partially discouraged when *P.javanicus* is growing on the grain, and coating a part of its surface from the surrounding soil, as the Control system (MMS+SG) with no fungal inoculum seems to have higher overall sorption of metals and release of P. Spent grain was washed in order to separate from attached soil particles and fungal biomass. This sample processing method, however, did not permit a recovery of fungal biomass samples for analysis. In consequence the content of metals and P in fungal biomass remains unknown. The difference between Control spent grain (MMS+SG) and *P.javanicus*-inoculated spent grain (all other microcosms) could be attributed to a preferential sorption/interaction of metals with the fungal biomass, when growing on the surface of the grain, preventing further sorption to the spent grain matrix in itself. Alternatively, the fungal biomass growing on the grain surface could just be acting as a physical barrier reducing direct contact between spent grain and the surrounding soil, and hence reducing slightly (in respect to un-inoculated grain in Control) the sorption and release of elements by the spent grain from and into the soil.

### 5.4.3 Spiked soil experiment

#### 5.4.3.1 Qualitative observations of microcosms overtime

As a follow up of the preliminary soil experiment, a more detailed experiment using Pb-Phen-B(a)p spiked garden soil was carried out to study the effect of selected amendment combinations (section 5.3.4.1.3) in soil contamination. Special emphasis was placed in qualitative observations, in order to assess the success of the soil inoculation.

The commercial garden soil used in this study, having a high water holding capacity (~50wt% moisture), unlike the preliminary experiment soil, did not liquefy when placed in the incubator at 150 rpm. Tables 5.6 and 5.7 show a summary of observations made over the incubation time of microcosms.

*Table 5.6. Summary of observations made over the incubation time of microcosms of the spiked soil experiment (part 1).*

| **Day of experiment** | **Observations** |
|---|---|
| 10 | There was substantial growth of *P.javanicus* on all replicates of S+SG+Pj, S+SSG+Pj and S+SP+SG+Pj, mostly focused on visible spent grain specifically (Figure 5.18-c, d and f). These treatments already seemed drier than all other microcosms. Treatments with inoculum but no additional spent grain amendment (S+Pj and S+SP+Pj), on the other hand, didn't appear to have any growth on the soil (Figure 5.18-b and e). All microcosms with inoculum still had visible fungi on the grain at the base of the flask. Growth of grey-greenish mould could be seen in spent grain in the S+SG treatment (Figure 5.18-a). |
| 15 | Treatments with visible fungi (S+SG+Pj, S+SSG+Pj and S+SP+SG+Pj) and mould (S+SG) seemed to have continued to dry, with no perceptible aqueous water left, and with very little increase in fungal growth from day 10. In consequence the mentioned further addition of water was performed. |
| 16 - 22 | After the water addition, fungal colonies in treatments S+SG+Pj, S+SSG+Pj and S+SP+SG+Pj continued to be visible on the soil-grain mixture for the following week and then appeared to begin to recede. |
| 44 | Fungal colonies initially visible had disappeared (Figure 5.19-c, d and f), leading to the re-inoculation from MEA plates. Approaches with repeated inoculations overtime in nutrient-amended soil, have shown to ensure adequate and sufficient concentration of the desired microorganism and the successful bioaugmentation (136). Though treatments S+Pj and S+SP+Pj had not shown visible growth of fungi in the soil at any time, they were also re-inoculated to maintain the same "inoculation conditions" as the other treatments. S+SG8 on the other hand, showed a cover of white filamentous (hairy-spider web aspect) fungi (Figure 5.19-a). |
| 60 | The newly inoculated fungi did not seem to have spread beyond the MEA plugs themselves. Active colonies of *P.javanicus* where, nevertheless, still visible from the base of the flask, in the original inoculation spent grain. Reduction in the water content of the soil in treatments with grain, however, appeared to have continued, hence the further addition of a small amount of water. A slight re-work of the soil to incorporate the water and increase aeration was also performed with sterile pipettes, to test if it would aid the spread out of *P.javanicus* from the MEA plugs into the soil. |
| 70 | As the re-inoculation from MEA plates did not appear to have had a successful outcome, a second attempt was carried out, this time from liquid (PDb) cultures (as described in the previous section). |
| 78 | Only in some of the flasks (S+Pj11, S+SG+Pj14, S+SP+Pj19, S+SP+Pj20, S+SP+Pj21, S+SG+SP+Pj22) the added fungi appeared to have grown slightly, though limited to its original inoculation colony, in the rest (PDb) colonies seem to have died off. S+SG treatments on the other hand, had a proliferation of fungus, S+SG8 as described before, while S+SG7 in the form of grey-greenish mould. |
| 102 | All originally inoculated replicates seemed to still have active colonies growing on the inoculum spent grain at the base of the flasks. While S+SG+SP+Pj treatments appeared to have the most, S+SSG+Pj had the least, with very little fungi still visible, and showing detachment from the bottom of the flask in replicate S+SSG+18b. In S and S+SP treatments, separation by grain size had occurred, with a layer of fine particles of a lighter colour visible in the bottom of the flask. |

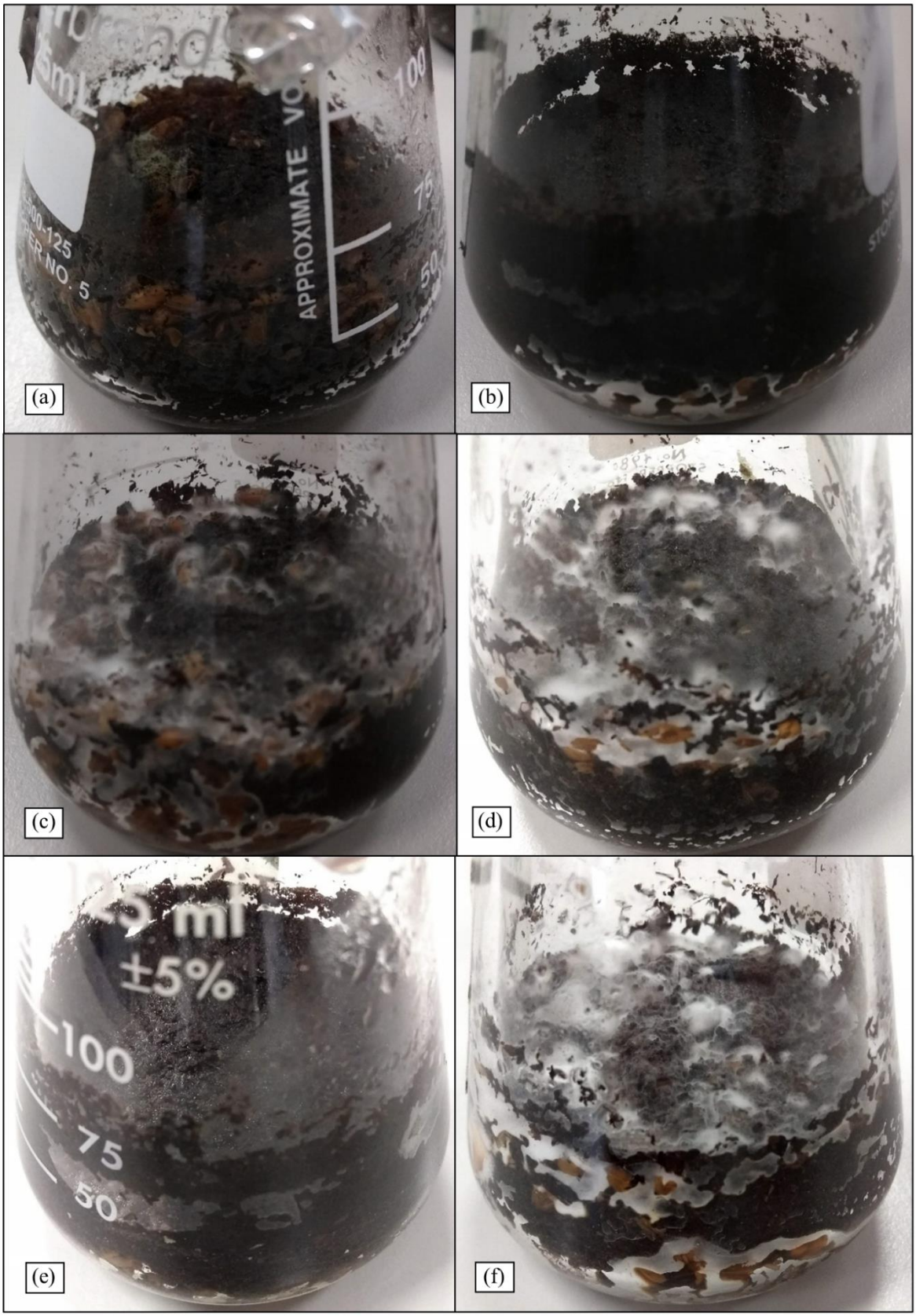



*Figure 5.18. Examples for day 10: (a) S+SG7, (b) S+Pj11, (c) S+SG+Pj13, (d) S+SSG+Pj18, (e) S+SP+Pj19 and (d) S+SP+SG+Pj22.*

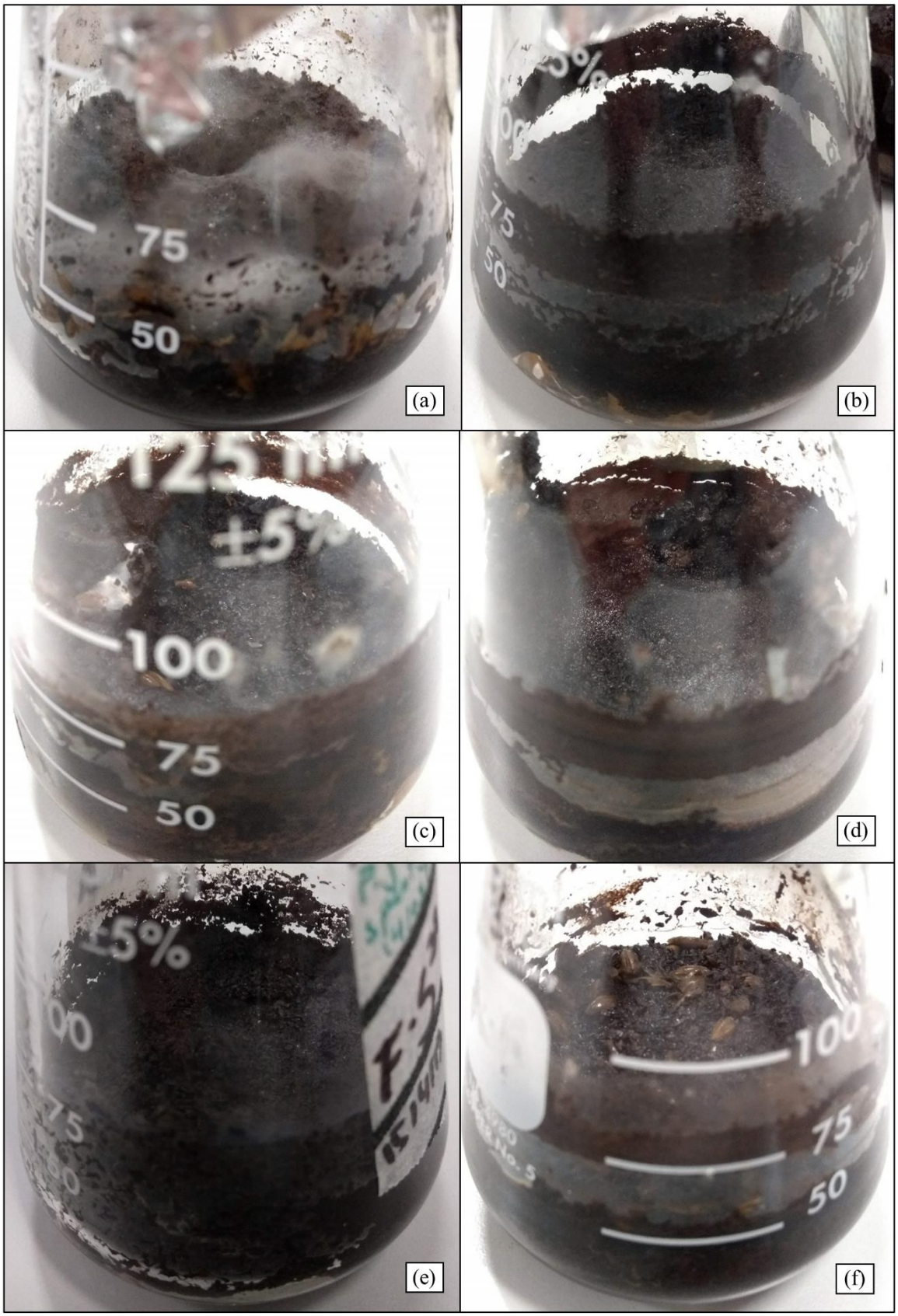



*Figure 5.19. Examples for day 44: (a) S+SG8, (b) S+Pj10, (c) S+SG+Pj14, (d) S+SSG+Pj17, (e) S+SP+Pj20 and (f) S+SP+SG+Pj23.*

*Table 5.7. Summary of observations made over the incubation time of microcosms of the spiked soil experiment (part 2).*

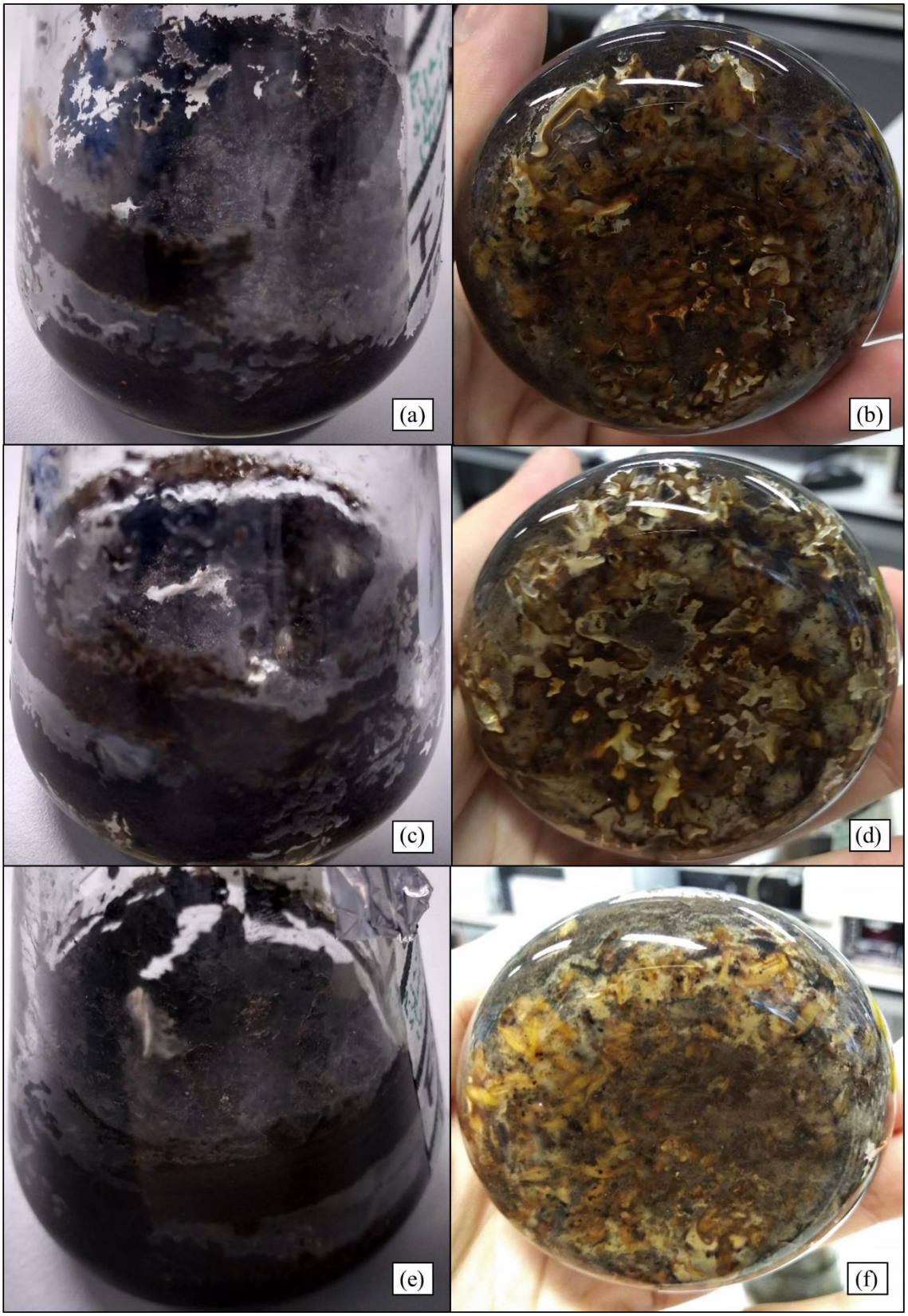
| Day of experiment | Observations |
|---|---|
| 108 | On S+SG treatments the separation by particle size was not as clear, there seemed to be some gradation, but no colour difference. In its place a layer of fungal growth had appeared in between the sediment in the base of the flask and the rest, this was visible as a whitish circumference, when looking from the base. |
| 109-150 | No further notable changes occurred. Control (S) and S+SP treatments remained largely unchanged until the end, other than the mentioned particle size gradation (Figure 5.20-a, b, c and d). Growth in S+SG7 and 8 appeared to have slowed down, beginning to die off towards the end of the experiment (Figure 5.20-e and f). S+SG9 did not show visible growth at any time during the experiment. All other treatments, with *P.javanicus* inoculums, only had visibly active fungal colonies in the original inoculum spent grain, at the base of the flasks (Figure 5.21 and Figure 5.22). Further small additions of sterile water had been made to all treatments at days 91 and 129 as available moisture appeared to have reduced. |

During the first 20 days, the use of spent grain (sterile or non-sterile) as an additional soil amendment, appeared to succeed in encouraging the dispersion and growth of *P.javanicus* within the soil. In contrast to soils with only inoculum, which never showed fungal growth in the soil. As visible colonies, other than in the inoculation spent grain, disappeared overtime, it is not known if the activity of *P.javanicus* in the soil completely stopped, rendering the inoculation ineffective overtime, or continued within the soil and grain mixture in a less perceptible manner.

It has been shown that introducing microbes into contaminated soil is mechanically difficult (113). It is not unusual for an inoculated microbial population to decline after a few weeks, as shown by previous bioaugmentation studies (75). As a result, contaminant removals may not differ significantly in bioaugmented and not bioaugmented microcosms (75). Bioaugmented microorganisms may be outcompeted by indigenous microorganisms, even after an initial population growth, and subsequent inoculation attempts may further unbalance the ecosystem in favour of native organisms (75). Protozoan grazing has also been identified as a cause of unsuccessful microbial inoculation (75). Poor survival of microbes added from liquid culture has also been observed (113), as occurred in the re-inoculation attempts during this experiment.

A study of biostimulation/augmentation of PAH contaminated soil with spent mushroom substrate (SMS) in laboratory microcosms (106) showed that treatments bioaugmented with non-sterile SMS showed development of filamentous fungi in the soil only at the beginning of the experiment. The hyphae lasted a few days and were not observed later because of their poor colonization (106). Initial higher activities of manganese peroxidase and laccase secreted by fungal mycelia in treatments amended with non-sterile SMS, declined with time


*Figure 5.20. Examples for different microcosm treatments, best photograph closest to the end of the experiment: (a) and (b) S1 at 142 days; (c) S+SP4 at 150 days; (d) S+SP4 at 142 days; (e) S+SG7 at day 129; (f) S+SG7 at day 142.*




*Figue 5.21. Examples for different microcosm treatments, best photograph closest to the end of the experiment: (a) S+Pj10 at 150 days; (b) S+Pj11 at day 142; (c) S+SG+Pj14 at 150 days; (d) S+SG+Pj14 at day 142; (e) S+SSG+Pj18 at day 150; (f) S+SSG+Pj17 at day 142.*

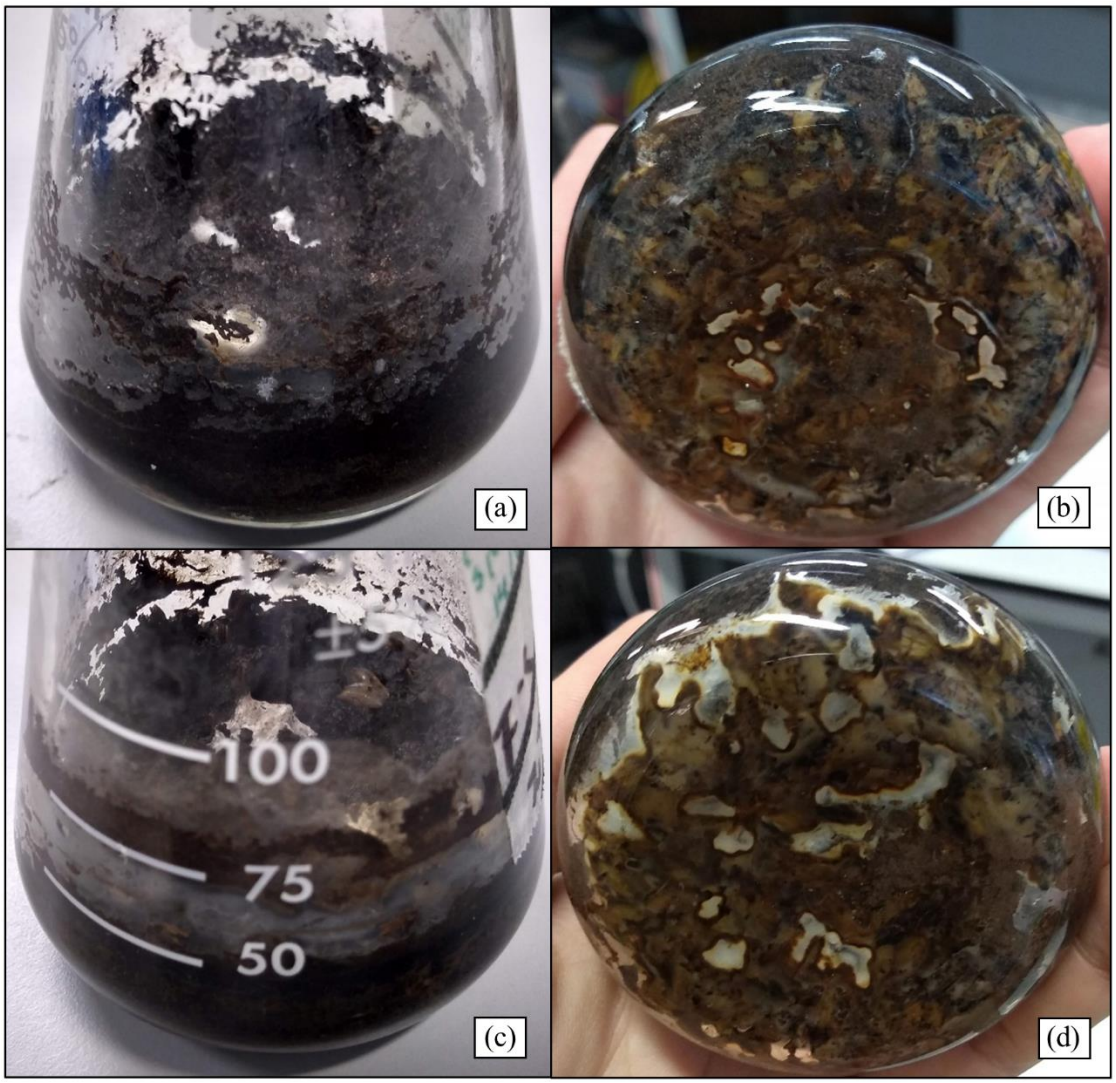



*Figure 5.22. Examples for different microcosm treatments, best photograph closest to the end of the experiment: (a) S+SP+Pj20 at 150 days; (b) S+SP+Pj19 at 142 days; (c) S+SP+SG+Pj23 at 150 days; (d) S+SP+SG+Pj22 at 142 days.*

because of the decrease of fungal population due to poor colonization, nutrition exhaustion and competition with indigenous microorganisms (106).

Some authors have stated that inoculations may be more effective in organic and mineral-poor soils or stressed soils, where the development of indigenous microflora was inhibited before bioaugmentation (75). In the preliminary soil experiment (section 5.4.2.1) there may have been less competition with indigenous microorganisms, as the soil was of poorer quality, while in spiked soil the competition may have been harsher.

It is worth noting, however, that none of the replicates with added non-sterile spent grain and inoculum (S+SG+Pj and S+SP+SG+Pj), even after visible *P.javanicus* colonies had receded, showed visible growth/proliferation of other organisms, such as those present in abundance in S+SG7 and 8. This indicates that *P.javanicus* may have still had a competitive advantage over other organism present in the grain (visible in S+SG treatment).

Unlike the preliminary experiment, the system was also opened on various occasions during this experiment. With the intention of encouraging further growth of the fungus, small modifications (namely the addition of small portions of sterile Milli-Q water and attempts of re-inoculation) were introduced in the system. This, in turn, may have contributed to the recession of visible colonies of *P.javanicus* overtime. The latter, as in the inoculum preparation experiment, shows sensitivity of the population and growth of *P.javanicus* to changes in the conditions of its medium, even in controlled systems that are comparatively much simpler to a real *in situ* top soil environment.

There being active fungal colonies, even if reduced, on the inoculum spent grain until the end of the experiment, leads us to believe that, though it may have started stronger and then reduced overtime, there is potential for the activity and metabolism of *P.javanicus* to have exerted effects in the soil matrix.

### 5.4.3.2 General soil properties

#### 5.4.3.2.1 % Moisture content and LOI

Percentage moisture and LOI was calculated on air dried soil, before spiking. Results are shown in Table 5.8. The average moisture content of the utilized garden soil was of 49.84 ± 2.28 % and LOI 14.71 ± 2.47 % in respect to air dried sample weight, leaving a 35.46 ± 3.72 % mineral residue. LOI changes to an average 29.33 ± 5.36 % with a mineral residue fraction of 70.67 ± 5.36 % when calculated in respect to the oven dry (105°C) sample weight, that is excluding moisture content from the total (100) percentage.

*Table 5.8: Moisture content and LOI of garden soil (before incubation).*

| **Initial soil weight (g)** | **Soil weight 105°C (g)** | **Moisture (g)** | **Moisture %** | **Soil weight 450°C (g)** | **LOI (g)** | **LOI (% isw[*1])** | **LOI (% 105[*2])** |
|---|---|---|---|---|---|---|---|
| 4.500 | 2.211 | 2.289 | 50.87 | 1.575 | 0.640 | 14.14 | 28.78 |
| 4.670 | 2.335 | 2.335 | 50.00 | 1.583 | 0.750 | 16.10 | 32.21 |
| 4.720 | 2.424 | 2.296 | 48.65 | 1.769 | 0.650 | 13.87 | 27.01 |
| **Average** | 2.323 | 2.307 | 49.84 | 1.642 | 0.681 | 14.71 | 29.33 |
| **error** | 0.217 | 0.049 | 2.276 | 0.223 | 0.126 | 2.474 | 5.358 |

**1 isw stands for initial soil weights, and refers to the LOI been calculated in respect to the sample weight (column 1) with LOI%+Moiture%+Residue= 100%. *2 %105 refers to the LOI been calculated from oven dried sample weight (column 2), excluding moisture, with LOI%+Residue = 100%.*

#### 5.4.3.2.2 pH

Table 5.9 shows a summary of average pH values for each treated soil after harvesting, as well as the garden soil before spiking (GS) and spiked garden soil before the beginning of the experiment (T0). Figure 5.23 shows all pH measurements in a box plot according to soil treatment. Finally, Table 5.10 has p-values calculated with the t-test, pairing pH values of

*Table 5.9. Average pH value per soil treatment.*

| | Average pH | error |
|---|---|---|
| **GS** | 6.195 | 0.221 |
| **T0** | 6.364 | 0.080 |
| **S** | 6.520 | 0.071 |
| **S+SP** | 6.216 | 0.074 |
| **S+SG** | 7.786 | 0.041 |
| **S+Pj** | 7.739 | 0.112 |
| **S+SG+Pj** | 7.692 | 0.158 |
| **S+SSG+Pj** | 7.758 | 0.053 |
| **S+SP+Pj** | 7.429 | 0.124 |
| **S+SP+SG+Pj** | 7.521 | 0.099 |

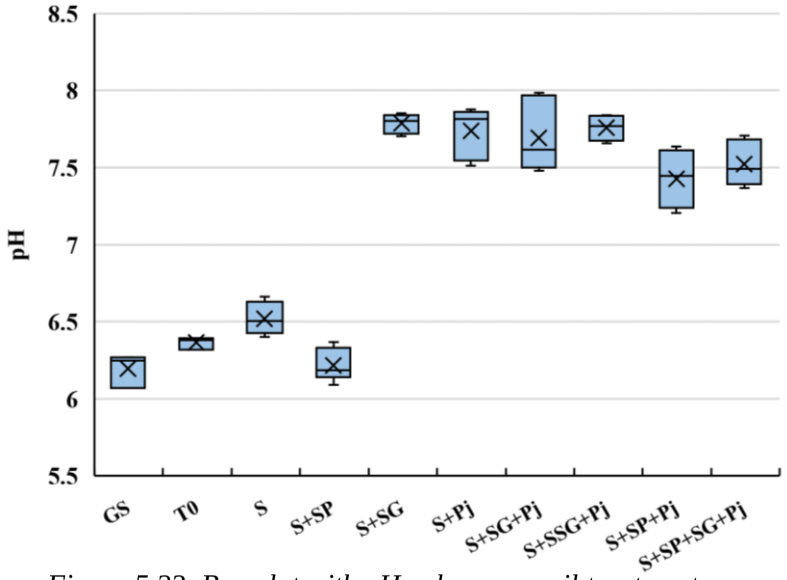



*Figure 5.23. Box plot with pH values per soil treatment.*

*Table 5.10. Summary of p-values obtained with t-Test: Two-Sample Assuming Unequal Variances.*

| | GS | T0 | S | S+SP | S+SG | S+Pj | S+SG+Pj | S+SSG+Pj | S+SP+Pj |
|---|---|---|---|---|---|---|---|---|---|
| **T0** | 0.0855 | | | | | | | | |
| **S** | 0.0195 | 0.0035 | | | | | | | |
| **S+SP** | 0.7836 | 0.0056 | 8.17E-06 | | | | | | |
| **S+SG** | 0.0017 | 7.31E-08 | 5.31E-14 | 4.66E-15 | | | | | |
| **S+Pj** | 7.47E-06 | 3.07E-10 | 1.12E-11 | 6.20E-13 | 0.4102 | | | | |
| **S+SG+Pj** | 2.87E-07 | 3.01E-08 | 1.40E-08 | 1.30E-09 | 0.2403 | 0.6052 | | | |
| **S+SSG+Pj** | 0.0002 | 1.16E-09 | 7.07E-15 | 3.83E-16 | 0.3854 | 0.7368 | 0.4037 | | |
| **S+SP+Pj** | 6.63E-06 | 8.55E-09 | 3.77E-09 | 1.18E-10 | 0.0001 | 0.0010 | 0.0123 | 0.0002 | |
| **S+SP+SG+Pj** | 6.84E-05 | 5.89E-10 | 1.57E-11 | 4.01E-13 | 0.0002 | 0.0059 | 0.0674 | 0.0006 | 0.2263 |

** p-values < α = 0.05, highlighted in grey*

all treatments with each other, in the bottom left side, and p-values calculated with Anova analysis on the top right side. Anova p values were calculated comparing all treatments together (second row) and in subgroups, constituted by the soil treatments on the header row above each consecutive cell.

Two main groups can be distinguished, soils with pH < 7 (GS, T0, S and S+SP) and soil with pH > 7 (S+SG, S+Pj, S+SG+Pj, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj). The common factor that soils with pH > 7 all have, is the presence of spent grain: as part of the inoculum (S+Pj and S+SP+Pj), only as a soil amendment (S+SG) or both (S+SG+Pj, S+SSG+P, and S+SP+SG+Pj). Soils with pH < 7 on the other hand have no spent grain in any form.

This differs from previous findings which have characterized spent grain as a slightly acidic material (pH 5–6.4) (146), causing reduction of soil pH when added as an amendment in calcareous soils (155). Crosier (2014) (153), however, also registered a slight increase in soil pH in agricultural soil amended with spent grain (pH 6.2) in relation to a control (pH 5.8).

Anova analysis of all soil pH values, with the null hypothesis that the mean pH value of all soil treatments, including non-incubated T0 soil and non-spiked garden soil (GS), were equal, produced a resulting p-value of 2.43E-44 < $\alpha = 0.05$. The null hypothesis was hence rejected, indicating that mean pH value of all soil treatments are not equal. With the t-Test, comparing all soil treatments with each other (Table 5.11), soil treatments GS, T0, S and S+SP (pH < 7) had significantly (p-value < $\alpha = 0.05$) lower pH values than all treatments with any form of spent grain and pH > 7 (S+SG, S+Pj, S+SG+Pj, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj). GS, T0 and S+SP had statistically equal pH values (p-value > $\alpha = 0.05$). Treatments S+SG, S+Pj, S+SG+Pj and S+SSG+Pj also all had statistically equal pH values (p-value > $\alpha = 0.05$), while treatment S+SP+Pj had slightly yet significantly (p-value < $\alpha = 0.05$) lower pH than the other treatments with spent grain and no superphosphate. Treatment S+SP+SG+Pj only had significantly lower pH than treatments S+SG and S+SSG+Pj.

Providers of superphosphate fertilizer (182) indicate that P fertilizer added to soil can affect soil acidity, depending on the pre-existing soil pH. Although the phosphate content (%) in superphosphate is represented in phosphate pentoxide ($P_2O_5$), this form of fertilizer adds P to soil mostly as dihydrogen phosphate ($H_2PO_4^-$) (182), which can acidify soil with a pH greater than 7.2, but has no effect on soil pH in acidic soils (182). The following reaction is used (182) to explain such effect:

$$Ca(H_2PO_2)_2 + 2H_2O \rightarrow CaHPO_4 + H^+ + H_2PO_4^-$$

While at lower pH the produced acidity is neutralized with other reactions such as (182):

$$CaHPO_4 + H_2O \rightarrow Ca^{2+} + H_2PO_4^- + OH^-$$

This could explain the slight pH difference of S+SP+Pj and S+SP+SG+Pj treatments with the other spent grain treated microcosms.

As no significant differences appear between S+SG treatment and treatments with inoculum, no change in pH can be specifically attributed to the inoculation of *P.javanicus*.

**5.5 Key findings**

- Brewery spent grain proved to be a good matrix for the growth of *P.javanicus.*
- Brewery spent grain proved to be a good matrix for use as an inoculum carrier and soil amendment to encourage the inoculation of microorganisms in a soil matrix.
- In preliminary soil experiments, sorption of Pb to the added brewery spent grain occurred, and was highest when grain was added with no *P.javanicus* inoculum, as the Control (MMS+SG) had 148.8 ± 2.897 mg of sorbed Pb per kg of soil, while the other microcosms ranged from 34.73 ± 2.222 to 57.02 ± 1.436 mg of sorbed Pb per kg of soil. This, however, when compared to BCR sequential extraction results (shown in detail in Chapter 6), did not appear to translate in a reduction of the more mobile fraction of Pb in Control soils in respect to microcosms with *P.javanicus* (P.jav 2 in particular).
- A reduction of the P concentration in spent grain used as a soil amendment indicates that P may have been released from the brewery spent grain into the soil system. The highest reduction of P concentration in amendment spent grain (and hence potential P release into the system) also occurred when grain was added with no *P.javanicus* inoculum (Control microcosm, MMS+SG).
- In Pb, Phen and B(a)p spiked soils, active fungal colonies were mostly visible during the first 20 days of incubation. Even if reduced (only visible on the inoculum spent grain at the base of the flask) towards the end of the experiment, there is potential for the activity and metabolism of *P.javanicus* to have exerted effects in the soil matrix.
- In Pb, Phen and B(a)p spiked soils, contrary to what previous published research has shown, the addition of brewery spent grain produced a significant increase in soil pH from 6.20 ± 0.22 - 6.52 ± 0.07 in microcosms without grain, to 7.43 ± 0.12 – 7.79 ± 0.04 in microcosms with any form of spent grain.

# CHAPTER 6 Chemical study of Pb and P in *P.javanicus*-soil laboratory microcosm experiments

## 6.1 Introduction

In Chapter 3, the ability of *P.javanicus* to biomineralize pyromorphite in liquid cultures spiked with Pb and an organic P source was shown. Changes in the pH of the culture media, from an initial average 7.10 ± 0.04, to pH 3.49 ± 0.06 at 10 days accompanied the capture of Pb in the fungal biomass as Pb-oxalate. Pb-oxalate was then replaced in pseudomorphism by pyromorphite, detected as the main mineral phase at 20 and 30 days of incubation. This change in mineralogy was accompanied with changes in the pH of the media, to pH 8.14 ± 0.09 and 8.52 ± 0.09 at 20 and 30 days respectively.

While phosphate amendments have been used for the *in situ* remediation of Pb-contaminated soil, through immobilization of Pb as pyromorphite (45, 56, 57, 63), limitations in the efficiency of this remediation strategy have been shown in soil with neutral to alkaline pHs, as more acidic pHs are required for the pre-existing Pb and P phases to be solubilized and react to form pyromorphite (45, 46, 57, 68, 70-72).

*P.javanicus* showed potential for the remediation of Pb-contaminated soil, through the biomineralization of Pb as pyromorphite, and its ability to change the pH in its microenvironment. The latter could promote the formation of pyromorphite in soils with added P but neutral pH, typical of urban environments, where the use of phosphate amendments alone have limited effects on Pb immobilization as pyromorphite.

Brewery spent grain is an agro-industrial waste product available in abundance and at no cost in Scotland (142, 145). Its rich nutritional content (143) (detailed in section 2.3) led to the selection of brewery spent grain as a potentially suitable matrix for the cultivation/growth of *P.javanicus*, to create a spent grain + *P.javanicus* inoculum for the bioaugmentation of soils with the fungus.

In Chapter 5, a preliminary soil experiment using real environmental soil samples from the Meat market site was carried out to evaluate the bioaugmentation approach using spent grain + *P.javanicus*. Due to the exploratory nature of this initial stage, the experiment was conceived as a qualitative observation-focused study, hence a simplified set up was devised. In this experiment, brewery spent grain proved to be a good matrix for use as an inoculum carrier and soil amendment to encourage the inoculation of microorganisms in a soil matrix.

A more detailed microcosm experiment was subsequently carried out, using Pb, Phen and B(a)p spiked commercial garden soils treated with different amendment combinations,

including *P.javanicus* and spent grain and superphosphate (Chapter 5). While active fungal colonies were mostly visible during the first 20 days of incubation, there is potential for the activity and metabolism of *P.javanicus*, combined with the addition of soil amendments, to have had effects in the soil matrix. Contrary to what previous published research has shown, however, the addition of brewery spent grain to soil produced a significant increase in soil pH (> 7), which may have had a detrimental effect, as limitations in the efficiency of phosphate amendments for Pb remediation have been shown in soil with neutral to alkaline pHs.

The soil matrixes used in each experiment were distinct. Real made ground soils obtained from the Meat market site were used in the first case and spiked commercial soil in the latter. By presenting results for both experiments together, common trends/effects caused by the inoculation of *P.javanicus* and use of spent grain as a soil amendment in different soils, with different properties and composition, can be identified.

BCR sequential extraction (section 2.1.4) was used to study changes on the fractionation of Pb and P (two main components of pyromorphite: $Pb_5(PO_4)_3X$; X= F, Cl, B or OH) associated to the different treatment combinations, in soils from both microcosm experiments. It has been shown that pyromorphite remains mostly insoluble over subsequent sequential extraction stages, remaining by the most part in the residual portion (82, 86). For this reason, and with the defined desired target of reducing Pb mobility-bioavailability, through *P.javanicus*-induced biomineralization of Pb as pyromorphite, special emphasis was placed in increases/decreases in the residual (BCR4) fraction of Pb and P.

As a complementary method, to study changes in Pb bioaccessibility, the UBM assay (section 2.1.5) was used. Low values of bioaccessible Pb have also been attributed to the presence of more stable Pb minerals such as pyromorphite (52, 91). For this reason, as with the BCR, emphasis was placed in increases/decreases in the residual (non-bioaccessible) fraction of Pb associated to different soil treatments.

In addition to the specific methodological quality controls used for each procedure, samples of the homogenized Meat market CP08 soil (section 4.4.4) (not used in microcosm experiments) were extracted alongside the experimental samples as a contrasting matrix. Inclusion of this ‘control’ matrix was considered to assist with identification of results that may be operational, rather than sample specific. Furthermore, results for CP08 samples were potentially beneficial for use in subsequent experimental studies.

## 6.2 Objectives

- Study the effects on Pb and P BCR-fractionation and Pb UBM bioaccessibility caused by soil amendments combined with the inoculation of *P.javanicus* in microcosms of the preliminary soil experiment and spiked soil experiment.
- In particular, determine if amendments were able to increase residual Pb in the BCR-fractionation (BCR4) and reduce gastric (Gc) and intestinal (Ist) Pb UBM-bioaccessibility.

## 6.3 Methodology

### 6.3.1 BCR sequential extraction

#### 6.3.1.1 Reagents, procedure and quality control

The methodology used was a modified version of Davidson *et al*. 1998 (81). Table 6.1 summarises the four steps used.

*Table 6.1. Regents and nominal targets for each BCR stage.*

| **Extraction step** | **Reagent (s)** | **Volume** | **Nominal target phase** |
|---|---|---|---|
| 1 | $CH_3COOH$ (0.11 mol $l^{-1}$) | 40 ml | Soil solution, carbonates, exchangeable metals |
| 2 | $NH_2OH$*HCl (0.1 mol $l^{-1}$) adjusted to pH 2 with $HNO_3$ | 40 ml | Iron/manganese oxyhydroxides |
| 3 | $H_2O_2$ (8.8 mol l-1) then $CH_3COONH_4$ (1.0 mol $l^{-1}$) adjusted to pH 2 with $HNO_3$ | 20 ml and 40 ml respectively | Organic matter, sulphides |
| 4 | Aqua regia (1:1 37% HCl: 70% $HNO_3$) | 10 ml | Remaining, non-silicate bound metals |

##### 6.3.1.1.1 Sample preparation

Stored, freeze-dried samples with spent grain amendment were sieved (<2 mm) to separate soil from large spent grains. The > 2 mm portion was stored separately for processing (section 5.3.3.3).

##### 6.3.1.1.2 Solution preparation

BCR 1 solution: a 0.11 mol $l^{-1}$ solution of acetic acid ($CH_3COOH$) was prepared by weighing 6.606 g of 99.81% $CH_3COOH$ (60.052 g $mol^{-1}$, Fisher Scientific, Loughborough, UK), by carefully pipetting the reagent with a Pasteur pipette into a glass beaker placed in a balance. The $CH_3COOH$ was transferred into a 1 l volumetric flask. To make sure all the acetic acid was transferred, the beaker was rinsed repeatedly with Milli-Q water that was then added to the volumetric flask. The solution was made up to 1 l with Milli-Q water and shaken manually.

BCR 2 solution: a 0.1 mol $l^{-1}$ hydroxylammonium chloride ($NH_2OH*HCl$) solution was prepared by dissolving 6.949 g of $NH_2OH*HCl$ (69.488 g $mol^{-1}$, VWR chemicals, Leicestershire, UK) in 1 l of Milli-Q water in a volumetric flask. pH was then adjusted to 2 with $HNO_3$ (ARISTAR®, VWR chemicals, Leuven, Belgium).

BCR3 solution: A 1.0 mol $l^{-1}$ solution of ammonium acetate ($CH_3COONH_4$, 77.083 g $mol^{-1}$, VWR chemicals, Leuven, Belgium) was prepared, dissolving 77.083g in Milli-Q water in a 1 l volumetric flask. pH was then adjusted to 2 with $HNO_3$.

BCR4 solution: aqua regia as described in section 4.3.2.3.1.

**6.3.1.1.3 Procedure**

From the <2mm fraction of all samples, 1.0 g dry weight was placed in 50 ml polypropylene centrifuge tubes.

40 ml of the BCR1 solution was pipetted into each tube (in replicates, as specified in 6.3.1.3.) Tubes were then placed, in a horizontal position, in an orbital incubator at 21ºC and 130 rpm for 16 hrs, after which they were centrifuged at 4000 rpm for 10 minutes. Supernatant was transferred to separate 50ml polypropylene centrifuge tubes with Pasteur pipettes for storage. Residual pellets were washed with 20ml of Milli-Q water, by shaking in the orbital incubator for 15 minutes, then centrifuging and the water discarded.

For the second extraction, BCR2 solution was added to the residual pellet, and the procedure was carried out as in BCR1.

For the third extraction, 10 ml of hydrogen peroxide ($H_2O_2$, 8.8 mol $l^{-1}$) was added to the residual pellet in the polypropylene tubes, covered with a watch glass and allowed to digest at room temperature for 1 hr. Tubes were then placed in the digestion block (DigiPREP Jr. ® SCP Science, Quebec H9X 4B6 Canada) at 85ºC for an hour, after which watch glasses were removed and the solution allowed to reduce to <3ml. The same procedure was then repeated for a second time. 40 ml of BCR3 solution was added to each tube and the rest of the procedure was carried out as in BCR1.

In BCR2 and 3 note was taken of the volume considering the small amount of Milli-Q water or $H_2O_2$ left from the previous step.

Finally, for the fourth extraction (BCR4), an aqua regia digestion of the residual pellet was carried out, as described in section 4.3.2.3.1.

**6.3.1.1.4 Quality control**

Two blank polypropylene tubes (containing no sample material) were included per BCR round, undergoing all stages as all other samples. Three replicates of a certified reference material (CRM: Sandy Clay 1 CRM049-50G Lot 010248 and Lot LRAB3033 Sigma-Aldrich, Laramie, Wyoming 82070, USA) were also included in each round. Although not certified for the BCR procedure, the sum off all BCR stages can be compared to the certified total metal concentration, and the repeatability of results on different replicates evaluated. As the goal was to compare the sequential extractions of our own sample with each other and not to other extractions performed elsewhere, the use of these standards was deemed sufficient.

#### 6.3.1.2 Calculations

For comparison between samples with different total concentrations, each BCR stage of a specific sample was transformed from mg $kg^{-1}$ to a % in relation to the addition of all BCR stages (mass balance) as follows:

$$\%\mathrm{BCRnx} = \frac{\mathrm{BCRnx} * 100}{\sum \mathrm{BCRx}}$$

With:

- %BCRnx: percentage of a specific element in BCR stage n (n= 1, 2, 3 and 4) in a sample x.
- BCRnx: mg $kg^{-1}$ of a specific element in BCR stage n (n= 1, 2, 3 and 4) in a sample x.
- ∑BCRx: addition of mg $kg^{-1}$ of a specific element in all BCR stages (BCR1 + BCR2 + BCR3 + BCR4) of a sample x.

An example of this calculation is given in Appendix CH6 A1.

The P/Pb molar ratio in each BCR stage was also calculated, for comparison with the stoichiometric P/Pb ratio required for the formation of pyromorphite (P/Pb = 3/5). For each sample, P/Pb was calculated considering P and Pb mg $kg^{-1}$ concentrations, sample weights, and the element's molar mass, as shown in the example in Appendix CH6 A2.

#### 6.3.1.3 Notes on the sample selection

In the first instance the BCR procedure was carried out in 2 replicates for each of the 24 microcosms from the spiked soil experiment, as well as for the 4 microcosms from the preliminary soil experiment, and in 6 replicates of the homogenized CP08 soil. However, issues in the extraction procedure, rendered those results invalid (data not shown). Due to

time limitations, the BCR extraction could not be repeated in its entirety, therefore a selected few representative samples were extracted as follows:

- From the preliminary soil experiment (section 5.3.3.2): P.jav2 (3 replicates) (SMMS+SSG+Pj) microcosm with sterile soil and spent grain mixture and *P.javanicus* inoculum. This represents an extreme where the growth of *P.javanicus* in the soil matrix was proven and profuse. Any effect *P.javanicus* could potentially have on the soil Pb speciation should be the most enhanced in these samples, and therefore easiest to detect through the sequential extraction. As a counterpart 3 replicates of the Control (MMS+SG) microcosm (with non-sterile soil and spent grain an no *P.javanicus* inoculum) were re-extracted.
- From the spiked soil experiment (section 5.3.4.1.3): 3 replicates from the "time zero" (T0) untreated spiked soil (not used in microcosms, and hence not incubated) and 2 replicates from each of the 3 S+SP+SG+Pj microcosms (soil treatment with spent grain, superphosphate and *P.javanicus* inoculum) were extracted, again representing two opposite extremes. If any of the amendments (fungal inoculum, spent grain or superphosphate) had produced a change in the soil Pb speciation, and more specifically the desired increase in residual Pb, it would show in the sequential extraction of these samples (when compared to the untreated soil).
- CP08 soil (contrasting matrix) (section 4.4.4): 4 replicates of the homogenized CP08 soil were re-extracted.

All repeated samples were extracted in one round. In consequence CRM quality control allowed the comparison of ∑BCR with certified concentrations, and repeatability between the 3 CRM replicates in each stage of one round of the BCR sequential extraction.

### 6.3.2 UBM bioaccessibility assay

#### 6.3.2.1 Reagents, procedure and quality control

The method was applied according to the official BARGE (Bioaccessibility Research Group of Europe) procedure instructions (UBM procedure for the measurement of inorganic contaminant bioaccessibility from solid matrices)(183).

##### 6.3.2.1.1 Sample preparation

A portion of the soil harvested from each microcosm was sieved (<250 μm) as indicated in the original method.

##### 6.3.2.1.2 Solution preparation

Reagents used and their respective brands are stated in Appendix CH6 A4.

Solutions were prepared the day before performing the assay, according to the recipe in Table 6.2, adjusting the weights and volumes depending on the required amount of each solution. The inorganic and organic solutions were prepared separately in volumetric flasks, combined in plastic bottles, and remaining ingredients (enzymes) were then added. Bottles were placed in an incubator and shaken at 200 rpm for 3 hrs. After removing from the incubator, pH was measured and if necessary, adjusted with NaOH 1 mol $l^{-1}$ or HCl 37% according to values given in Table 6.2. Bottles were stored in the fridge at 4ºC overnight.

*Table 6.2. Weights and volumes of reagents required for the preparation of 500ml of each UBM solution.*

| | **Reagents** | **Saliva (mg)** | **Gastric (mg)** | **Duodenal (mg)** | **Bile (mg)** |
|---|---|---|---|---|---|
| **Inorganic (250 ml) [IO]** | KCl | 448 | 412 | 282 | 188 |
| | $NaH_2PO_4$ | 444 | 133 | - | - |
| | KSCN | 100 | - | - | - |
| | $Na_2SO_4$ | 285 | - | - | - |
| | NaCl | 149 | 1376 | 3506 | 2630 |
| | $CaCl_2*2H_2O$ | - | 200 | - | - |
| | $NH_4Cl$ | - | 153 | - | - |
| | $NaHCO_3$ | - | - | 2803.5 | 2893 |
| | $KH_2PO_4$ | - | - | 40 | - |
| | $MgCl_2*6H_2O$ | - | - | 25 | - |
| | NaOH (1 mol $l^{-1}$) | 0.9 ml | - | - | - |
| | HCl (37%) | | 4.15 ml | 90 µl | 90 µl |
| **Organic (250 ml) [O]** | Urea | 100 | 42.5 | 50 | 125 |
| | Glucose | - | 325 | - | - |
| | Glucuronic acid | - | 10 | - | - |
| | Glucosamine hydrochloride | - | 165 | - | - |
| **Enzymes (250+250 ml) [IO+O]** | Alpha amylase | 72.5 | - | - | - |
| | Mucin | 25 | 1500 | - | - |
| | Uric acid | 7.5 | - | - | - |
| | Bovine serum albumin | - | 500 | 500 | 900 |
| | Pepsin | - | 500 | - | - |
| | $CaCl_2*2H_2O$ | - | - | 100 | 111 |
| | Pancreatin | - | - | 1500 | - |
| | Lipase | - | - | 250 | - |
| | Bile | - | - | - | 3000 |
| | **pH** | 6.5 ± 0.5 | 1.1 ± 0.1 | 7.4 ± 0.2 | 8 ± 0.2 |

**Target pH of each solution is included at the bottom of the table.*

### 6.3.2.1.3 Procedure

Four replicates of 0.6 g per sample (2 for the gastric [Gc] phase and 2 for the intestinal [Ist] phase) were weighed into their respective tubes. For gastric (Gc) samples 50ml polypropylene centrifuge tubes were used, while for intestinal (Ist) samples DigiTUBEs ®,

as they can hold the required solution volume. All tubes were tagged with the sample name followed by Gc or Ist for gastric and intestinal respectively.

On the days the assay was performed, all 4 solutions were removed from the fridge, and placed in an incubator at 37ºC and 200 rpm for an hour prior to use. 9 ml of the saliva solution were pipetted into each tube, tubes were shaken manually for 10 s, and then 13.5 ml of gastric solution was added. After shaking again, pH was checked and adjusted, when necessary, with NaOH 1 mol $l^{-1}$ or HCl 37% to 1.20±0.05. The volume of NaOH or HCl used was noted, to adjust the final volume. All the tubes where then placed in an end-over-end shaker at 25 rpm, and the shaker placed inside an incubator at 37ºC for an hour.

After removal from the incubator, pH was checked to be <1.5 in all tubes. Gc (gastric) samples were centrifuged at 4500 rcf for 15 minutes and the supernatant transferred with Pasteur pipettes to separate 50ml polypropylene centrifuge tubes. 500 µl of $HNO_3$ were added to acidify the extracts, and tubes were then placed in the fridge at 4ºC (for analysis Gc extracts were later diluted, 1 to 10 ml with 5% $HNO_3$). 20 ml of Milli-Q water were added to the residue for washing by handshaking, then tubes re-centrifuged and the water discarded (using Pasteur pipettes).

In Ist samples 27 ml of duodenal solution and 9ml of bile fluid were added. After gently shaking, pH was checked and adjusted, when necessary, with NaOH 1 mol $l^{-1}$ or HCl 37% to 6.30±0.5. Tubes were then placed back in the end-over-end shaker within the incubator at 37ºC for 4 hrs. After removing tubes from the shaker, the final pH was measured and noted. The supernatant was retrieved as described for the Gc step, and stored in DigiTUBEs ® (SCP Science, Quebec H9X 4B6 Canada).

As suggested by the BARGE procedure, 1 ml of $HNO_3$ was added to intestinal solutions, however, as a result all extracts (including blanks) showed signs of precipitated material. For this reason, and to dissolve the precipitated material, a further digestion of the Ist extract was performed as a modification to the original method. DigiTUBEs ® containing the extracts were placed in the digestion block (DigiPREP Jr. ® SCP Science, Quebec H9X 4B6 Canada) at 95ºC. Once enough headspace was available, 5ml of $H_2O_2$ were added, and the mixture allowed to reduce to ~3ml. 10 ml of $HNO_3$ were then added and watch glasses placed over the tubes for 2 hours, after which watch glasses were removed and solution allowed to reduce to ~3ml again. The volume was made up to 50ml with 5% HNO3 and tubes stored in the fridge at 4ºC.

The occurrence of precipitated material in the Ist solution at the end of the procedure and after the addition of the $HNO_3$, has been encountered by other researchers (verbal

communication (184)). Upon consultation with the BARGE team in the British Geological Survey (by (184)), a direct analysis of the Ist solution with no further digestion to dissolve the precipitates, was deemed correct practice in the context of risk assessment (184). This information was received by the author after the completion of this thesis, and hence the inclusion of the additional digestion step in the procedure was kept in the methodology description.

Soil residues from both Gc and Ist extracts were digested with aqua regia, as described in section 4.3.2.3.1.

Twenty-two out of 24 microcosms from the spiked soil experiment and all 4 from the preliminary soil experiment were extracted (2 Gc and 2 Ist replicates each as described above). The homogenized CP08 soil was extracted in 6 replicates for both Gc and Ist phases.

#### 6.3.2.1.4 Quality control

On each UBM round (usually composed of 4 samples, 2 Gc + 2 Ist replicates each) a Gc blank and an Ist blank were included, as well as a CRM (Sandy Clay 1 CRM049-50G Lot 019005 and Lot LRAB3033 Sigma-Aldrich, Laramie, Wyoming 82070, USA) with its respective 2 Gc and 2 Ist replicates. As for the BCR, although not certified for this specific procedure, the CRM allowed comparison with the total metal concentration, and assessment of repeatability over the various rounds the procedure was carried out.

#### 6.3.2.1.5 Reference matrix

In the context of risk assessment of contaminated soil, and as recommended by the UK Environment Agency, the use of reference matrixes is applied to bioavailability (*in vivo*) data rather than bioaccessibility (*in vitro*) data (185). *In vivo* bioavailability involves administering contaminated soil to a live animal and measuring the resultant concentration in a number of body compartments (e.g. blood, liver, kidney and bone) (185). In this case the absolute bioavailability (ABA) of a chemical in soil is a direct measure of the proportion of an intake dose that is absorbed and reaches the target compartments (185). In assessing risks from soil contamination, the information of interest is whether the absolute bioavailability of the chemical in a soil sample (ABAsoil) is greater or less than that measured in the media (or reference matrix) used for toxicological studies (ABAtox) (185). The relationship between these two bioavailabilities is also known as the relative bioavailability of a chemical (RBAsoil,tox) (185).

When a reliable site-specific RBAsoil,tox value has been determined for a specific pathway (e.g. direct oral ingestion), it may be used for calculating average daily exposure values, with

the relative bioavailability for other pathways (e.g. inhalation and dermal) (185). The UK Environment Agency (185) defines the relationship between the relative bioavailability of a chemical in a specific soil (RBAsoil,tox) and its *in vitro* bioaccessibility, with the following formula:

$$\mathrm{RBAsoil,tox} = \frac{\mathrm{ABAsoil}}{\mathrm{ABAtox}}, \qquad \mathrm{ABAsoil} = \mathrm{F_B} * \mathrm{F_A} * \mathrm{F_H}$$

Where, the absolute bioavailability of a chemical in soil (ABAsoil) corresponds to the bioaccessible fraction of the chemical in soil ($F_B$) multiplied by the fraction of the solubilized (or bioaccessible) chemical that is adsorbed or transported across the gastrointestinal wall into systemic circulation ($F_A$); and the fraction of the adsorbed chemical that passes through the liver intact and enters systemic circulation ($F_H$) (185). The relative bioavailability (RBAsoil,tox) then corresponds to all this 3 factors (including bioaccessibility) divided by the absolute bioavailability in the reference matrix (ABAtox) (185).

In the *in vivo* validation study for the UBM procedure (Denys *et al*, 2012 (90), discussed in section 2.1.5), however, in order to compare *in vivo* bioavailability with *in vitro* bioaccessibility, a relative value in respect to a reference matrix (e.g. Pb-acetate) was calculated for both the *in vivo* (juvenile swine model) bioavailability and *in vitro* UBM-bioaccessibility. While, a value for "relative bioaccessibility" is not normally used in applications of the UBM procedure in the context of risk assessment or other uses (the total or absolute bioaccessibility is used directly), in this study a reference matrix (such as that used by Denys *et al*, 2012 (90)) was included for comparative purposes. Having used $Pb(NO_3)_2$ -spiked soil in the main microcosm experiment, the use of $Pb(NO_3)_2$ alone as a reference matrix, allowed to distinguish a "background" of bioaccessibility produced by this readily soluble Pb compound alone in the UBM-procedure, versus when spiked in soil (after aging [T0] and incubated with different treatments). In other words, it aided in distinguishing operational effects of subjecting $Pb(NO_3)_2$ -spiked soil to the UBM procedure.

Additionally, in Denys *et al*, 2012 (90), by using a reference matrix, an equivalency in the magnitude of relative gastric (Gc) and intestinal (Ist) bioaccessibility was established in 16 different contaminated soil samples (detailed in section 2.1.5). This was considered as an added benefit of the use of $Pb(NO_3)_2$ as a reference matrix in this study, that may allow to draw comparisons between the magnitudes of Gc and Ist bioaccessibility, when normalised to the respective Gc and Ist bioaccessibility of the reference matrix.

As in (90), 0.5 ml of a solution with the Pb-reference matrix was added into tubes right before the beginning of the assay, and then the procedure was carried out. The concentration

of the prepared solution was calculated to provide the same amount of Pb by adding 0.5 ml in the tube as would be in the system if 0.6 g of soil theoretically spiked with 1000 mg $kg^{-1}$.

In practice 38.8 mg of $Pb(NO_3)_2$ was dissolved in 20ml of Milli-Q water, and 0.5 ml of the solution added to eight tubes (4 Gc + 4 Ist). The exact weight of the 0.5 ml per tube was noted, and then used to calculate concentrations.

#### 6.3.2.2 Calculations

Gc available Pb in all cases was calculated as a percentage:

$$\%\text{Gc nx} = \frac{\text{Gc nx} * 100}{\text{Gc nx} + \text{GcRes nx}}$$

With:

- %Gc nx: percentage of an element x present in the gastric phase for a sample n.
- Gc nx: concentration (mg $kg^{-1}$) of an element x in the gastric phase for a sample n.
- GcRes nx: concentration (mg $kg^{-1}$) of an element x in the residual pellet after gastric digestion, for a sample n.

Equivalently Ist available Pb was calculated:

$$\%\text{Ist nx} \frac{\text{Ist nx} * 100}{\text{Ist nx} + \text{IstRes nx}}$$

With:

- %Ist nx: percentage of an element x present in the intestinal phase for a sample n.
- Ist nx: concentration (mg $kg^{-1}$) of an element x in the intestinal phase for a sample n.
- IstRes nx: concentration (mg $kg^{-1}$) of an element x in the residual pellet after intestinal digestion, for a sample n.

When normalizing against the Pb reference matrix, the formula was changed to:

$$\%\text{RM Ist nx} = \frac{\text{Ist nPb} * 100}{(\text{Ist nPb} + \text{IstRes nPb}) * (\frac{\text{RM Ist\%}}{100})}$$

With

- %RM Ist nPb: percentage of Pb present in the intestinal phase for a sample normalized to the reference matrix.

- Ist nPb: concentration (mg $kg^{-1}$) of Pb in the intestinal phase for a sample n.
- IstRes nPb: concentration (mg $kg^{-1}$) of Pb in the residual pellet after intestinal digestion, for a sample n.
- RM Ist%: average Ist available Pb in percentage, calculated from the 4 reference matrix replicates.

Calculation for the Gc phase would be made analogously. An example of this calculation is given in Appendix CH6 A3.

### 6.3.4 ICP-OES analysis

Analysis was carried out in a Perkin Elmer Optima 7300 DV ICP-OES.

A Pb-P calibration line (1, 5, 10, 15, 20 and 25 mg $l^{-1}$) was prepared from appropriate standards (PlasmaCAL ICP/ICPMS Standard – Lead 1000 µg $ml^{-1}$, catalogue number 140-051-82x. Lot: S190814018; PlasmaCAL ICP/ICPMS Standard – Phosphorus 1000 µg $ml^{-1}$, catalogue number 140-050-15x. Lot: S190819014, both SCP Science, Quebec H9X 4B6 Canada). A 1 mg $l^{-1}$ standard was placed every 10 samples as a quality control check for instrumental drift, as well as 5% $HNO_3$ blanks, approximately every 15 samples. Three wavelengths were measured per element, and the best value was selected according to the $R^2$ of the calibration line, consistency of 1 mg $l^{-1}$ standards and RSD. For Pb the selected wavelength was 220.353 nm and for P an average between wavelengths 177.495 nm and 213.618 nm. Raw data is included in Appendix CH6 S1.

### 6.3.5 Statistics

All statistical analysis was carried out in Microsoft Excel (2016). Errors were calculated using the t-distribution confidence interval formula, appropriate for populations under 30:

$$X \pm t_{n-1} \frac{s}{\sqrt{n}}$$

Where

X= average value for a group of measurements

n= sample size (number of measurements)

n-1= degrees of freedom

s= standard deviation

t= constant from the t Table

The two-tails probability was set at p=0.05

All remaining analysis was done using the Data Analysis package (e.g. correlations) Statistical significance was evaluated with the "t=Test: two sample assuming unequal variance" and "Anova: single factor", with significance assumed at a P-value < 0.05.

## 6.4 Results and discussion

### 6.4.1 Lead (Pb) and Phosphate (P) BCR sequential extraction

A summary of results for the BCR sequential extraction of soil samples is presented in Table 6.3, showing averaged values for CP08, Control (MMS+SG), P.jav2 (SMMS+SSG+Pj), T0 and S+SP+SG+Pj of: % of total Pb and P in each BCR stage (1, 2 3 and 4); the sum of Pb and P in all BCR stages in mg $kg^{-1}$ (∑BCR); Pb and P concentration from pseudo-total (aqua regia) digestion for comparison; average results for the CRM (quality control), with certified confidence interval and prediction interval for comparison. The average concentration of both elements in operational blanks is also shown for each BCR stage. The error is replaced by standard deviation in the total and blank concentrations, as in most cases only two replicates were averaged. The complete data set is included in the Appendix CH6 S2. Concentrations obtained with pseudo-total aqua regia digestion for preliminary soil experiment soil are shown in Appendix CH5 A2.

For Pb, there is generally good agreement between the total sum of BCR stages (∑BCR) and total concentrations obtained with pseudo-total aqua regia digestion (Total), with the Total value being within the error confidence interval of the ∑BCR value (Control, P.jav2, S+SP+SG+Pj) or overlapping SD intervals (S). The addition of stages for the CRM also falls within the certified prediction interval.

The only exception is the homogenised CP08 soil, which may be attributable to sample heterogeneity. In the latter 2 out of 4 replicates showed an unusually high total concentration of Pb. The pseudo-total aqua regia digestion, averaging 669.69 ± 80.20 mg $kg^{-1}$ was carried out in 6 replicates (section 4.4.4), and is in agreement with the average value of 671.72 ± 74.88 mg $kg^{-1}$. For the BCR procedure, however, replicates 2 and 3 had a value of ∑BCR of 1132.11 and 1286.98 mg $kg^{-1}$ respectively, well above the average pseudo-total concentration, while replicates 1 and 4, with 570.74 and 649.03 mg $kg^{-1}$, were closer to the average pseudo-total concentration. Replicates 2 and 3 also showed a different distribution of Pb in the different BCR stages, in relation to replicates 1 and 4, as detailed in Figure 6.1.

*Table 6.3. Summary of results of the BCR sequential extraction.*

| **Pb** | **n** | **BCR1 (%)** | **error (%)** | **BCR2 (%)** | **error (%)** | **BCR3 (%)** | **error (%)** | **BCR4 (%)** | **error (%)** | **∑BCR (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **Total (mg kg$^{-1}$)** | **SD** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CP08** | 4 | 2.353 | *2.446* | 33.20 | *6.560* | 41.69 | *1.340* | 22.75 | *7.593* | 909.7 | *486.9* | 669.7 | *80.20* |
| **Control** | 3 | 4.315 | *1.172* | 34.48 | *3.764* | 25.66 | *5.492* | 35.55 | *7.146* | 315.2 | *41.98* | 333.8 | *0.607* |
| **P.jav2** | 3 | 2.197 | *1.116* | 33.75 | *1.263* | 37.10 | *3.113* | 26.95 | *2.685* | 328.9 | *74.85* | 357.1 | *15.23* |
| **T0** | 3 | 1.545 | *0.300* | 42.51 | *2.251* | 40.34 | *4.249* | 15.61 | *2.967* | 1294 | *51.21* | 1205 | *53.54* |
| **S+SP+SG+Pj** | 5 | 1.539 | *0.346* | 38.06 | *1.506* | 43.76 | *2.411* | 16.65 | *1.442* | 1178 | *51.16* | 1205 | *53.54* |
| | | | | | | | | | | | | **CI** | **PI** |
| **CRM** | 6 | 51.09 | *1.123* | 41.78 | *0.563* | 1.879 | *0.350* | 5.244 | *1.621* | 321.0 | *10.44* | 333-347 | 283-398 |

| **P** | **n** | **BCR1 (%)** | **error (%)** | **BCR2 (%)** | **error (%)** | **BCR3 (%)** | **error (%)** | **BCR4 (%)** | **error (%)** | **∑BCR (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **Total (mg kg$^{-1}$)** | **SD** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CP08** | 4 | 0.748 | *0.828* | 14.75 | *1.190* | 40.02 | *1.894* | 44.49 | *1.957* | 1386 | *105.7* | 645.8 | *37.69* |
| **Control** | 3 | 0.958 | *0.648* | 14.19 | *0.883* | 24.17 | *5.175* | 60.68 | *5.872* | 1890 | *247.7* | 879.0 | *1.487* |
| **P.jav2** | 3 | 2.512 | *0.531* | 16.95 | *1.121* | 35.28 | *3.301* | 45.26 | *3.904* | 1597 | *97.84* | 802.2 | *25.78* |
| **T0** | 3 | 48.43 | *1.880* | 23.90 | *0.292* | 17.06 | *0.552* | 10.62 | *2.649* | 4022 | *377.6* | 3403 | *231.9* |
| **S+SP+SG+Pj** | 5 | 54.57 | *1.107* | 20.48 | *0.495* | 15.47 | *0.686* | 9.477 | *0.596* | 4471 | *164.3* | - | - |
| | | | | | | | | | | | | **CI** | **PI** |
| **CRM** | 6 | 8.790 | *0.797* | 12.10 | *0.936* | 49.220 | *0.675* | 29.89 | *0.834* | 1145 | *46.89* | 411-477 | 274-615 |
| | | **mg kg$^{-1}$** | ***SD*** | **mg kg$^{-1}$** | ***SD*** | **mg kg$^{-1}$** | ***SD*** | **mg kg$^{-1}$** | ***SD*** | | | | |
| **Blank (Pb)** | 2 | -0.073 | *0.040* | -0.045 | *0.006* | -0.028 | *0.012* | -0.050 | *0.004* | | | | |
| **Blank (P)** | 2 | 0.060 | *0.025* | 0.059 | *0.110* | 15.21 | *0.343* | 0.135 | *0.050* | | | | |

**CRM were ran both in the Pb-P ICP-OES analysis and in the multielement analysis. Average values were obtained from results from both analyses. For Pb in both analyses the wavelength used was the same (220.353 nm). For P on the other hand, in multielement analysis wavelength 177.495 nm was used and in Pb-P analysis the average of 177.495 nm and 213.618 nm. BCR1 extracts of the 3 CRM replicates were an exception, as wavelength 213.618 nm had negative values, so only 177.495 nm was used.*

*Table 6.4. Summary of BCR P results adjusted with the Blank background.*

| P | n | BCR1 (%) | error | BCR2 (%) | error | BCR3 (%) | error | BCR4 (%) | error | ∑BCR (mg kg$^{-1}$) | error | Total (mg kg$^{-1}$) | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CP08** | 4 | 1.250 | *1.391* | 24.59 | *2.029* | 0.000 | *0.000* | 74.16 | *1.443* | 831.9 | *54.22* | 645.8 | *37.69* |
| **Control** | 3 | 1.263 | *0.827* | 18.73 | *2.429* | 0.000 | *0.000* | 80.00 | *2.353* | 1435 | *109.3* | 879.0 | *1.487* |
| **P.jav2** | 3 | 3.876 | *0.641* | 26.21 | *3.085* | 0.000 | *0.000* | 69.92 | *2.480* | 1034 | *97.10* | 802.2 | *25.78* |
| **T0** | 3 | 57.07 | *1.784* | 28.16 | *0.254* | 2.244 | *1.386* | 12.52 | *3.210* | 3413 | *139.46* | 3402.6 | *231.9* |
| **S+SP+SG+Pj** | 5 | 63.16 | *1.152* | 23.70 | *0.622* | 2.172 | *0.797* | 10.97 | *0.710* | 3862 | *125.5* | - | - |
| | | | | | | | | | | | | **CI** | **PI** |
| **CRM** | 6 | 17.31 | *1.583* | 23.84 | *1.881* | 0.000 | *0.000* | 58.85 | *1.100* | 581.1 | *22.82* | 411-477 | 274-615 |

*Table 6.5. Average P/Pb molar ratios for each BCR stage.*

| P/Pb molar ratio | n | BCR1 | error | BCR2 | error | BCR3 | error | BCR4 | error | Total | error |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **CP08** | 4 | 6.231 | *10.06* | 5.270 | *3.357* | 0.000 | *0.000* | 21.82 | *4.975* | 6.861 | *3.591* |
| **Control** | 3 | 9.071 | *6.550* | 16.52 | *0.902* | 0.000 | *0.000* | 68.72 | *7.993* | 30.42 | *2.331* |
| **P.jav2** | 3 | 39.71 | *31.62* | 16.41 | *2.958* | 0.000 | *0.000* | 55.06 | *13.76* | 21.27 | *6.751* |
| **T0** | 3 | 653.4 | *33.79* | 11.70 | *1.448* | 0.984 | *0.643* | 14.11 | *0.597* | 20.80 | *2.140* |
| **S+SP+SG+Pj** | 5 | 888.6 | *174.1* | 13.60 | *0.824* | 1.218 | *0.415* | 14.55 | *0.621* | 25.51 | *0.695* |

Blanks show negative values at all BCR stages, suggesting no background interference affecting results.

P on the other hand, shows values of ∑BCR well above respective Total concentrations, as the CRM is almost double the higher limit of the prediction interval. When looking at the results of the Blank, BCR3 stage stands out with an average 15.21 mg $kg^{-1}$. While none of the utilised reagents used in the BCR3 stage have P present as anion traces, blanks from the discarded BCR procedure also showed a background of P consistently in the BCR3 extract (Appendix CH6 A5). This would suggest that even if not stated in the certificate of analysis, one of the two reagents used in this stage ($CH_3COONH_4$ and $H_2O_2$) must have had P impurities. Concentrations obtained for Blanks with ICP-OES are not adjusted with a dilution factor, unlike real samples which are adjusted according to the sample weight (~1g) and final volume (~40 ml for BCR3), hence amplifying the background.

For this reason, concentration values for the BCR3 stage in all samples were adjusted by subtracting the average P mg $kg^{-1}$ in the Blanks, amplified according to each specific sample weight and final volume (example in Appendix CH6 A6). ∑BCR and % of all BCR stages were adjusted accordingly (Table 6.4). By doing this, in sample groups CP08, Control, P.jav2 and the CRM, P concentrations were brought to negative values in BCR3, and subsequently adjusted to zero.

∑BCR remained higher, yet much closer to Total P concentrations in CP08, Control and P.jav2. In T0, ∑BCR and Total, after the adjustment, were within each other's error and SD intervals. S+SP+SG+Pj average ∑BCR came closer to the Total P value for T0. Pseudo-total aqua regia digestion was only performed in T0 spiked soils, and not in soils with added superphosphate, hence the difference ∑BCR (S+SP+SG+Pj) > T0 Total was expected. ∑BCR P in the CRM, which was double the expected concentration in the original value, fell within the prediction interval after adjusting.

As adjusted P results are more representative of the true distribution of P than unadjusted data, for the purpose of interpretation, all further data analysis was carried out with adjusted results.

Figures 6.1 and 6.2 show the % of total Pb and P, respectively, present in each BCR stage, for individual samples (not averaged values). Comparisons are made for samples belonging to the same experiment (Control with P.jav2 and T0 with S+SP+SG+Pj) by connecting the same BCR stages in samples from the same experiment with segmented lines, to indicate

positions/changes respect to one another (marked with a red star when there are statistical differences).

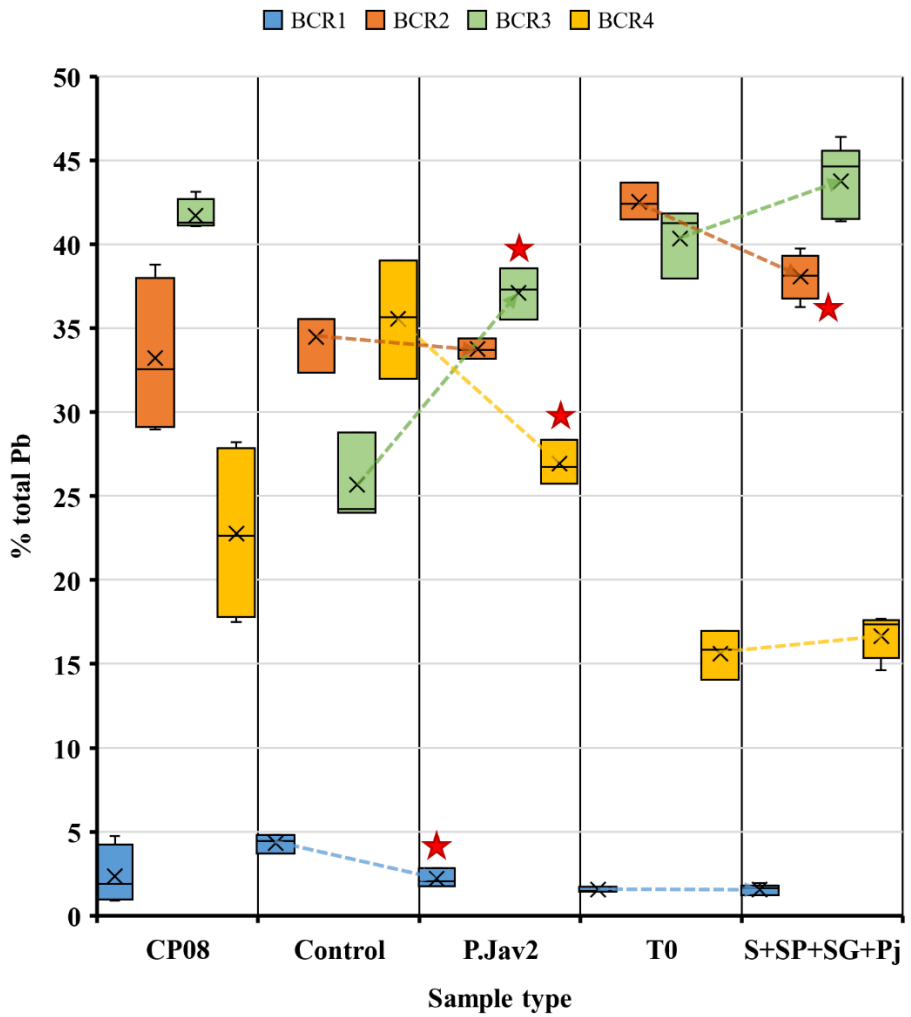



*Figure 6.1. Box plot with %s of total Pb in each BCR stage for CP08, Control (MMS+SG), P.jav2 (SMMS+SSG+Pj), T0 and S+SP+SG+Pj: BCR1in blue, BCR2 in orange, BCR3 in green and BCR4 in yellow. Segmented lines connect the same BCR stage/fraction in samples from the same microcosm experiment (i.e. Control with P.jav2 and T0 with S+SP+SG+Pj), to highlight an increase or reduction of the %Pb in BCR stages, associated to soil treatment. The red star indicates the increase/reduction is statistically significant.*

The P/Pb molar ratio (considering Blank-adjusted P concentrations in BCR3) is displayed in Table 6.5 and Figure 6.3, for comparison with the stoichiometric P/Pb ratio required for the formation of pyromorphite (P/Pb = 3/5).

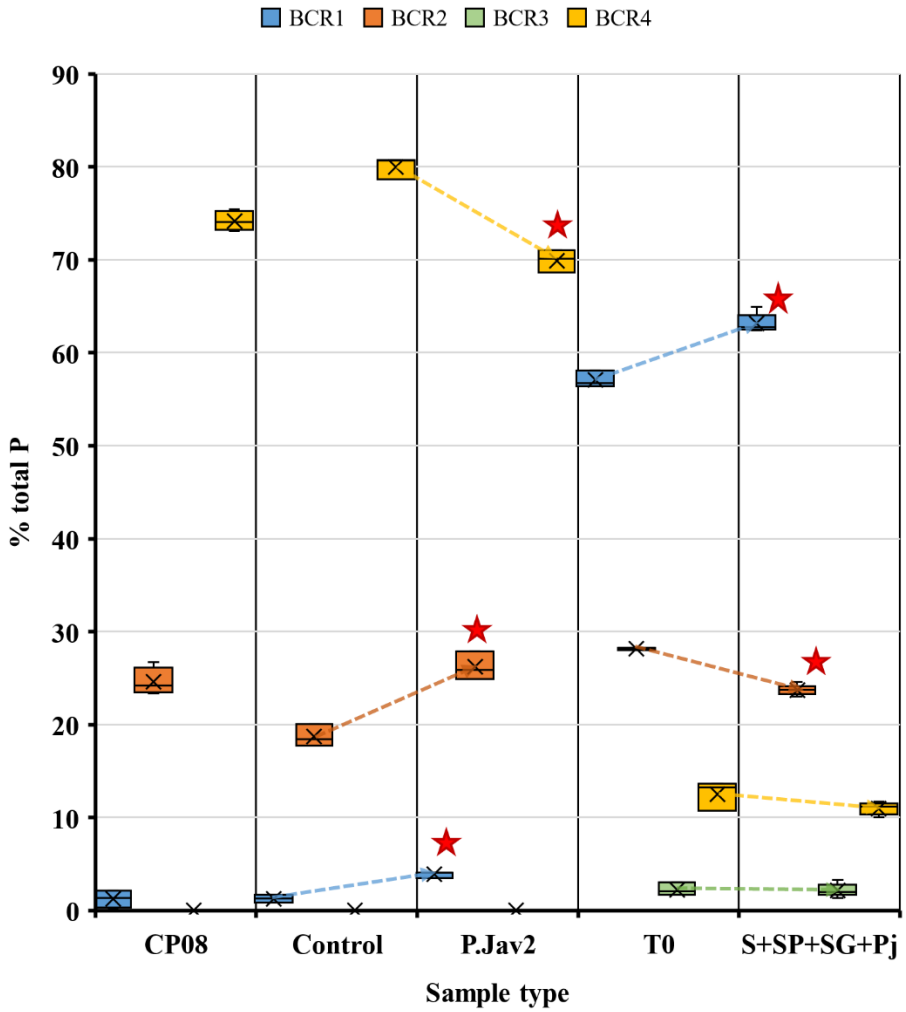



*Figure 6.2. Box plot with %s of total P in each BCR stage for CP08, Control (MMS+SG), P.jav2 (SMMS+SSG+Pj), T0 and S+SP+SG+Pj: BCR1in blue, BCR2 in orange, BCR3 in green and BCR4 in yellow. Segmented lines connect the same BCR stage/fraction in samples from the same microcosm experiment (i.e. Control with P.jav2 and T0 with S+SP+SG+Pj), to highlight an increase or reduction of the %P in BCR stages, associated to soil treatment. The red star indicates the increase/reduction is statistically significant.*

In order to assess if comparisons and changes between samples, observed in the % total Pb or P or mol P/mol Pb ratio (Figures 6.1, 6.2 and 6.3) are statistically significant, t-Tests (Two-Sample Assuming Unequal Variances, $\alpha$=0.05) were carried out comparing Pb and P at each BCR stage, between Control (MMS+SG) samples and P.jav2 (SMMS+SSG+Pj) samples, and T0 samples with S+SP+SG+Pj samples. Resulting p values are shown in Table 6.6, with those below the $\alpha$=0.05 value, and hence considered significant, highlighted in grey.

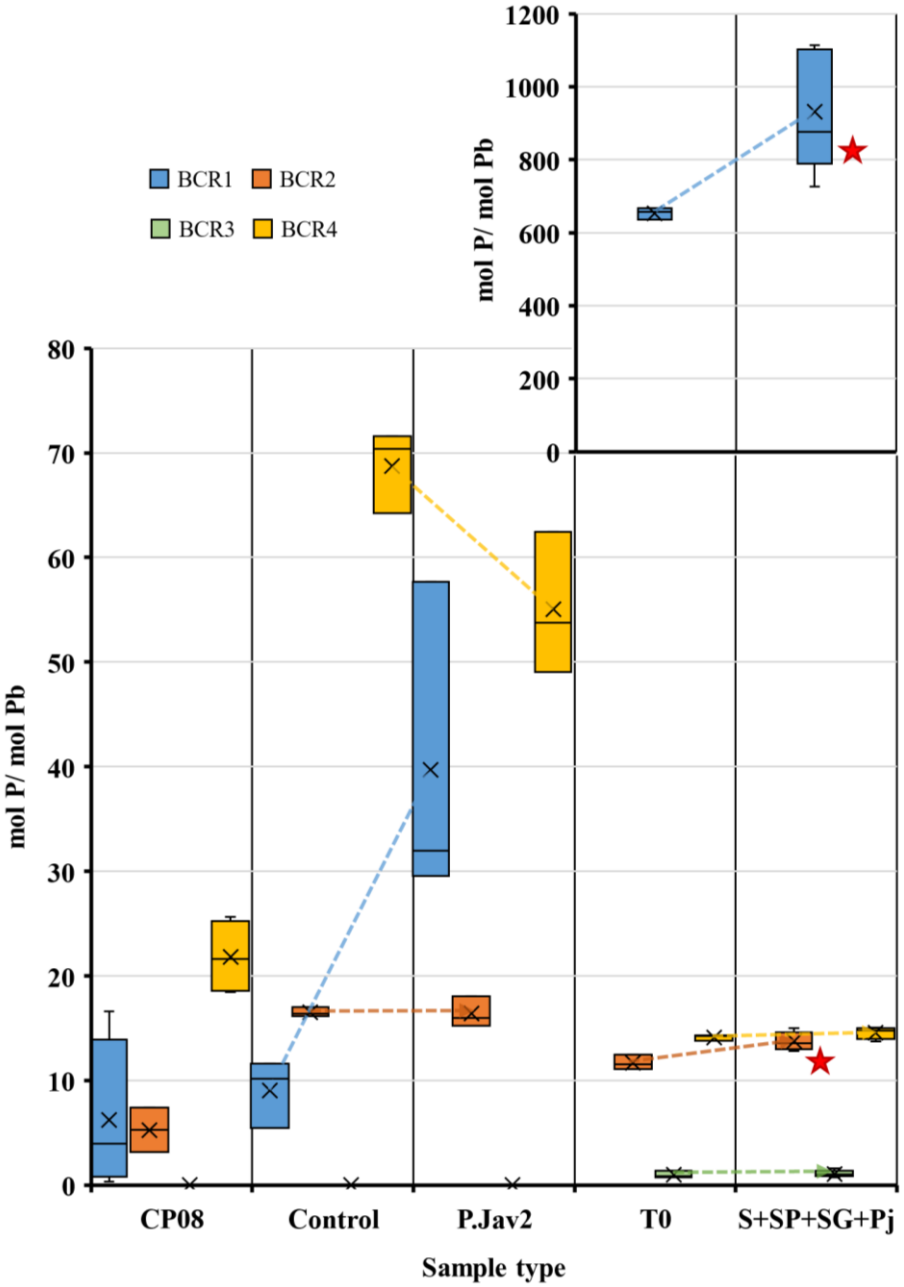



*Figure 6.3. Box plot with P/Pb molar ratio in each BCR stage for CP08, Control (MMS+SG), P.jav2 (SMMS+SSG+Pj), T0 and S+SP+SG+Pj: BCR1in blue, BCR2 in orange, BCR3 in green and BCR4 in yellow. Segmented lines connect the same BCR stage/fraction in samples from the same microcosm experiment (i.e. Control with P.jav2 and T0 with S+SP+SG+Pj), to highlight an increase or reduction of the P/Pb molar ratio in BCR stages, associated to soil treatment. The red star indicates the increase/reduction is statistically significant. The smaller plot in the top-right shows the P/Pb molar ratio in BCR1, for samples T0 and S+SP+SG+Pj with a different Y-axis scale.*

*Table 6.6. t-Tests p values carried out on BCR results.*

| t-Test: Two-Sample Assuming Unequal Variances (α=0.05) | | |
|---|---|---|
| **P(T<=t) two-tail** | **Control-P.jav2** | **T0-S+SP+SG+Pj** |
| **BCR1 Pb** | 0.0100 | 0.9712 |
| **BCR2 Pb** | 0.5871 | 0.0039 |
| **BCR3 Pb** | 0.0078 | 0.0919 |
| **BCR4 Pb** | 0.0287 | 0.3693 |
| **BCR1 P** | 0.0009 | 0.0003 |
| **BCR2 P** | 0.0026 | 1.25E-05 |
| **BCR3 P** | - | 0.8927 |
| **BCR4 P** | 0.0005 | 0.2460 |
| **BCR1 P/Pb** | 0.0794 | 0.0203 |
| **BCR2 P/Pb** | 0.9121 | 0.0154 |
| **BCR3 P/Pb** | - | 0.6860 |
| **BCR4 P/Pb** | 0.0570 | 0.2004 |

For samples from the spiked soil experiment, BCR2 and 3 have the highest % of the total Pb, with BCR2= 42.5 ± 2.3 % and BCR3= 40.3 ± 4.2 % for T0, and BCR2= 38.1 ± 1.5 % and BCR3= 43.8 ± 2.4 % for S+SP+SG+Pj. BCR4 follows with around 15% and finally BCR1 with ~1.5%. Though subtle, a few differences can be made between the time zero and treated soil. While BCR1 and 4 remained unchanged (no increase in residual Pb in treated soil) (Figure 6.1), BCR2 associated Pb (nominal target phases: iron/manganese oxyhydroxides) appears to be reduced in favour of BCR3 associated Pb (nominal target phases: organic matter, sulphides) (Figure 6.1) in treated soil. When it comes to statistical significance however, only the change in BCR2 is significant ($p = 0.0039$).

For P in soils from the spiked soil experiment, BCR1 has the clear majority with >57% in both sample groups (Table 6.4), followed by BCR2 with between 23 and 29%, then BCR4 with between 10 and 13% and finally BCR3 with < 3%. When comparing between T0 and treated soil (Figure 6.2), BCR3 and 4 remain unchanged, while BCR1 associated P is increased in treated soil ($p = 0.0003$) in detriment of BCR2 which is reduced ($p = 1.25E-05$).

The overall molar P/Pb ratio is increased in treated soils (S+SP+SG+Pj) in respect to S soils, as a product of the addition of superphosphate. This in the individual BCR stages is only reflected in the BCR1 and 2 stages, that have significantly higher ratios in treated soil ($p = 0.020$ in BCR1 and 0.015 in BCR2). The significant increase in BCR1 P/Pb (Figure 6.3) as well as BCR1 P% (Figure 6.2) in S+SP+SG+Pj, indicates that the added superphosphate remained in the more mobile (BCR1) stage. This in turn produces the proportional reduction in %BCR2 P (Figure 6.2). As Pb was reduced in BCR2, this translates in the enhanced P/Pb in this stage.

Samples from the preliminary soil experiment have a more even distribution of Pb in BCR2, 3 and 4, with BCR1 being the lowest at <5%. While BCR2 is not different in the Control (MMS+SG) and P.jav2 (SMMS+SSG+Pj), BCR4 and 1 have statistically significant lower % Pb in P.jav2 than in the Control ($p = 0.029$ in BCR4 and 0.010 in BCR1), in benefit of BCR3 which has an average % Pb of 37.11 ± 3.11 % in P.jav2 and 25.66 ± 5.492 % in the Control ($p = 0.0783$).

P distribution also shows statistically significant differences between Control (MMS+SG) and P.jav2 (SMMS+SSG+Pj) in all BCR stages, with exception of BCR3 where P concentrations are undetectable when adjusted with the Blank background. With BCR4 being the predominant stage, it reduces P from 80.00 ± 2.35 % in Control, to 69.92 ± 2.48 % in P.jav2 ($p= 0.0005$), mostly in benefit of BCR2 (18.73 ± 2.43 % in Control and 26.21 ± 3.09 % in P.jav2, $p= 0.0026$) but also BCR1 (1.263 ± 0.83 % in Control and 3.88 ± 0.64 % in P.jav2, $p= 0.0009$). Hence mobilization from the residual phase would not only appear to be occurring for Pb, but also for P.

In Chapter 3 the ability of *P.javanicus* to biomineralize Pb as pyromorphite was shown. It has been shown that pyromorphite remains mostly insoluble over subsequent sequential extraction stages, remaining by the most part in the residual portion (82, 86). The aim of treating the soil via bioaugmentation with *P.javanicus* was to increase residual (BCR4) Pb, as it represents the least mobile and potentially bioavailable fraction, hence reducing potential negative impacts to human health. It would appear, however, that in the preliminary soil experiment, the activity of *P.javanicus* in the soil, rather than increasing the residual Pb, mobilized Pb and P from the BCR4 fraction towards the organic matter and sulphides targeted fraction (BCR3).

While total P and P/Pb are higher in Control (MMS+SG) than in P.jav2 (SMMS+SSG+Pj), both the overall and individual P/Pb molar ratio for separate BCR stages (with exception of BCR3) are sufficient for the stoichiometric formation of pyromorphite ($P/Pb > 0.6$) in both cases.

In CP08 samples, utilized as comparative environmental un-treated matrix, besides the heterogeneities described before (observable in Figure 6.1), BCR 2 and 3 have in average higher % of Pb (33.20 ± 6.56 and 41.69 ± 1.34 % respectively) followed by BCR 4 (22.75 ± 7.59 %) and finally BCR 1 (2.35 ± 2.45 %). P does not reflect the same heterogeneities as Pb (Figure 6.2). As in soils from the preliminary soil experiment, BCR4 is dominant, with 74.16 ± 1.44 %, followed by BCR2 (24.59 ± 2.03 %) and BCR1 (1.25 ± 1.39 %). BCR3,

also like Control and P.jav2, has negative values after subtracting the Blank background. Molar ratio (overall and for each BCR stage) are shown in Table 6.5 and Figure 6.3.

#### 6.4.1.1 Statistical correlations

To complement, and reinforce, observations made with the graphing of results and evaluation of statistical significance, correlations were calculated amongst various parameters for samples from the two experiments. In addition to BCR % and ∑BCR for Pb and P, as well as P/Pb ratios of each sample, correlations with the presence or absence of SP+SG+Pj treatment in the spiked soil experiment and *P.javanicus* inoculum in the preliminary soil experiment, were evaluated by assigning a value of +1 when treatment was present and -1 when not. Correlations were calculated for each parameter against all parameters with the "Correlations" tool in Microsoft Excel (2016), as shown in a detailed example in appendix CH6 A7.

Resulting correlation coefficients for samples from the spiked soil experiment are shown in Table 6.7. Positive correlation coefficients higher than 0.5 are marked with pink and negative correlation coefficients lower than -0.5 with blue.

As can be seen in Table 6.7 treated soils from the spiked soil experiment (S+SP+SG+Pj) mostly have positive correlation coefficients with parameters associated to the addition of superphosphate, and consequent increase of overall P content, as well as P in the more available fraction. This also relates to some of the observed negative correlations, which reflect the proportional reduction of BCR2 and BCR4 associated P, respective to BCR1 P.

A moderate positive correlation between S+SP+SG+Pj and Pb associated to the BCR3 fraction, and corresponding negative correlation with BCR2 Pb, can also be observed. The slight dilution of Total Pb, associated to the addition of amendments to soils, is also reflected with a negative correlation coefficient. No correlation is apparent between amendment of soil and the % of Pb present in BCR1 and 4, further suggesting that the desired increase in residual Pb was not achieved by the treatment of soil (superphosphate, spent grain or *P.javanicus* inoculum).

Other positive and negative correlation coefficients between BCR%, Total and P/Pb molar ratio parameters, shown in Table 6.7, reflect the same changes in overall P and Pb, as well as BCR-stage specific concentrations differences described above.

Resulting correlation coefficients for samples from the preliminary soil experiment are shown in Table 6.8. As before, positive correlation coefficients higher than 0.5 are marked with pink and negative correlation coefficients lower than -0.5 with blue.

*Table 6.7. Correlation coefficients for BCR results of the spiked soil experiment.*

| Spiked soil experiment | BCR1 Pb | BCR2 Pb | BCR3 Pb | BCR4 Pb | ∑BCR Pb | BCR1 P | BCR2 P | BCR3 P | BCR4 P | ∑BCR P | BCR1 P/Pb | BCR2 P/Pb | BCR3 P/Pb | BCR4 P/Pb | Total P/Pb | Pj |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **BCR1 Pb** | 1.000 | | | | | | | | | | | | | | | |
| **BCR2 Pb** | -0.120 | 1.000 | | | | | | | | | | | | | | |
| **BCR3 Pb** | 0.107 | -0.869 | 1.000 | | | | | | | | | | | | | |
| **BCR4 Pb** | -0.162 | -0.182 | -0.320 | 1.000 | | | | | | | | | | | | |
| **Total Pb** | -0.342 | 0.643 | -0.292 | -0.588 | 1.000 | | | | | | | | | | | |
| **BCR1 P** | 0.086 | -0.907 | 0.793 | 0.160 | -0.742 | 1.000 | | | | | | | | | | |
| **BCR2 P** | 0.087 | 0.910 | -0.749 | -0.282 | 0.722 | -0.976 | 1.000 | | | | | | | | | |
| **BCR3 P** | -0.493 | 0.002 | 0.317 | -0.533 | 0.375 | -0.002 | -0.020 | 1.000 | | | | | | | | |
| **BCR4 P** | -0.129 | 0.663 | -0.831 | 0.386 | 0.389 | -0.781 | 0.686 | -0.474 | 1.000 | | | | | | | |
| **Total P** | 0.037 | -0.926 | 0.903 | -0.011 | -0.565 | 0.931 | -0.902 | 0.244 | -0.868 | 1.000 | | | | | | |
| **BCR1 P/Pb** | -0.645 | -0.588 | 0.469 | 0.319 | -0.396 | 0.678 | -0.790 | 0.395 | -0.498 | 0.670 | 1.000 | | | | | |
| **BCR2 P/Pb** | 0.415 | -0.895 | 0.712 | 0.234 | -0.793 | 0.825 | -0.771 | -0.094 | -0.659 | 0.850 | 0.373 | 1.000 | | | | |
| **BCR3 P/Pb** | -0.500 | -0.171 | 0.401 | -0.367 | 0.138 | 0.204 | -0.233 | 0.965 | -0.596 | 0.421 | 0.577 | 0.099 | 1.000 | | | |
| **BCR4 P/Pb** | 0.753 | -0.671 | 0.693 | -0.215 | -0.453 | 0.565 | -0.444 | -0.269 | -0.502 | 0.555 | -0.156 | 0.726 | -0.224 | 1.000 | | |
| **Total P/Pb** | 0.226 | -0.896 | 0.693 | 0.309 | -0.875 | 0.947 | -0.916 | -0.069 | -0.719 | 0.893 | 0.593 | 0.937 | 0.161 | 0.588 | 1.000 | |
| **Pj** | -0.013 | -0.889 | 0.665 | 0.393 | -0.831 | 0.960 | -0.983 | -0.057 | -0.637 | 0.858 | 0.754 | 0.812 | 0.174 | 0.446 | 0.951 | 1.000 |

*Table 6.8. Correlation coefficients for BCR results of the preliminary soil experiment.*

| Preliminary soil exp. | BCR1 Pb | BCR2 Pb | BCR3 Pb | BCR4 Pb | ∑BCR Pb | BCR1 P | BCR2 P | BCR3 P | BCR4 P | ∑BCR P | BCR1 P/Pb | BCR2 P/Pb | BCR3 P/Pb | BCR4 P/Pb | Total P/Pb | Pj |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **BCR1 Pb** | 1.000 | | | | | | | | | | | | | | | |
| **BCR2 Pb** | 0.152 | 1.000 | | | | | | | | | | | | | | |
| **BCR3 Pb** | -0.933 | -0.210 | 1.000 | | | | | | | | | | | | | |
| **BCR4 Pb** | 0.884 | -0.021 | -0.969 | 1.000 | | | | | | | | | | | | |
| **Total Pb** | -0.153 | -0.642 | 0.310 | -0.191 | 1.000 | | | | | | | | | | | |
| **BCR1 P** | -0.904 | -0.426 | 0.918 | -0.821 | 0.225 | 1.000 | | | | | | | | | | |
| **BCR2 P** | -0.917 | -0.198 | 0.989 | -0.963 | 0.375 | 0.897 | 1.000 | | | | | | | | | |
| **BCR3 P** | | | | | | | | | | | | | | | | |
| **BCR4 P** | 0.932 | 0.262 | -0.991 | 0.945 | -0.344 | -0.942 | -0.993 | | 1.000 | | | | | | | |
| **Total P** | 0.885 | 0.026 | -0.974 | 0.994 | -0.181 | -0.861 | -0.969 | | 0.960 | 1.000 | | | | | | |
| **BCR1 P/Pb** | -0.908 | -0.184 | 0.762 | -0.686 | -0.085 | 0.884 | 0.743 | | -0.795 | -0.718 | 1.000 | | | | | |
| **BCR2 P/Pb** | -0.078 | -0.015 | -0.213 | 0.288 | -0.663 | 0.082 | -0.279 | | 0.190 | 0.251 | 0.426 | 1.000 | | | | |
| **BCR3 P/Pb** | | | | | | | | | | | | | | | | |
| **BCR4 P/Pb** | 0.684 | 0.602 | -0.823 | 0.712 | -0.750 | -0.798 | -0.857 | | 0.859 | 0.733 | -0.512 | 0.433 | | 1.000 | | |
| **Total P/Pb** | 0.691 | 0.389 | -0.876 | 0.828 | -0.683 | -0.753 | -0.907 | | 0.885 | 0.834 | -0.443 | 0.585 | | 0.958 | 1.000 | |
| **Pj** | -0.917 | -0.305 | 0.954 | -0.893 | 0.270 | 0.975 | 0.958 | | -0.982 | -0.925 | 0.857 | -0.062 | | -0.833 | -0.824 | 1.000 |

The inoculation and proliferation of *P.javanicus* in soil (P.jav2: SMMS+SSG+Pj) correlates with parameters that suggest the mobilization of P from the residual phase (BCR4) towards BCR1 and 2, and Pb from the residual (BCR4) and most mobile phase (BCR1) to BCR3 (organic matter targeted phase).

Negative correlations between the presence of *P.javanicus* and Total P and Total P/Pb, rather than reflecting an effect of the fungal activity, are consequence of a degree of heterogeneity between the soils used in each microcosm system. Although theoretically replicates of an homogenized soil mixture, Control (MMS+SG, 1435.02 ± 271.58) showed higher total P concentrations than P.jav2 (SMMS+SSG+Pj, 1033.99 ± 97.61).

In order to assess if any correlation trends derived from the inoculation of *P.javanicus* in soil, transcended individual experiments, correlation coefficients were calculated grouping all soils together (data not shown). Through this exercise, the only correlation coefficient > 0.5 and common effect observed in both experiments, pairs the addition of *P.javanicus* with BCR3 associated Pb (0.584).

### 6.4.2 Lead UBM bioaccessibility

A summary of results of the UBM bioaccessibility assay is presented in Table 6.9, with averaged values for each sample type (CP08 homogenised soil, preliminary soil experiment, spiked soil experiment) as well as the reference matrix (RefMtx) and CRM.

Both the average Gc+Res and Ist+Res values for the CRM fall within the certified prediction interval of 283 to 398 mg $kg^{-1}$ (confidence interval: 333 to 347 mg $kg^{-1}$).

As the solutions used in the UBM have P in their preparation, resulting in a high background and distortion of the resulting data, no analysis of the P results was included in this section.

Figures 6.4 and 6.5 show box plots with data from all samples, resulting from the Gc and Ist phases respectively. In Figure 6.4 the first graph (Figure 6.4-a) illustrates the % of total Pb in solution in the Gc phase for each different soil treatment or soil type. As it can be noted, the reference matrix (RefMtx) shows a Pb bioaccessibility of almost 100% (99.93 ± 0.13 %), indicating that ~0 % of Pb introduced directly to the Gc-phase solution as $Pb(NO_3)_2$, precipitated (refer to section 6.3.2.1.5). In consequence, no normalization to the RefMtx of the Gc-Pb bioaccessibility in soil samples, for comparison with the Ist phase, was made. The second graph (Figure 6.4-b) illustrates Pb concentration (mg $kg^{-1}$) according to soil treatment/type, resulting from the addition of the Gc phase with its respective residue. The final graph (Figure 6.4-c) shows the % of total Pb in solution in the Gc phase for the CRM, on each round of the assay.

*Table 6.9. Summary of results for the UBM bioaccessibility assay.*

| | | Pb | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **n** | **Gc %** | **error (%)** | **Gc+Res (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **Ist%** | **error (%)** | **Ist+Res (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **Ist% RefMtxNorm** | **error (%)** |
| CP08 | 6 | 60.69 | *1.044* | 827.4 | *20.80* | 11.14 | *2.351* | 763.0 | *17.41* | 26.56 | *5.604* |
| P.jav1+3 | 4 | 77.50 | *3.592* | 464.2 | *24.30* | 2.422 | *1.110* | 456.3 | *7.721* | 5.774 | *2.646* |
| P.jav 2 | 2 | 67.56 | *40.82* | 448.1 | *1.157* | 1.558 | *5.370* | 449.9 | *62.28* | 3.715 | *12.80* |
| Control | 2 | 70.02 | *7.691* | 431.2 | *6.292* | 1.592 | *4.652* | 431.5 | *30.36* | 3.795 | *11.09* |
| T0 | 7 | 90.01 | *0.552* | 1228 | *112.5* | 6.983 | *1.360* | 1141 | *60.66* | 16.65 | *3.241* |
| S | 6 | 87.40 | *2.133* | 1191 | *138.9* | 17.34 | *1.336* | 1118 | *83.55* | 41.33 | *3.186* |
| S+SP | 6 | 87.83 | *1.342* | 1339 | *85.37* | 11.24 | *7.081* | 1229 | *40.54* | 26.80 | *16.88* |
| S+SG | 6 | 86.34 | *0.511* | 1278 | *50.83* | 7.685 | *3.232* | 1168 | *83.55* | 18.321 | *7.706* |
| S+Pj | 6 | 86.02 | *0.994* | 1335 | *72.92* | 12.23 | *2.169* | 1167 | *47.91* | 29.16 | *5.171* |
| S+SG+Pj | 6 | 85.97 | *1.201* | 1276 | *55.89* | 11.17 | *1.482* | 1087 | *23.15* | 26.64 | *3.533* |
| S+SSG+Pj | 6 | 84.76 | *2.149* | 1246 | *74.36* | 8.515 | *0.422* | 1103 | *110.0* | 20.30 | *1.006* |
| S+SP+Pj | 4 | 85.89 | *1.192* | 1326 | *91.92* | 11.69 | *4.618* | 1243 | *52.89* | 27.88 | *11.01* |
| S+SP+SG+Pj | 4 | 85.98 | *0.657* | 1272 | *105.0* | 9.012 | *2.077* | 1085 | *33.22* | 21.49 | *4.952* |
| RefMtx | 4 | 99.93 | *0.133* | 1135 | *37.88* | 41.95 | *4.595* | 1021 | *32.89* | 100.0 | |
| CRM | 18 | 81.02 | *0.906* | 315.9 | *9.803* | 3.262 | *1.126* | 323.2 | *31.10* | 7.777 | *2.684* |

***Note that samples P.jav2 and Control have only 2 replicates (2) which impacts on high Gc% and Ist% errors. From the preliminary soil experiment: Control = MMS+SG; P.jav 1 & 3 = MMS+SG+Pj and P.jav2 = SMMS+SSG+Pj. Gc % refers to the % of total Pb in solution in the gastric phase; Gc+Res refers to the total Pb concentration (mg kg-1) resulting from the addition of the Gc phase and respective residue (aqua regia digestion); Ist% refers to the % of total Pb in solution in the intestinal phase; Ist+Res refers to the total Pb concentration (mg kg-1) resulting from the addition of the Ist phase and respective residue (aqua regia digestion); Ist RefMtxNorm refers to the % of Pb in solution in the intestinal phase normalized to the reference matrix (Ist RefMtxNorm). The error is included for each of these values, and the number of replicates per sample type (n) is displayed in a separate column.*

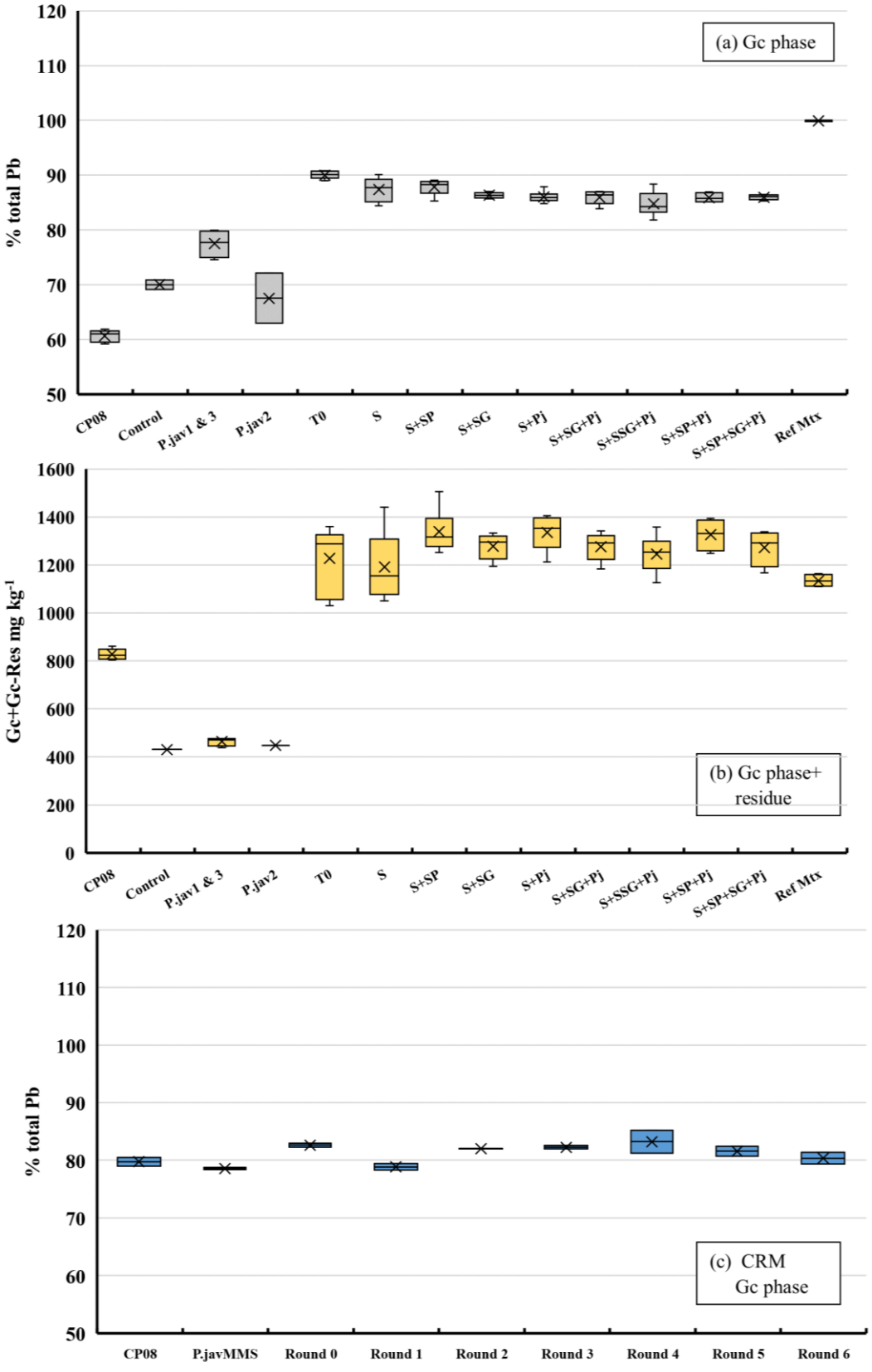



*Figure 6.4. Box plots for the gastric (Gc) phase of the UBM bioaccessibility assay. From the preliminary soil experiment: Control = MMS+SG; P.jav 1 & 3 = MMS+SG+Pj and P.jav2 = SMMS+SSG+Pj.*

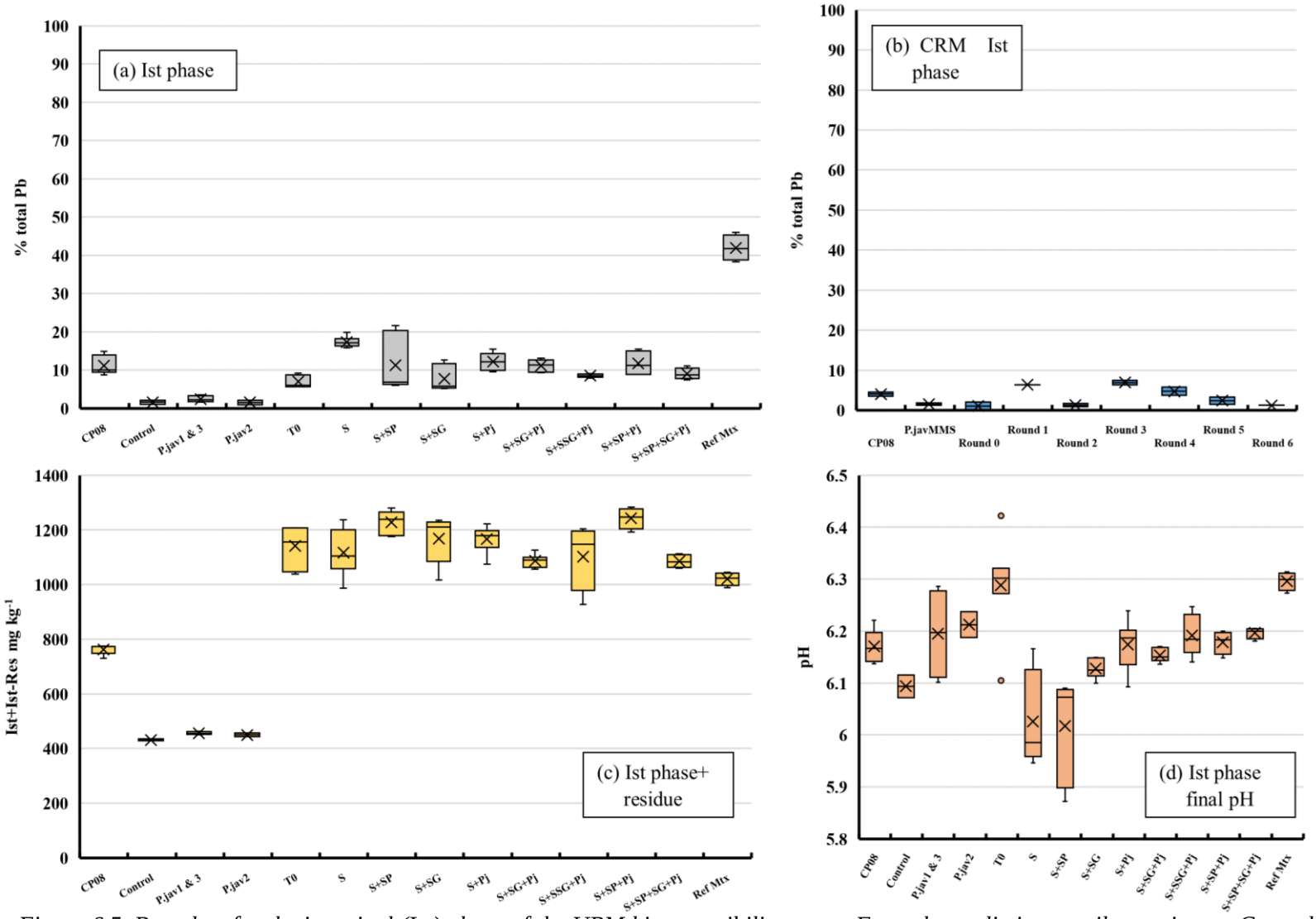



*Figure 6.5. Box plots for the intestinal (Ist) phase of the UBM bioaccessibility assay. From the preliminary soil experiment: Control = MMS+SG; P.jav 1 & 3 = MMS+SG+Pj and P.jav2 = SMMS+SSG+Pj.*

*Table 6.10. CRM-UBM round with their respective samples.*

| CRM-UBM round | Samples |
|---|---|
| CP08 | All CP08 samples |
| P.javMMS | P.jav1 &3, P.jav2 and Control samples |
| Round 0 | T0 samples |
| Round 1 | S 1, 2 and 3; S+SP 4 |
| Round 2 | S+SP 5 and 6; S+SG 7 and 8 |
| Round 3 | S+SG 9; S+Pj 10, 11 and 12 |
| Round 4 | S+SG+Pj 13, 14 and 15; S+SSG+Pj 16 |
| Round 5 | S+SSG+Pj 17 and 18; S+SP+Pj 19 and 20 |
| Round 6 | S+SP+SG+Pj 22 and 23; RefMtx 1, 2,3 and 4 |

Certain variability is observed, with an average Pb Gc-bioaccessibility for all CRMs of 81.02 ± 0.906 % (Table 6.9), where the error corresponds only to a 1.118% of the main average Gc-bioaccessibility (%) value, showing a good repeatability. As UBM rounds overlap different sample groups, the corresponding CRM to samples digested in a specific round is shown in Table 6.10.

In Figure 6.5, the first graph (Figure 6.5-a) illustrates the % of total Pb in solution in the Ist phase for each different soil treatment or soil type. In the Ist phase, the reference matrix (RefMtx) shows a Pb bioaccessibility of 41.95 ± 4.60 % (Ist % values normalized to the RefMtx as described in section 6.3.2.2 are included in table 6.9). The second graph (Figure 6.5-b) shows the % of total Pb in solution in the Ist phase for the CRM, on each round of the assay. The pairing of each CRM with soil samples is the same as in the Gc phase (Table 6.10). The variability in the % of bioaccessible Pb in the CRM on the Ist phase is higher than in the Gc phase, with an error of 1.126, which corresponds to a 34.51 % of the average of 3.262 %. The third graph (Figure 6.5-c) shows Pb concentration (mg $kg^{-1}$) according to soil treatment or soil type, resulting from the addition of the Ist phase with its respective residue. The final graph (Figure 6.5-d) shows the final pH value for each sample (grouped by soil treatment/ soil type), measured on the Ist supernatant at the end of the assay.

To support the interpretation of results, the statistical significance of the difference in %Gc and %Ist amongst soil treatments belonging to the same experiment was assessed. For the spiked soil experiment the t-test (two-sample assuming unequal variance, $\alpha = 0.05$) was used to compare the control soils (S) to each soil treatment, as well as the T0 soil. Anova (single factor, $\alpha = 0.05$) was used to assess differences of all soil treatments as a group. All resulting p values are displayed in Table 6.11, with those indicating statistical significance ($< \alpha = 0.05$) highlighted in grey.

*Table 6.11. p-values for t-test and Anova analysis of UBM results for spiked soil experiment samples.*

| t-Test: Two-Sample Assuming Unequal Variances (α=0.05) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **P(T<=t) two-tail** | **T0** | **S** | **S+SP** | **S+SG** | **S+Pj** | **S+SG+Pj** | **S+SSG+Pj** | **S+SP+Pj** | **S+SP+SG+Pj** |
| **S (Gc)** | 0.0324 | | 0.7031 | 0.2986 | 0.2106 | 0.2064 | 0.0675 | 0.1767 | 0.1812 |
| **S (Ist)** | 7.54E-08 | | 0.1038 | 0.0003 | 0.0015 | 0.0000 | 0.0000 | 0.0333 | 0.0733 |
| **Anova: Single Factor (α=0.05)** | | | | | | | | | |
| **P-value** | **T0** | **S** | **S+SP** | **S+SG** | **S+Pj** | **S+SG+Pj** | **S+SSG+Pj** | **S+SP+Pj** | **S+SP+SG+Pj** |
| **Gc** | 1.93E-06 | | | | | | | | |
| **Ist** | 5.76E-05 | | | | | | | | |

*Table 6.12. p-values for t-test and Anova analysis of UBM results for preliminary soil experiment samples.*

| **t-Test: Two-Sample Assuming Unequal Variances (α=0.05)** | | | | |
|---|---|---|---|---|
| **P(T<=t) two-tail** | | **Control** | **P.jav 2** | **P.jav1+3** |
| **Gc** | **P.jav 2** | 0.6885 | | |
| | **P.jav1-3** | 0.0087 | 0.2824 | |
| **Ist** | **P.jav 2** | 0.9701 | | |
| | **P.jav1-3** | 0.3331 | 0.3535 | |
| **Anova: Single Factor (α=0.05)** | | | | |
| **P-value** | | **Control** | **P.jav 2** | **P.jav1+3** |
| **Gc** | | 0.0436 | | |
| **Ist** | | 0.3956 | | |

**Control = MMS+SG; P.jav 1 & 3 = MMS+SG+Pj and P.jav2 = SMMS+SSG+Pj.*

For the preliminary soil experiment the t-test (two-sample assuming unequal variance, α = 0.05) was used to assess difference between all 3 treatments (Control [MMS+SG] = soil + spent grain; P.jav2 [SMMS+SSG+Pj] = sterile soil + sterile spent grain + *P.javanicus* inoculum; P.jav1+3 [MMS+SG+Pj] = non-sterile soil + non-sterile spent grain + *P.javanicus* inoculum) against each other in pairs. Anova (single factor, α = 0.05) was used to assess differences amongst the 3 different treatments as a group. All resulting p values are displayed in Table 6.12, with those indicating statistical significance (< α = 0.05) highlighted in grey.

Correlations were also evaluated, for the two experiments separately (analogously to the example shown in appendix CH6 A7), as well as all samples as a whole. Correlation coefficient were calculated by pairing Gc% and Ist% with different selected parameters, shown in Table 6.13.

### 6.4.2.1 Spiked soil experiment

As Figure 6.4-a shows, the Gc% bioaccessible Pb did not show a great variation amongst all soil treatments, with average percentages ranging from 84.76 ± 2.15 (S+SSG+Pj) to 90.01 ± 0.552 (T0), or 87.83 ± 1.342 (S+SP) if only considering soils after incubation. This % of Pb bioaccessibility is quite high relative to registered soil Pb bioaccessibilities shown in section

2.1.5, with only a 10-15% difference to the RefMtx, spiked directly in solution into the UBM fluids. As the same compound ($Pb(NO_3)_2$) was used for the RefMtx and to spike the soil, this low difference (10% at time zero) could be indicating that after spiking and ageing the soil, most of the Pb remained in its soluble form, only decreasing slightly in incubated soils.

*Table 6.13. Correlation coefficients for results of the UBM biaccessibility assay.*

| Correlations | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gc% | | | | Ist% | | | |
| | All | Spiked Soil | Prel. exp | CRM | All | Spiked Soil | Prel. exp | CRM |
| CRMGc % | 0.517 | -0.017 | | | | | | |
| Gc+Res | 0.701 | -0.402 | 0.428 | -0.508 | | | | |
| Ist% | 0.196 | -0.166 | 0.520 | 0.099 | | | | |
| Pj | 0.092 | -0.577 | 0.343 | | -0.129 | -0.013 | 0.309 | |
| SG | -0.036 | -0.470 | | | -0.464 | -0.309 | | |
| SP | 0.242 | -0.021 | | | 0.125 | 0.019 | | |
| SG+Pj | -0.024 | -0.447 | 0.343 | | -0.301 | -0.155 | 0.309 | |
| Inc time | | -0.643 | | | | 0.342 | | |
| P | 0.906 | -0.021 | 0.540 | | 0.377 | 0.019 | 0.755 | |
| CRMIst % | | | | | 0.751 | 0.748 | | |
| Ist+Res | | | | | 0.471 | -0.113 | 0.414 | -0.220 |
| pH | | | | | -0.502 | -0.620 | -0.197 | -0.169 |

**Column "All" considers Pb bioaccessibility data (Gc% and Ist% respectively) for both experiments together; column "Spiked soil" considers Pb bioaccessibility data (Gc% and Ist% respectively) only for samples from the spiked soil experiment; column "Prel. Exp" considers Pb bioaccessibility data (Gc% and Ist% respectively) only for samples from the preliminary soil experiment; finally, column "CRM" considers Pb bioaccessibility data (Gc% and Ist% respectively) for the certified reference material. Row "CRM Gc%" refers to the Pb Gc% in the CRM extracted simultaneously to a sample as quality control; row "Gc+Res" refers to the respective total Pb (mg $kg^{-1}$) for each Gc sample resulting from Pb in the Gc phase + Pb in the residue; row "Ist%" refers to the corresponding intestinal Pb bioaccessibility for each Gc sample; row "Pj" refers to the presence or absence of P.javanicus inoculum (assigned by a +1 or -1 respectively) in a soil sample; row "SG" refers to the presence or absence spent grain amendment (assigned by a +1 or -1 respectively) in a soil sample; row SP refers to the presence or absence of superphosphate amendment (assigned by a +1 or -1 respectively) in a soil sample; row "SG+Pj" refers to the presence or absence of P.javanicus inoculum plus additional spent grain amendment (assigned by a +1 or -1 respectively) in a soil sample; row "Inc time" refers to samples that have (+1) or have not (-1) been incubated as microcosms; row "P" refers to the total P concentration of each sample; row "CRM Ist%" refers to the Pb Ist% in the CRM extracted simultaneously to a sample as quality control; row Ist+Res refers to the respective total Pb (mg $kg^{-1}$) for each Ist sample resulting from Pb in the Ist phase + Pb in the residue; finally row "pH" refers to the final pH value measured in the Ist solution after extraction. Positive correlation coefficients > 0.5 are marked in pink and negative correlation coefficients < -0.5 in blue.*

When assessing the differences in %Gc of all treatments, including T0, with Anova analysis the p value is low (7.538E-08) and indicates that not all %Gc are statistically equal. With the t-Test, only the T0-S (time zero and incubated control) pair appears to be significantly different ($0.032 < p = 0.05$), while all other treatments, compared to S (control), have a p

value > 0.05, so are not significantly (statistically) different. This is indication that the incubation time, with added soil solution, played a more significant role than any soil amendment/treatment, in decreasing (if only slightly) Gc bioaccessible Pb.

Of all correlations made with %Gc the only 2 parameters that show a level of correlation >0.5 are: incubation time (coefficient -0.643), in agreement with the analysis of statistical significance, and the presence of *P.javanicus* inoculum (-0.577).

Intestinal (Ist) bioaccessibility of Pb is more variable, spanning from 6.98 ± 1.36 % (T0) to 17.34 ± 1.336 % (S). %Ist values are quite low, but the RefMtx showing an intestinal bioaccessibility of 41.95 ± 4.60 % of total Pb, indicates that approximately 58% of total Pb becomes inaccessible even when adding $Pb(NO_3)_2$ alone, directly into the Ist UBM solution. As previously mentioned in section 2.1.5, the higher pH values of the Ist solution, in combination with the presence of other elements (i.e. P) in solution are the likely cause of the reduction of bioaccessibilty of the RefMtx in the Ist phase. In consequence, when normalizing to the reference matrix (RefMtx), the magnitude of "relative bioaccessibility" becomes higher (16.65 ± 3.24 % to 41.33 ± 3.19 %) than that of direct %Ist.

Denys *et al*. (2012) (90) applied the UBM procedure in a variety of soil samples, for validation of the UBM procedure against an *in vivo* juvenile swine model, using a reference matrix for normalization of both *in vivo* bioavailability and UBM-bioaccessibility (refer to section 2.15 and 6.3.2.1.5). After normalization against the reference matrix ("relative bioaccessibility"), Gc and Ist Pb bioaccessibilities were not statistically different, and hence were both validated with the *in vivo* study (90). In our case however, the relative-Ist% of Pb (i.e. normalized with the RefMtx) remains significantly lower to Pb Gc% bioaccessibility (70.69 ± 6.210 % lower, in average), and hence the two parameters (Pb Gc% and relative-Pb Ist%[RefMtx normalized]) continue to deliver different Pb bioaccessibility values for a given sample. The high input of P in solution derived from the spiked garden soil (treated or not with superphosphate) at the near neutral pH of the Ist solution, most likely caused the Pb Ist% to remain lower than Pb Gc% after normalization with the reference matrix, contrary to what Denys *et al*. (2012) (90) observed.

Anova analysis of all soil treatments as a group indicates that not all %Ist are statistically equal (p-value < $\alpha = 0.05$). With the t-Test, differences in Ist% Pb bioaccessibility between the control soils (S) and all other treatments are significant (p value < 0.05), with exception of S+SP ($p = 0.104$) and S+SP+SG+Pj ($p = 0.073$).

Analysis of correlations with Pb Ist% included both parameters related to differences in soil treatment (e.g. presence of spent grain, *P.javanicus* or superphosphate amendments) and

operational parameters related to the UBM procedure (e.g. final pH of the Ist solution and Pb %Ist in the corresponding CRM), to identify which of the two was responsible for statistical differences in Pb Ist% in the different soil samples. The analysis of correlations shows, that these differences in Ist% are most likely operational.

The presence of none of the individual amendments (SG, SP and Pj), in any combination, showed a correlation with the Ist% variation. Neither did incubation time (T0 vs incubated treatments) or total concentrations of P and Pb (Ist+Res), although the last two were very similar in all treatments, so it was expected that they would not have a numerical correlation.

Furthermore, CRM Ist% had a correlation coefficient of 0.748 and pH of -0.620 with Ist%. pH is adjusted individually in each sample, and for this reason it is impossible to have the exact same pH in all cases. Eeach individual sample also has an influence in the final solution pH. It would appear then that a higher final pH (representing pH during the extraction) would produce lower Pb intestinal bioaccessibility. As CRMs have their own pH, which if higher or lower will not necessarily mean higher or lower pH in the samples of the respective rounds (CRM Ist% in fact did not show a correlation with final pH, correlation coefficient of -0.169), the positive correlation between CRM Ist% and sample Ist% shows that other factors (other than pH) varying from round to round may be causing these differences. Although the same recipe is followed every time, and temperature in the incubator, end over end rotation speed, and digestion time are set to be the same, a certain degree of variability is inevitable. Concentrations of reagents in solution will never be exactly the same; times may vary depending on the human factor of handling samples after the end of the process; temperature in the incubator may be affected by the ambient temperature in the lab; etc. The soil samples themselves also add variability. Therefore, correlation coefficients appear to be showing that the intestinal phase of the UBM process is quite sensitive to these small variations.

If a sample has a high Pb Gc%, when the Ist solutions are added to the sample the majority of the soil Pb will already be in solution. For this reason, conditions, elements and components present in the Ist solution are more likely to determined Pb Ist bioaccessibility, than the pre-existing soil Pb-phases, permitting a higher impact of operational variations (measured as final pH and CRM Ist%) on Pb Ist%, compared to Pb Gc%.

When comparing results from the UBM procedure and BCR sequential extraction, the residual (non-bioaccessible) Pb in the gastric phase of T0 (9.993 ± 0.552 %) samples is significantly (p-value = 0.023) lower than the BCR4 (residual) Pb in the same sample (15.61 ± 2.967). In S+SP+SG+Pj residual (non-bioaccessible) Pb in the gastric phase (14.02 ± 0.657

%) while also significantly (p-value = 0.009) lower, is closer in % to the BCR4 (residual) Pb in the same sample (16.65 ± 1.442). If the solution pH is taken in consideration, pH in the 3 first BCR stages (≥ 2) is higher than the pH in the gastric phase (< 1.5), so Pb phases soluble at the solution pH of BCR1, 2 and 3 are likely to be soluble at the pH of the Gc solution. It would appear that a further portion of Pb (~ 5.6 % in T0 and ~ 2.6 % in S+SP+SG+Pj), present in the BCR4 residual stage was soluble and hence bioaccessible in the UBM Gc phase. The pH difference (≥ 2 – 1.5 >) may account for this. Gc bioaccessible Pb is slightly, yet significantly (p-value = 2.42E-6), higher in T0 than in S+SP+SG+Pj (discussed earlier as correlated to incubation time), however, this difference is not reflected in results from the BCR procedure, with BCR4 Pb in T0 and S+SP+SG+Pj being statistically equal (p-value = 0.369). As the variations observed in the Pb intestinal bioaccessibility (Ist%) were deemed to be most influenced by operational factors (and not pre-existing soil phases), no correlations were made with BCR sequential extraction.

#### 6.4.2.2 Preliminary soil experiment

Pb bioaccessibility in the Gc phase is comparatively lower in samples from the preliminary soil experiment, in relation to the spiked soil experiment. However, they are still high, ranging from 67.56 ± 40.82 % (P.jav2 = SMMS+SSG+Pj) to 77.50 ± 3.59 % (P.jav1+3 = MMS+SG+Pj). With the t-test (Table 6.12) only the pair Control (MMS+SG) and P.jav1+3 have a p value (0.0087) that indicates statistically significant differences. The Anova p value (0.0436) also indicates significance when comparing all samples as a group. This could be implying, that the inoculation of *P.javanicus* with non-sterile spent grain amendment, which produced growth of an active microbial population in the soil (section 5.4.2.1), made Pb more bioaccessible (in the Gc phase) than in soil with only spent grain amendment (Control). In P.jav2, where *P.javanicus* was inoculated in sterile soil with spent grain, although the average %Gc is lower than in the Control, there is no statistically significant difference, hence no apparent change in bioaccessibility.

Besides the difference observed between P.jav1+3 (MMS+SG+Pj) and Control (MMS+SG), no notable correlations were found between Gc% and any of the tested parameters (Table 6.13).

In the intestinal phase, Pb bioaccessibility is on average 1.56 ± 5.37 % (3.17 ± 12.80 normalized to RefMtx) in P.jav2, 1.59 ± 4.65 % (3.80 ± 11.09 normalized to RefMtx) in Control and 2.422 ± 1.11 % (5.77 ± 2.65 normalized to RefMtx) in P.jav1+3. Differences in Ist% between samples don't appear as statistically significant with t-test and Anova analysis. The only parameter that appears correlated to Ist% is the total P concentration (correlation

coefficient 0.755). As all these samples were digested in one round, the incidence of subtle variations in the conditions of the specific UBM round, signified by CRMIst% cannot be assessed within this sample group.

When comparing results from the UBM procedure and BCR sequential extraction, the residual (non-bioaccessible) Pb in the gastric phase of Control (29.98 ± 7.69 %, MMS+SG) samples is statistically equal (p-value = 0.085) to the BCR4 (residual) Pb in the same sample (35.55 ± 7.146). In P.jav2 (SMMS+SSG+Pj) residual (non-bioaccessible) Pb in the gastric phase (32.44 ± 40.82 %, n=2, SD = 4.542) is also statistically equal (p-value = 0.444) to the BCR4 (residual) Pb in the same sample (26.95 ± 2.685, n=3, SD = 1.081). Gc bioaccessible Pb is statistically equal (p-value = 0.688) in Control and P.jav2, however, BCR4 is significantly (p-value = 0.029) lower in P.jav2 in respect to Control. This could be an indication that ultimately the reduction of BCR4 residual Pb caused by introducing *P.javanicus* in the P.jav2 soil, did not make a difference when it comes to gastric bioaccessibility. Again, as with other samples the variations observed in the Pb intestinal bioaccessibility (Ist%) were deemed to be most influenced by operational factors (and not pre-existing soil phases), so no correlations were made with BCR sequential extraction.

#### 6.4.2.3 CP08 samples

CP08 samples have an average gastric Pb bioaccessibility of 60.69 ± 1.04 %, the lowest from all samples, and an intestinal bioaccessibility of 11.12 ± 2.35 % (26.56 ± 5.60 % normalized to the RefMtx) similar to that of spiked soil, and higher to that of soils from the preliminary experiment. The different soil matrices had distinct results for gastric bioaccessibility, suggesting that an operational component of the results in this stage is unlikely. As discussed before in relation to variations registered in the CRM, however, this would not seem to be the case in the Ist phase.

Comparing with results from the BCR, residual Pb (BCR4 = 22.75 ± 7.59 %) is significantly (p-value = 0.010) lower than Gc residual (non-bioaccessible) Pb (39.31 ± 1.044), indicating that a portion of ~ 16.6 % of the Pb that was extracted within the first 3 BCR stages, remained insoluble in the gastric phase, besides the pH being lower, in contrast to samples from the preliminary and spiked soil experiments. This shows a clear distinction of the CP08 soil matrix, with the other Meat market soil samples used in microcosms, indicating that even if residual BCR4 is significantly lower (p-value with Control BCR4 of 0.013), non-bioaccessible Gc Pb can be higher (p-value with Control Pb Gc% of 0.010). It is worth noting, however, that although the pH is lower in the gastric phase, exposure time is a lot

higher in the BCR stages with 16 hours (plus the $H_2O_2$ digestion in BCR3) versus 1 hour in the Gc phase.

#### 6.4.2.4 Trends across different soil matrixes

In order to identify common trends amongst the 3 different soil matrixes resulting from the bioaccessibility assay, correlations of %Gc and %Ist with the selected parameters were assessed for all samples together (Table 6.13).

A high correlation coefficient appears for %Gc phase with total P concentration (0.906). This appears to be due to the fact that samples with higher Pb %Gc also have higher total P concentrations (spiked soils specifically). However, the correlation is not causal, as a higher P concentration should not produce a higher Pb gastric bioaccessibility.

The parameter Gc+Res had a correlation coefficient of 0.701 with Pb %Gc, which would indicate that soils with higher Pb concentrations would also have a higher Gc bioaccessibility. CP08 soils had higher Pb concentrations than soils from the preliminary soil experiment, yet a lower Gc Pb bioaccessibilty. Hence, the 0.701 correlation coefficient may be indicating variations within each soil group (intra-group), where replicates with higher Pb mg $kg^{-1}$ also had higher %Gc, rather than between different groups (inter-group).

CRMIst% correlated with a coefficient of 0.751 to Ist%, further suggesting an operational component causing variation in the intestinal bioaccessibility of Pb, even when assessed across different soil matrices. Final pH of the solution however, had a relatively low coefficient of -0.502, confirming that the pH would not be entirely responsible for this operational variation.

### 6.5 Key findings

- The desired increase in the BCR-residual (BCR4) Pb fraction was not achieved by amending Pb-spiked soils with spent grain, superphosphate or *P.javanicus* inoculum.
- Bioaugmentation with *P.javanicus* appears to have encouraged an increase in BCR3 (organically bound target phase) Pb in both microcosm experiments in comparison to non-inoculated soil. In the preliminary soil experiment BCR3-Pb increased significantly by ~ 11 % in the sterile *P.javanicus* inoculated microcosms (P.jav2 = SMMS+SSG+Pj) in respect to the non-sterile spent grain + soil (Control = MMS+SG) microcosms, and had a reduction of ~ 9 % and ~ 2 % in BCR4 and BCR1 respectively. In the spiked soil experiment, only a significant change in BCR2-Pb occurred between T0 spiked soil and S+SP+SG+Pj treated soil, with a decrease of ~

4 %. While not statically significant, an increase of ~ 3 % in BCR3-Pb partly accounts for the reduction in BCR2-Pb.

- P in preliminary soil microcosms appears to have been mobilised from the BCR4 residual phase in sterile microcosms inoculated with *P.javanicus* (P.jav2 = SMMS+SSG+Pj), in respect to the microcosm with only spent grain (Control = MMS+SG). A significant reduction of ~10 % in BCR4-P was balanced with an increase of ~ 7 % and ~3 % in BCR2-P and BCR1-P respectively.
- No significant changes in Pb gastric (Gc) bioaccessibility were caused by the amendment of Pb-spiked soils with spent grain, superphosphate or *P.javanicus* inoculum, in respect to the unamended soils (S).
- In soils from the preliminary soil experiment, Pb gastric bioaccessibility significantly increased by ~ 7.5 % in microcosms with non-sterile spent grain and *P.javanicus* inoculum (P.jav1 + 3 = MMS+SG+Pj) in respect to the microcosm with no *P.javanicus* inoculum (Control = MMS + SG).
- Intestinal Pb-bioaccessibility appears to vary mainly due to operational effects and hence is not a good representation of the effects of soil treatments.
- If changes in the residual BCR4 Pb and residual non-bioaccessible Pb are considered as a representation of the possible immobilization of Pb as pyromorphite in soil, it would appear that the ability of *P.javanicus* to biomineralize pyromorphite was not easily transferred to a soil matrix, and the limitations of P amendments in soils with neutral to alkaline pH were not overcome with bioaugmentation in microcosm experiments.

# CHAPTER 7 Effect on Phen and B(a)p concentrations of selected amendments in the spiked soil laboratory microcosm experiment

## 7.1 Introduction

A common contamination profile found in urban soil is that of Pb and PAHs (1, 4, 6-8). The Old Glasgow Meat market is an example of this type of contamination, with Pb, total PAHs (16 priority contaminants USEPA) and B(a)p surpassing selecting soil guideline values, in data provided by Glasgow City Council (section 1.3).

In Chapter 3, the ability of *P.javanicus* to biomineralize pyromorphite in liquid cultures spiked with Pb and an organic P source was shown. Chapter 3 also showed that *P.javanicus* was tolerant to the presence of B(a)p and Phen in culture media. No detectable Phen remained dissolved in the liquid culture media after 20 days of incubation in *P.javanicus* cultures. Sorption by fungal biomass appeared to be the main process responsible for the removal of Phen from solution in spiked *P.javanicus* liquid cultures, as total Phen mass in biomass at 20, 30 and 50 days of cultivation remained statistically unchanged.

Although biosorption, in the context of soil, may not reduce human bioavailability, it can potentially reduce Phen environmental availability to groundwater, plants and other soil organisms, whilst avoiding the formation of undesirable by-products (171). Degradation of biosorbed Phen by cultured fungi overtime may also occur (139).

The Phen sorption capacity of *P.javanicus* could be further exploited for co-remediation of Pb and PAHs by combining the fungus with complementary amendments that can promote biodegradation through biostimulation of the native microbiota.

Brewery spent grain is an agro-industrial waste product available in abundance and at no cost in Scotland (142, 145). Its rich nutritional content (143) (detailed in section 2.3) led to the selection of brewery spent grain as a potentially suitable matrix for the cultivation/growth of *P.javanicus*. As a lignocellulosic material (143), the use of spent grain as a soil amendment may also have potential in stimulating the activity of native lignin degrading microorganisms, known to play a role in the degradation of PAHs (section 1.3.2).

Experiments were carried out to determine the suitability of brewery spent grain as a carrier for bioaugmentation and a soil amendment for biostimulation. Pb, Phen and B(a)p spiked soils treated with different amendment combinations, including *P.javanicus* and spent grain, were studied in microcosms.

In culture experiments (Chapter 3), sorption of Phen by *P.javanicus* was detected at the studied time frame of 70 days. With an extended incubation time of 150 days and the inclusion of spent grain amendment, both processes of sorption and degradation were taken into consideration, for the spiked soil experiment.

Accelerated solvent extraction (ASE) and gas chromatography- mass spectrometry (GC-MS) analysis was carried out on harvested soils from the spiked soil experiment, to study the effects that bioaugmentation and biostimulation with *P.javanicus* and spent grain amendments (in different combinations, section 5.3.4) may have had on soil Phen and B(a)p concentrations.

## 7.2 Objectives

- Study the effects on Phen and B(a)p soil concentrations of bioaugmentation with *P.javanicus* and biostimulation with brewery spent grain in different combinations in Phen-B(a)p-Pb spiked microcosms of the spiked soil experiment (section 5.3.4).

## 7.3 Methodology

### 7.3.1 ASE extraction

#### 7.3.1.1 Soil sample processing

Stored freeze dried samples with spent grain amendment were sieved (<2 mm) to separate the soil from spent grains.

#### 7.3.1.2 Solution preparation

A 500 μg $ml^{-1}$ surrogate solution was prepared by weighing 10 mg of anthracene-d10 (Sigma-Aldrich, St. Louis, MO 63103) and 10 mg of chrysene-d12 (Sigma-Aldrich, St. Louis, MO 63103), in a headspace vial. 20 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG) was then added and the vial sealed with a Teflon cap.

A 500 μg $ml^{-1}$ internal standard (IS) solution was prepared by weighing 10 mg of phenanthrene-d10 (Sigma-Aldrich, St. Louis, MO 63103), in a headspace vial, and adding 20 ml of toluene (Fisher Scientific, Leicestershire, LE11 5RG).

#### 7.3.1.3 Extraction protocol

ASE extraction was carried out as described in section 3.3.4.2.2 (subsection Extraction protocol), with 1.0 g samples, 0.5 g of sodium sulphate and 50 μl of surrogate solution and internal standard.

Two replicates from each microcosm were extracted, as well as seven replicates of time zero (pre-incubation) spiked soil.

### 7.3.2 GC-MS analysis

GC-MS analysis was performed at the Environmental laboratory, in the department of Civil & Environmental Engineering, University of Strathclyde (G1 1XQ, Glasgow), by technical staff as described in section 3.3.4.2.2. Each sample extract was analysed in duplicate.

Recoveries were calculated with the surrogate anthracene-d10 for Phen and chrysene-d12 for B(a)p, and analyte concentrations adjusted accordingly, as described in section 3.3.4.2.2 (subsection GC-MS analysis).

### 7.3.3 Statistics

All statistical analysis was carried out in Microsoft Excel (2016). Errors were calculated using the t-distribution confidence interval formula, appropriate for populations under 30:

$$X \pm t_{n-1} \frac{s}{\sqrt{n}}$$

Where

X= average value for a group of measurements

n= sample size (number of measurements)

n-1= degrees of freedom

s= standard deviation

t= constant from the t Table

The two-tails probability was set at p=0.05

All remaining analysis was done using the Microsoft Excel (2016) Data Analysis package (e.g. correlations). Statistical significance was evaluated with the "t=Test: two sample assuming unequal variance" and "Anova: single factor", with significance assumed at a P-value < 0.05.

## 7.4 Results and discussion

Recoveries of anthracene-d10 (Table 7.1) ranged from 42.8 % to 69.3 %, with an average 57.5 %. Seventeen out of 110 analytical results of Phen were excluded due to injection errors, recovery anomalies or isolated anomalous concentration results (e.g. replicates with very low Phen concentrations, different to the rest) and quantification issues, leaving a total

of 93 analytical results (complete data set in Appendix CH7 S1). Chrysene-d12 recoveries ranged from 51.8 % to 84.2 % with an average of 67.0 %. As for Phen, from 110 analytical results for B(a)p eight were excluded, leaving a final 102 (complete data set in Appendix CH7 S1).

*Table 7.1. Summary of recoveries from ASE extraction and GC-MS analysis of soil samples.*

| | **Recovery anthracene-d10 (%)** | **Recovery chrysene-d12 (%)** |
|---|---|---|
| **Average** | 57.47 | 66.98 |
| **error** | *1.064* | *1.140* |
| **Min** | 42.79 | 51.81 |
| **Max** | 69.25 | 84.22 |
| **n** | 93 | 102 |
| | **Excluded** | **Excluded** |
| **Min** | 37.77 | 47.85 |
| **Max** | 271.1 | 275.2 |
| **Total n** | 110 | 110 |

*Under "Excluded" is the information of excluded analytical results. "n" corresponds to the number of analytical results.*

A summary of obtained Phen and B(a)p concentrations per soil treatment type is shown in Table 7.2. Average concentrations with the respective error are included, as well as the percentage that each average value represents in respect to the average Phen and B(a)p concentrations in the control soil treatment (%S) and time zero spiked soil (%T0).

*Table 7.2. Summary of Phen and B(a)p concentrations per soil treatment type.*

| | **Phen** | | | | | **B(a)p** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **n** | **Average (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **%S** | **%T0** | **n** | **Average (mg kg$^{-1}$)** | **error (mg kg$^{-1}$)** | **%S** | **%T0** |
| **T0** | 14 | 33.43 | *1.654* | 119.8 | 100.0 | 14 | 25.23 | *0.709* | 119.6 | 100.0 |
| **S** | 8 | 27.90 | *1.365* | 100.0 | 83.47 | 12 | 21.09 | *0.477* | 100.0 | 83.59 |
| **S+SP** | 12 | 30.34 | *1.143* | 108.7 | 90.77 | 12 | 21.08 | *0.570* | 99.97 | 83.56 |
| **S+SG** | 8 | 28.51 | *0.899* | 102.2 | 85.29 | 8 | 17.99 | *0.627* | 85.29 | 71.29 |
| **S+Pj** | 11 | 25.94 | *2.293* | 92.97 | 77.61 | 12 | 18.74 | *0.382* | 88.86 | 74.28 |
| **S+SG+Pj** | 9 | 28.98 | *1.182* | 103.8 | 86.68 | 9 | 18.46 | *0.581* | 87.51 | 73.14 |
| **S+SSG+PJ** | 11 | 29.62 | *0.740* | 106.2 | 88.61 | 11 | 18.22 | *0.391* | 86.41 | 72.23 |
| **S+SP+Pj** | 8 | 25.21 | *0.762* | 90.34 | 75.41 | 12 | 19.17 | *0.493* | 90.90 | 75.99 |
| **S+SP+SG+Pj** | 12 | 30.68 | *1.602* | 110.0 | 91.78 | 12 | 18.93 | *0.853* | 89.75 | 75.02 |

*"n" corresponds to the number of analytical results per soil treatment type.*

Figure 7.1 shows box plots with obtained concentrations of Phen per soil treatment (a) and B(a)p per soil treatment (b), adjusted with the recovery (as described in section 3.3.4.2.2 subsection GC-MS analysis).

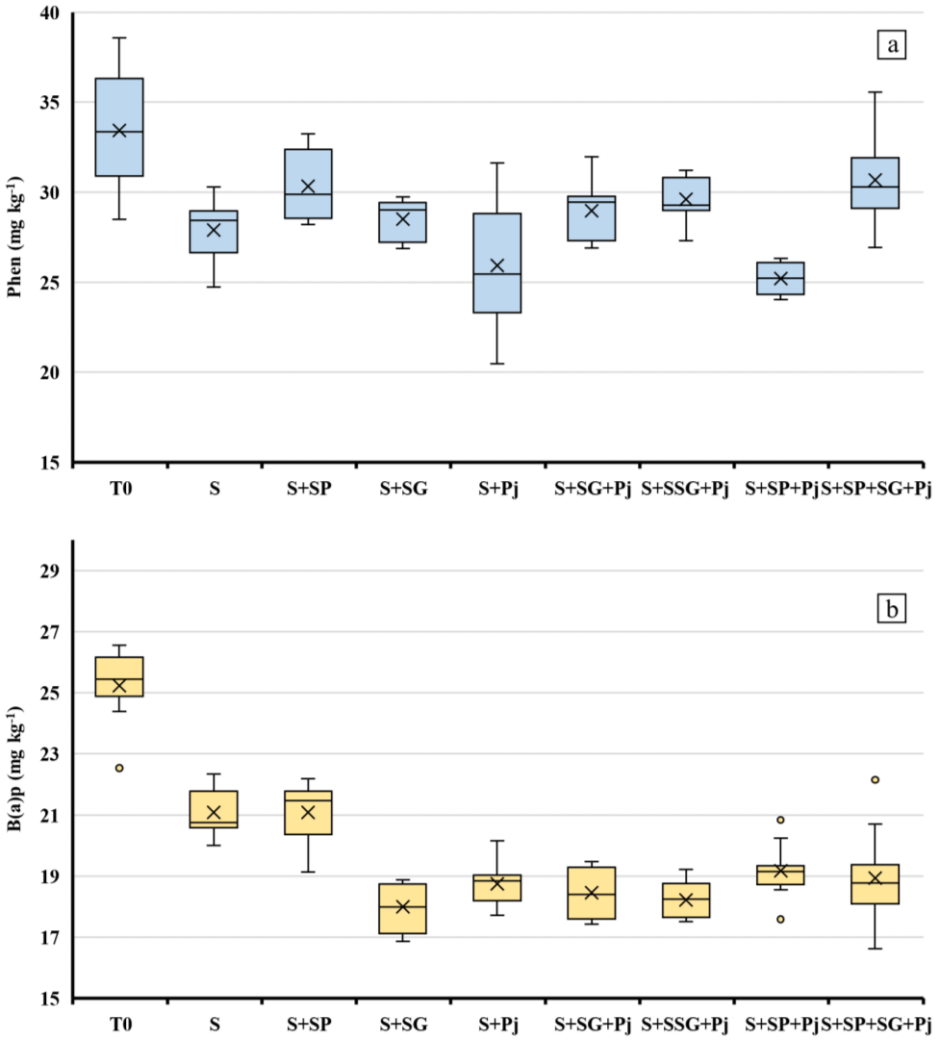



*Figure 7.1. Box plots with concentrations of Phen (a) and B(a)p (b) per soil treatment. Note that the two plots have different scale in the Y axis, but both begin at 15 mg kg$^{-1}$.*

*Table 7.3. p-values obtained with a t-Test: Two-Sample Assuming Unequal Variances two-tail (α=0.05)*

| | | T0 | S | S+SP | S+SG | S+Pj | S+SG+Pj | S+SSG+PJ | S+SP+Pj |
|---|---|---|---|---|---|---|---|---|---|
| Phen | S | 2.24E-05 | | | | | | | |
| | S+SP | 0.0041 | 0.0090 | | | | | | |
| | S+SG | 3.13E-05 | 0.4259 | 0.0146 | | | | | |
| | S+Pj | 2.19E-05 | 0.1357 | 0.0024 | 0.0442 | | | | |
| | S+SG+Pj | 0.0001 | 0.2126 | 0.0913 | 0.5046 | 0.0240 | | | |
| | S+SSG+PJ | 0.0004 | 0.0337 | 0.2780 | 0.0560 | 0.0070 | 0.3352 | | |
| | S+SP+Pj | 3.30E-08 | 0.0029 | 3.71E-07 | 2.32E-05 | 0.5287 | 5.62E-05 | 7.02E-08 | |
| | S+SP+SG+Pj | 0.0198 | 0.0109 | 0.7199 | 0.0229 | 0.0021 | 0.0847 | 0.2240 | 9.08E-06 |
| B(a)p | S | 9.77E-10 | | | | | | | |
| | S+SP | 1.25E-09 | 0.9853 | | | | | | |
| | S+SG | 4.94E-13 | 3.78E-07 | 4.35E-07 | | | | | |
| | S+Pj | 2.90E-13 | 6.83E-08 | 7.89E-07 | 0.0432 | | | | |
| | S+SG+Pj | 4.77E-13 | 8.37E-07 | 1.37E-06 | 0.2486 | 0.3894 | | | |
| | S+SSG+PJ | 7.29E-14 | 4.30E-09 | 4.43E-08 | 0.4976 | 0.0581 | 0.4861 | | |
| | S+SP+Pj | 7.80E-13 | 6.27E-06 | 2.30E-05 | 0.0057 | 0.1604 | 0.0596 | 0.0046 | |
| | S+SP+SG+Pj | 2.57E-11 | 0.0002 | 0.0003 | 0.0730 | 0.6790 | 0.3420 | 0.1337 | 0.6090 |

*p-values < α = 0.05, and hence considered significant, are highlighted in grey*

Table 7.3 contains p-values for statistical significance calculated with t-Test analysis, comparing all treatment types (and T0 spiked soil) with each other, for both Phen and B(a)p concentrations.

*Table 7.4. Correlation coefficients for Phen and B(a)p concentrations with selected parameters.*

| Microcosms | Parameter | [Phen] | [B(a)p] |
|---|---|---|---|
| | **[Phen]** | 1 | |
| | **[B(a)p]** | 0.598 | 1 |
| | **pH** | -0.444 | -0.762 |
| **T0 vs all others** | **Time** | -0.533 | -0.825 |
| **S+Pj, S+SG+Pj, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj vs all others** | **Pj** | -0.350 | -0.620 |
| **S+SG, S+SG+Pj and S+SP+SG+Pj vs all others** | **SG** | 0.052 | -0.406 |
| **S+SG vs all others** | **SG(-Pj)** | -0.075 | -0.251 |
| **S+SP, S+SP+Pj and S+SP+SG+Pj vs all others** | **SP** | -0.026 | -0.116 |
| **S+SG, S+Pj, S+SG+Pj, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj vs all others** | **SG/SSG** | -0.405 | -0.778 |
| **S+SG, S+SG+Pj, S+SSG+Pj, and S+SP+SG+Pj vs all others** | **(+)SG/SSG** | 0.072 | -0.545 |
| **S+SG+Pj and S+SP+SG+Pj vs all others** | **SG+Pj** | 0.107 | -0.286 |
| **S+SSG+Pj vs all others** | **SSG+Pj** | 0.036 | -0.266 |
| **S+SG+Pj, S+SSG+Pj and S+SP+SG+Pj vs all others** | **SG/SSG+Pj** | 0.119 | -0.427 |

**Correlation coefficients > 0.5 are highlighted in pink and those < -0.5 in blue. [Phen] and [B(a)p] stand for Phen and B(a)p soil concentrations respectively; pH stands for soil pH measured in each microcosm; Time refers to microcosms incubated for 150 days in comparison to T0 spiked soil. Remaining parameters refer to the presence (+1) or absence (-1) of an individual amendment (SG, SSG, SP or Pj) or a combination of them. The first column indicates which microcosms have this amendment or combination of amendments present. Pj refers to P.javanicus inoculum; SG refers to non-sterile spent grain; SG (-Pj) refers to presence of non-sterile spent grain but no fungal inoculum; SP refers to superphosphate; SG/SSG refers to any form of spent grain, as part of the inoculum, non-sterile amendment or sterile amendment; (+)SG/SSG refers to sterile or non-sterile spent grain as additional soil amendment, that is excluding addition of grain only as part of the inoculum; SG+Pj refers to the combination of P.javanicus inoculum and additional non-sterile spent grain soil amendment; SSG+Pj refers to the combination of P.javanicus inoculum and additional sterile spent grain soil amendment; finally SG/SSG+Pj to the combination of P.javanicus inoculum and additional sterile or non-sterile spent grain amendment.*

Finally, Table 7.4 shows correlation coefficients (obtained analogously to the example shown in appendix CH6 A7) comparing Phen [Phen] and B(a)p [B(a)p] concentrations with

selected parameters and the presence or absence of an individual amendment (SG, SSG, SP or Pj) or a combination of them.

Anova analysis indicates that in regards to Phen concentrations all tested groups are different statistically (p value of $2.03E^{-12}$). With t-Test analysis, Phen concentrations appear to have significantly decreased after 150 days of incubation in respect to the time zero spiked soil (p-value for all treatments including S are $< \alpha = 0.05$, compared to T0 and correlation coefficient of [Phen] and Time of -0.533), with all treatments (Table 7.2) having average concentrations that correspond to < 91.8 % of the average T0 concentration. In respect to the control microcosms (S) however, only two treatments have lower average concentrations, S+Pj (93.0 % of average S concentration) and S+SP+Pj (90.3 % of average S concentration). Only S+SP+Pj is statistically different to S (p-value of 0.0029). All other treatments have higher average concentrations, though only S+SP, S+SSG+Pj and S+SP+SG+Pj appear as statistically different to S (p-values of 0.0090, 0.0337 and 0.0109 respectively). Correlation coefficients in Table 7.4 show no correlation of any amendment combination with a reduction of Phen concentrations.

In liquid culture experiments (Chapter 3), *P.javanicus* biomass proved very effective in sorbing Phen in solution in the media, but in the present matrix, systematic separation of any potential fungal biomass present in the soil matrix after freeze drying was not possible, and hence any fungal biomass-sorbed Phen would still appear accounted for in the soil extraction and analysis.

While treatments S+Pj and S+SP+Pj appear to have lower average Phen concentrations than the control, and other treatments, it is difficult to attribute this difference to any specific parameter or treatment variation. What distinguishes these two treatments from the rest is the inclusion of *P.javanicus*-spent grain inoculum, with no further addition of spent grain amendment. Unlike in other treatments with *P.javanicus* inoculum as well as the S+SG treatment, no fungal growth or colonization was visible within the soil at any point of the experiment. Though this does not necessarily imply that no growth occurred, it most likely means it was at least less prolific than in other inoculated treatments. Furthermore, although no additional spent grain amendment was present in these treatments, effects in the soil system from the inoculum spent grain were still noted, particularly in the soil pH which increased in respect to treatments with no form of spent grain (section 5.4.3.2.2). The further addition of P in S+SP+Pj, is shared by S+SP and S+SP+SG+Pj treatments, and as was shown in Chapter 6 was a minimal addition in relation to the overall P soil content. It is also worth noting, that S+SP+Pj was the only one of the two treatments with lower Phen concentrations

relative to the control (S) which are statistically significant, and also had a lower amount of usable analytical results (n = 8, out of 12 extracted and analysed) in comparison with some of the other treatments, as 4 of the results were discarded due to Phen quantification issues in the extraction replicates.

Anova analysis of B(a)p concentrations also shows that all tested groups are different statistically (p value of $4.38E^{-37}$), that is the intragroup variances are smaller than the intergroup variance. A significant reduction of B(a)p concentrations in all incubated microcosms in respect to T0 soils is shown by t-Test analysis (p-value for all treatments including S are $< \alpha = 0.05$ and correlation coefficient of [B(a)p] and Time of -0.825), with average concentrations corresponding to < 83.6 % of the T0 average value. Amongst incubated microcosms, all treatments, with the exception of S+SP, have significantly lower B(a)p concentrations than the control S (averages < 90.9 % of the S average concentration). All these treatments have in common the presence of spent grain in one form or another, as part of the inoculum, non-sterile amendment or sterile amendment (correlation coefficient between [B(a)p] and SG/SSG of -0.778). This would indicate, that rather than the inoculation of *P.javanicus*, the addition of spent grain contributed to the reduction of B(a)p concentrations in soil. The treatment with the lowest average concentration is S+SG, which corresponds to soil with non-sterile spent grain amendment and no *P.javanicus* inoculum, further suggests the spent grain addition is linked to the B(a)p reduction. t-Test analysis shows that when compared to other treatments with added spent grain, S+SG had significantly lower concentrations than treatments S+Pj and S+SP+Pj, which only had addition of sterile spent grain from the *P.javanicus* inoculum. When comparing treatments S+Pj, S+SG+P, S+SSG+Pj, S+SP+Pj and S+SP+SG+Pj no significant differences in B(a)p concentrations were detected between treatments with only sterile spent grain in respect to those with non-sterile spent grain addition. Coefficients comparing [B(a)p] with soil pH (-0.762) and Pj (-0.620) show a certain degree of correlation, however these are likely an indirect effect of the correlation with SG/SSG, as all microcosms with some form of spent grain addition showed an increase in soil pH, and 5 out of 6 treatments with some form of spent grain also had been inoculated with *P.javanicus*.

As with the liquid culture experiments in Chapter 3, metabolite identification in GC-MS chromatograms was planned, to establish the occurrence of PAHs degradation, however, the Covid-19 lockdown made it impossible to access the technical equipment and assistance required to accomplish the analysis within the time frame of the project.

In summary, it would appear that incubation time had an effect on both Phen and B(a)p concentrations, though more markedly for B(a)p, with some PAH degradation potentially occurring in all soils overtime. While the different treatment combinations seemed to have no distinctive effect on Phen concentrations, the addition of spent grain (sterile or non-sterile) appears to relate to a reduction of soil B(a)p concentrations.

Certain amounts of PAH loss can occur due to abiotic processes such as volatilisation (107). However, had that been the case, a higher time related concentration reduction would have been expected from the more volatile Phen (boiling point at 340°C and vapour pressure of $1.21*10^{-4}$ mm Hg at 25 °C (160)) in respect to B(a)p (boiling point at 495°C and vapour pressure of $5.49*10^{-9}$ mm Hg at 25 °C (159)), which is not readily volatile, and stable as a solid in the experimental conditions (104). Another form of abiotic PAH oxidation results from exposure to UV light (99, 100, 104) but for this to occur, significant doses need to be applied, and microcosms were cultivated in the dark.

It is hence quite possible that the time related concentration reduction of both PAHs was the result of natural attenuation, caused by the metabolic activity of the soil microbial population.

The significantly lower B(a)p concentrations in microcosms with spent grain additions (as inoculum or amendment) could be the result of sorption to coarse spent grain particles (excluded from the sample extraction to reduce sample heterogeneity issues) or due to biostimulation of the microbial population resulting from the addition of spent grain, causing an increased degradation rate.

Studies have shown that PAHs can be immobilized in soil through sorption onto different types of materials like activated carbon, biochar and cellulose, amongst others (138, 140, 171). Several researchers have reported that biochar is a good sink for PAHs in water and soils, with removal efficiency of about 1 order of magnitude higher than reported data for soil/sediment organic matter, showing an enhanced affinity of pollutants to biochar (124).

Agro-industrial wastes, like spent grain, contain biopolymers such as lignin, cellulose and hemicellulose and the lignin fraction can act as an absorber of organic pollutants (e.g., PAH) in soil (128). Lignins have been shown to have a much higher sorption affinity for hydrophobic organic compounds than chitins and celluloses due to their high hydrophobic C content and low polarity (186). If lignins are present in solid waste applied to soil in large quantity, mobility and bioavailability of hydrophobic organic compounds could be reduced (186).

Organic matter in soil or sediment plays a similar role as an immiscible solvent with water, in terms of partitioning (75). For this reason the tendency of organic contaminants to accumulate in biosolids can be reasonably estimated from the octanol-water partition coefficients ($K_{OW}$) of the contaminants, with a log $K_{OW} > 4.0$ indicating a high sorption potential (75, 124, 187). The reported octanol-water partition coefficients (log $K_{OW}$) of B(a)p are relatively high, ranging from 5.97 to 6.35 (104), while Phen has a log $K_{OW}$ of 4.46 (160). Though still higher than the proposed 4.0 (187), Phen's log $K_{OW}$ is lower than that of B(a)p, which could translate to a preferential sorption of B(a)p on the spent grain in respect to Phen; causing the observed reduction of B(a)p concentration on treatments with spent grain, which was not detected in the case of Phen concentrations. Preferential sorption of HMW PAHs compared to LMW PAHs has been reported in soil amended with sewage sludge biochar, highlighting their high affinity to sorbents (124).

Though sorption of PAHs may hinder their immediate degradation (187-189), the longer term fate of sorded PAHs to spent grain may still allow for degradation, while reducing their availability and hence toxicity to potential receptors through volatilization (187), or ingestion/inhalation of fine contaminated soil particles (187).

The lignin, cellulose and hemicellulose content of agroindustrial waste can also stimulate the biodegradation of petrogenic hydrocarbons through increased growth and metabolic activity of soil microflora (100, 103, 119, 128). Physically, these residues can also improve soil properties, in terms of aeration, moisture, nutrition and structural properties, all of which may lead to the acceleration of hydrocarbon bioremediation (128).

Being rich in lignin, spent grain has the potential to facilitate co-metabolism of PAHs by stimulating extracellular enzymatic activity known to assist PAH degradation through cleaving (100, 103, 119, 189) (i.e. lignin-degrading system: lignin peroxidase, manganese peroxidase; phenoloxidases like laccases; $H_2O_2$-producing enzymes and the intracellular cytochrome P-450 system (103, 119)), either from the native soil microbial population (biostimulation (189)) or species inoculated with the grain (bioaugmentation (189)).

In general it is considered that resistance to biodegradation increases with the number of rings and molecular weight of PAHs, particularly in structures with more than 4 aromatic rings (99, 100, 102, 103). For this reason, it would have been expected that an enhanced degradation caused by the biostimulation effect of spent grain, would have preferentially affected Phen concentrations, rather than B(a)p. Previous research has shown, however, that in some cases the use of lignocellulosic material as a co-substrate for PAH biodegradation, may result in preferential degradation of heavier PAHs (101, 112).

Cavalcanti *et al*. (2019 (101)) studied the association of biostimulation with residues from the processing of cotton, peanut or sesame cakes and bioaugmentation with bacterial strains *Pseudomonas aeruginosa* and *Burkholderia cepacia*, in soil contaminated by a lubricating oil mixture containing approximately 5 mg $kg^{-1}$ PAH (Phen and pyrene) (101). After 60 days incubation at room temperature, between 65 and 80 % of the phenanthrene and pyrene was removed (soxhlet extraction using dichloromethane and GC-MS analysis of samples of the soil + cake mixture) (101). It was noted that the use of cakes as the co-substrate for degradation through cometabolism promoted preferential consumption of the more persistent PAH (pyrene over Phen) (101). This phenomenon was partly attributed to an increase of Phen half-life when it is associated with other PAHs in soils, whereas for pyrene this does not appear to occur (101). Some studies of kinetics of pyrene biodegradation have revealed that its removal is more efficient in the presence of LMW-PAH, such as fluoranthene and Phen (101).

These result were compared to previous research with co-substrates in the bioremediation of soils contaminated with 8 mg/kg of the 16 priority PAHs (101). The use of glycerol produced a preferential degradation of heavy molecular weight (HMW) PAHs, most of them between 20 and 40%, some above 50% and none less than 10%; as did raw glycerol which resulted in the removal of not less than 60% of all HMW-PAHs (101).

Combined bioaugmentation-biostimulation studies with fungal species *Agaricus bisporus* and sterile spent mushroom waste, also proved more effective in removing 5 and 6-ring PAHs, in particular B(a)p, over 3 ringed PAHs (112). The best reduction in this microcosm experiment was observed for B(a)p and dibenz(ah)anthracene despite being higher molecular weight PAHs (112). The latter was explained by their low ionization potential (IP) values (i.e., 7.12 and 7.38 respectively) which would make them highly susceptible to oxidation by both laccase and manganese peroxidase (MnP) (112).

While the soil used in this experiment was spiked with the contaminants, in true aged contaminated soils the pre-exposed native microbial community with increased hydrocarbon oxidizing potential (100, 103) could be further encouraged with the addition of spent grain. In real contaminated soils consisting of poor quality made-ground, the difference between natural attenuation and spent grain-biostimulation would also likely be greater.

Agroindustrial residues also have their own associated microbial community which may contribute to the degradation of hydrocarbons (128). This was notable in particular in S+SG replicates. When *P.javanicus* inoculum was added, the reduction in B(a)p concentration was not statistically different in treatments with sterile grain (S+Pj, S+SSG+Pj, S+SP+Pj) to

those with non-sterile grain (S+SG+Pj, S+SP+SG+Pj), hence no contribution in this reduction can be assigned to the microbial population added to the system through the non-sterile grain, in these microcosms. Treatment S+SG, however, though slight, showed the largest average % reduction in respect to the control (S), and statistically different to other treatment with spent grain according to Anova analysis.

As presented in Chapter 5, in S+SG microcosms growth of other fungi/microorganisms was abundantly visible in the amendment spent grain and from the base of the flasks (section 5.4.3.1). In microcosms with non-sterile grain and *P.javanicus* inoculum, besides the visible growth of *P.javanicus* during the first part of the experiment, no apparent growth of these other organisms was observed at any point. Furthermore, even though the visible growth of *P.javanicus* reseeded after the first weeks of the experiment, the initial prevalence of *P.javanicus* may have hindered the activity of the other microorganisms present in the spent grain. This could explain a further reduction in B(a)p concentrations being caused by microorganisms present in non-sterile grain, only in treatment S+SG.

### 7.5 Key findings

- The inoculation of *P.javanicus* in Phen-B(a)p-Pb spiked soil did not appear to have an effect on the concentration of Phen and B(a)p overtime.
- The amendment of Phen-B(a)p-Pb spiked soil with sterile and non-sterile brewery spent grain produced a significant decrease in B(a)p concentration after 150 days of incubation, in comparison to microcosms with no added spent grain.
- Sorption of B(a)p to the spent grain and/or a stimulation of the biodegradative activity of the native soil microbial population through the addition of spent grain, may be the cause of the significant decrease in B(a)p concentration.

# CHAPTER 8. Amendment of soil with brewery spent grain and *P.javanicus* inoculum under environmental temperature and rainfall conditions: a pot experiment

## 8.1 Introduction

Urban soils present distinctive characteristics, compared to rural soils, resulting from high anthropogenic pressure (1-4). Contamination levels of urban soils tend to be higher, even in the absence of heavy industry, showing significant increases in the concentrations of a number of metals and other compounds that can pose a hazard to health (2, 4, 5). Sources of contamination in the urban context are multiple: industrial uses, demolition of buildings, atmospheric deposition from motor vehicles, domestic and industrial burning of fossil fuels, incinerators, particles of paint from houses and other structures (1, 4, 6, 7). Historic use of waste (ash, building rubble, tar, sludge and mineral materials) as fill in sites, for levelling, has also been an important source of contamination (1, 12) The physico-chemical parameters of these soils are dominated by their past and current uses, which can lead to compaction of deeper layers, low biodiversity, presence of fragments of various materials and high concentrations of pollutants (2-4). Due to the latter, urban topsoil and subsoil tend to be largely artificial and complex matrixes (6).

Sustainable development encompasses sustainable methods of urban brownfield soil restoration-rehabilitation (20). Bioremediation is a good example of sustainable in situ remediation (10). Phytoremediation, which uses plants, is an in situ decontamination approach that has shown promise for addressing both organic and inorganic contaminants (8). Another example of bioremediation is the introduction or stimulation of indigenous pollutant-degrading microorganisms (i.e. bacteria and fungi) (10).

These methods can be applied in synergy, combining processes on site to deliver several useful services and improvements in the long term, for example combining gentle bioremediation and biomass cultivation, or some other form of intervention such as green infrastructure (20).

The efficiency of bioaugmentation is determined by many biotic and abiotic factors (102). The selected microorganisms must not only be able to degrade or biomineralize contaminants but also successfully compete with indigenous microflora (102) and adapt to soil conditions (75). Poor soil conditions, common in contaminated soil and made ground, can often prevent successful inoculation of additional microorganisms and sustain their growth. Addition of available nutrients (biostimulation) is commonly necessary to achieve a successful inoculation (75).

In the context of sustainable development, combining bioremediation with waste re-use and recycling (e.g. beneficial re-use of composts or agro-industrial residues) as a source of nutrients, has significant promise (20, 28).

As described in the literature review, brewery spent grain is a waste product available in abundance and at no cost in Scotland (142, 145). Its rich nutritional content (143) (detailed in section 2.3) lead to the selection of brewery spent grain as a potentially suitable matrix for the cultivation/growth of a fungal species (*P.javanicus*), to create a spent grain + *P.javanicus* inoculum for the bioaugmentation of soils (Chapter 5). As a lignocellulosic material (143), the use of spent grain as a soil amendment may also have potential in stimulating the activity of native lignin degrading microorganisms, known to play a role in the degradation of PAHs (section 1.3.2).

All previous experiments, both in culture media and soil microcosms (Chapters 3 and 5), were carried out under the controlled conditions of laboratory incubators, and in closed systems. For this reason a pot experiment was designed to test the brewery spent grain-*P.javanicus* bioaugmentation approach, under real environmental conditions of temperatures and precipitation, and in open non-sterile systems, representing a closer approximation to *in situ* soil conditions than laboratory microcosms. By using a pot experiment set up, the effects of the selected amendments on the development of vegetation in poor quality urban soil could be assessed.

## 8.2 Objectives

- Assess the effect on vegetation cover and root growth caused by the use of brewery spent grain as a soil amendment in poor quality brownfield –type soil.
- Assess the feasibility of bioaugmentation of soil with *P.javanicus*-spent grain inoculums, in an open system exposed to real environmental conditions of temperature and precipitation, by identifying qualitative changes in the soil microbial population.
- Assess the effect on vegetation cover and root growth caused by bioaugmentation with *P.javanicus.*

## 8.3 Methodology

### 8.3.1 Soil preparation

Soil was collected from excavated made ground left after the construction of a new car park in the premises of the Scottish Enterprise Technology Park, adjacent to SUERC (Scottish

Universities Environmental Research Centre) in East Kilbride (G75 0QF). After collection soil was dried at 105°C for 8 hours, sieved (< 2mm) and homogenized.

### 8.3.2 Spent grain inoculum preparation

Spent grain inoculum was prepared by placing 250 g of spent grain and 250 ml of Milli-Q water in a 2.5 l glass Pyrex® dish covered with another 2.5 l dish, attached with aluminium foil and autoclave tape (as shown in Figure 8.1-a). The container was then autoclaved (121°C for 15 minutes) and inoculated from a 10 days MEA *P.javanicus* culture, as described in section 5.3.2. The spent grain inoculum preparation was incubated at 20ºC and 150 rpm in the dark for 40 days (Figure 8.1-b and c).

### 8.3.3 Experimental set up

Volume portions of 200 ml of the prepared soil were distributed in plastic plant pots. Filter paper was placed at the bottom of the pots to avoid loss of soil through the holes in the pot base. In three of the pots, 50 ml of non-sterile spent grain were added and thoroughly mixed in with the soil (S+SG treatment). In three other pots, 50ml of a mixture of spent grain-*P.javanicus* inoculum and non-sterile spent grain, in a ratio of 3:2, were added and thoroughly mixed in (S+SG+Pj treatment). This gave a grain: soil volume ratio of 0.25, lower than the 0.315 volume ratio adapted to a weight ratio in the spiked soil experiment (section 5.3.4.1.3), and used in previous studies of soil amendment with spent grain (153). Such reduction in the SG: soil ratio was implemented in order to test if success in the bioaugmentation strategy, and positive effects on vegetation growth, were attained with lower amendment additions, more efficient at a larger scale.

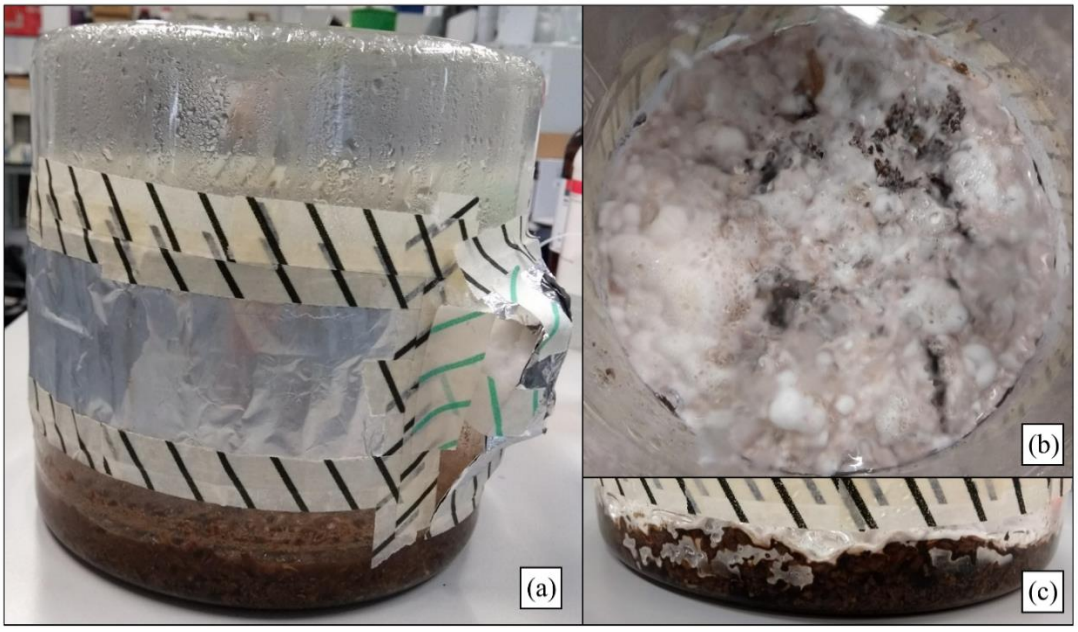



*Figure 8.1. Spent grain inoculum preparation. (a) Set up at time zero; (b) fungal growth at day 40 from above and (c) in profile.*

Controls were prepared by leaving three pots only with soil (S treatment). As there was excess soil for 2 remaining pots, these were prepared as an alternative control with soil and inoculation of *P.javanicus* direct from a 10 day old PDb liquid culture, by transferring a small portion of fungal biomass into the pot (S+Pj treatment).

*Figure 8.2. Pots set up on rooftop terrace.*

Pots were placed in an alternate order over cooler boxes in the rooftop terrace of the Rankine Building at the University of Glasgow (Oakfield Ave, Glasgow G12 8LT). To avoid pots from falling over, due to wind, they were stabilized with a string support (Figure 8.2).

#### 8.3.3.1 Sowing of native UK grass and wild flower seeds

The soil and respective amendments were left without further intervention for 30 days, allowing any weed species potentially dormant in the collected soil to appear. As the latter did not occur, on day 30 a mixture of seeds of native UK species was sown into the soil. The seed mixture added into each pot consisted of: 6 seeds of *Cynosurus cristatus* (crested dogstail, perennial grass), 6 seeds of *Fetusca rubra* (slender creeping red-fescue, perennial grass), 3 seeds of *Leucanthemum vulgare* (oxeye daisy, wild flower), and 2 seeds of *Centaurea nigra* (common knapweed, knapweed) (Emorsgate Seeds, Limes Farm, Tilney All Saints, King's Lynn, Norfolk PE34 4RT).

Pots were monitored for a further 56 days (after seeds were sown), after which time above ground vegetation cover was harvested. For logistical reasons the soil and below ground root biomass was returned to the pots, placed back in the rooftop terrace and harvested a week later.

#### 8.3.3.2 Summary of temperature and rainfall in Glasgow over the experimental period

Temperature and precipitation conditions over the period of time the experiment was carried out are summarised in Table 8.1. Rainfall data was obtained from the online Scottish Environmental Protection Agency (SEPA) database. Data from the two nearest station (Dalmarnock STW latitude 55.8374507, longitude -4.216965486; Killermont Rainguage

latitude 55.91108458, longitude -4.305014408) was averaged. Temperature data was obtained from the Met Office National Climate Information Centre for the Scotland W region.

*Table 8.1: Summary of rainfall and temperature conditions during the experiment.*

| Month | Dates (days) | Number of days | Rainfall (mm) | Average minimum (°C) | Average maximum (°C) |
|---|---|---|---|---|---|
| **June** | 5th to 30th | 26 | 35.1 | 8.6 | 16.5 |
| **July** | 1st to 31st | 31 | 91.8 | 11.5 | 18.7 |
| **August** | 1st to 31st | 31 | 163.9 | 11.4 | 18.0 |
| **September** | 1st to 6th | 6 | 20.2 | 8.6 | 15.4 |

### 8.3.4 Sample processing

#### 8.3.4.1 Above ground vegetation cover

Pot walls were cut open with scissors, to expose the soil surface. All above ground vegetation (grass and wild flower plants) was harvested using scissors to cut the base of each stem. Biomass was thoroughly washed with Milli-Q water, making sure no losses were produced in the process (Figure 8.3). The remaining soil and below ground root biomass were transferred into new pots, tagged and returned to the rooftop terrace, awaiting further processing.

Biomass from each sample pot was then placed on pre-weighed porcelain crucibles. An initial moist biomass weight was recorded, and crucibles were then placed in an oven at 50°C for 12 hours. Crucibles were then removed, allowed to cool to room temperature, and the final weight recorded.

*Figure 8.3. Example images of above ground vegetation harvesting.*

### 8.3.4.2 Soil culturing

In order to assess the influence *P.javanicus* inoculums and non-sterile spent grain had in the soil microbiota, soil MEA plate cultures were prepared.

Plant pots were cut open and the soil removed and divided in four portions with a previously sterilized metallic spatula. Three 2 g (moist weight) soil samples were taken from different portions of each pot and placed in sterile 25 ml glass bottles, with rubber caps and 5 ml of sterile Milli-Q water. All bottles were then shaken in an orbital incubator at 190 rpm for 30 minutes and allowed to settle. With sterile syringes 0.3 ml of the water was extracted from each bottle and spread on MEA plates. All plates (33 in total) were then incubated at 21°C in the dark.

### 8.3.4.3 Below ground root biomass

After the process described in the previous section was complete, the total content of each pot (soil and root biomass) was submerged in a plastic bowl with Milli-Q water, and manually disaggregated. Soil was allowed to settle in the bottom of the bowl to separate it from the root biomass. The root biomass was then washed repeatedly to remove remaining soil particles and/or spent grain. After washing, the recovered biomass was placed in pre-weighed porcelain crucibles.

*Figure 8.4. Main root biomass harvesting.*

An initial moist weight was recorded, and crucibles were placed in an oven at 50°C for 35 hours. Samples were removed from the oven, allowed to cool and weighed (Figure 8.4). An

additional step was added to remove any remaining dry soil or grain, by mechanically (by hand) disaggregating the dry biomass, and disposing of any released soil and grain particles. Loss of some fine roots during the different cleaning steps was inevitable, and for this reason the final recovered below-ground root biomass was referred to as the main root biomass, and its weight was considered as an approximate of the true total, rather than an absolute value.

## 8.4 Results and discussion

### 8.4.1 Observations on the evolution of soil mixtures and vegetation in pots

During the first 10 days, the only notable difference between soil controls and all other treatments, was that controls appeared quite dry and disaggregated (Figure 8.5-a), while in treated soils the water holding capacity seemed to have improved, and soils were moist and forming aggregates (Figure 8.5-b and c).


*Figure 8.5. Examples of soils at 9 days. (a) Soil control; (b) S+Pj treatment; (c) S+SG treatment.*

*Figure 8.6. Example of S+SG+Pj treatment at 19 days, with apparent fungal growth in white tones.*

After 19 days, soil with spent grain-*P.javanicus* inoculum showed some signs of possible dispersion of the fungus, with growth of a white coloured organism occurring in soil aggregates, observable on the soil surface (Figure 8.6).

No further notable changes occurred until day 30, when seeds were sown into the soil. From this point onwards the day count was reset, considering the seed sowing as day 0. After 10 days, shoots of both grass and wildflowers were visible in all pots (Figure 8.7). Soils amended with spent grain already appeared to have more abundant plant growth, and S+SG treatment had begun to show a mossy cover on the soil surface (Figure 8.7-b).

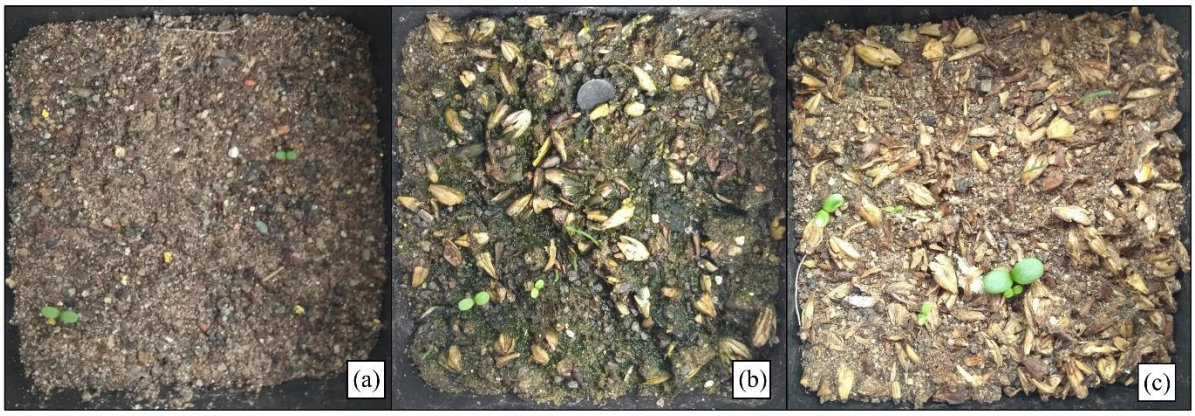



*Figure 8.7. Examples of soils 10 days after seed sowing. (a) Soil control; (b) S+SG treatment; (c) S+SG+Pj treatment.*

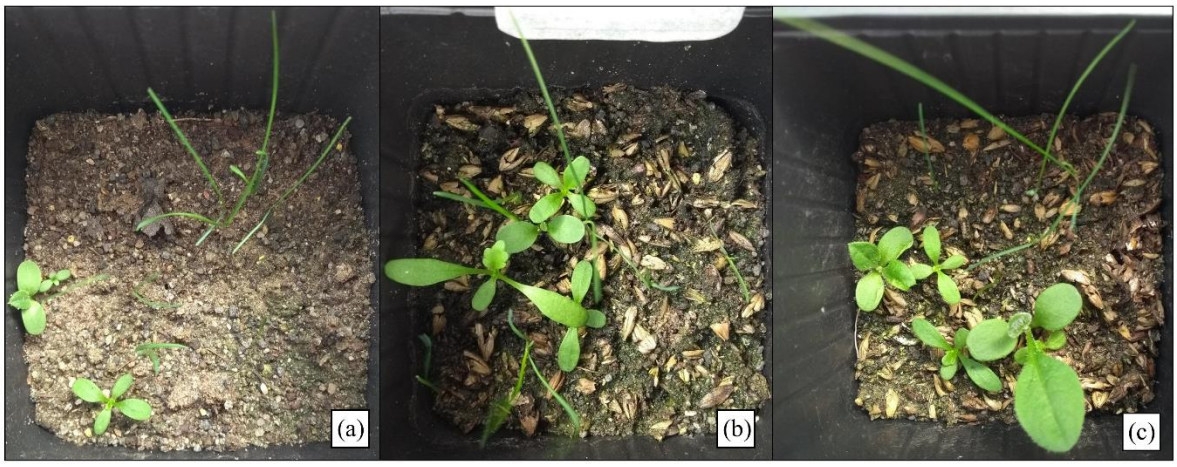



*Figure 8.8. Examples of soils 21 days after seed sowing. (a) S+Pj treatment; (b) S+SG treatment; (c) S+SG+Pj treatment.*

Observed growth rate continued to be faster in grain amended soils, particularly noticeable with wild flower species (Figure 8.8 at 21 days). A shoot count per pot was carried out at day 10 and 21 (Table 8.2) to determine germination rate. This measurement became increasingly difficult to perform from this point onward, as growth became more abundant and individual shoots were harder to distinguish from one another.

Grass germination rate on average was not greatly different in pots with different treatments at both 10 and 21 days, with a slightly higher rate in the control at day 21. Wildflowers on day 10 had similar average germination rates in S, S+SG and S+SG+Pj treatments, and a lower rate in S+Pj treatment. At day 21, both S+SG and S+SG+Pj treatments had average wildflower germination rates, of 86.7%, while S and S+Pj treatments had averages of 60%.

*Table 8.2. Shoot count per pot with the respective germination rate (GR) in %, and treatment average, for days 10 and 21 after seed sowing.*

| **Day** | **10** (15/07/19) | | | | **21** (26/07/19) | | | |
|---|---|---|---|---|---|---|---|---|
| **Pot type** | **Grass shoots** | **GR (%)** | **Wildflower shoots** | **GR (%)** | **Grass shoots** | **GR (%)** | **Wildflower shoots** | **GR (%)** |
| **S 1** | 1 | 8.3 | 1 | 20.0 | 9 | 75.0 | 2 | 40.0 |
| **S 2** | 2 | 16.7 | 2 | 40.0 | 6 | 50.0 | 2 | 40.0 |
| **S 3** | 8 | 66.7 | 4 | 80.0 | 10 | 83.3 | 5 | 100 |
| **$S_A$** | 3.7 | 30.6 | 2.3 | 46.7 | 8.3 | 69.4 | 3 | 60.0 |
| **S+Pj 1** | 1 | 8.3 | 1 | 20.0 | 8 | 66.7 | 3 | 60.0 |
| **S+Pj 2** | 7 | 58.3 | 1 | 20.0 | 7 | 58.3 | 3 | 60.0 |
| **$S+Pj_A$** | 4 | 33.3 | 1 | 20.0 | 7.5 | 62.5 | 3 | 60.0 |
| **S+SG 1** | 6 | 50.0 | 3 | 60.0 | 7 | 58.3 | 4 | 80.0 |
| **S+SG 2** | 5 | 41.7 | 2 | 40.0 | 8 | 66.7 | 4 | 80.0 |
| **S+SG 3** | 2 | 16.7 | 2 | 40.0 | 7 | 58.3 | 5 | 100 |
| **$S+SG_A$** | 4.3 | 36.1 | 2.3 | 46.7 | 7.3 | 61.1 | 4.3 | 86.7 |
| **S+SG+Pj 1** | 7 | 58.3 | 0 | 0.0 | 7 | 58.3 | 4 | 80.0 |
| **S+SG+Pj 2** | 2 | 16.7 | 5 | 100 | 5 | 41.7 | 5 | 100 |
| **S+SG+Pj 3** | 3 | 25.0 | 3 | 60.0 | 8 | 66.7 | 4 | 80.0 |
| **$S+SG+Pj_A$** | 4 | 33.3 | 2.7 | 53.3 | 6.7 | 55.6 | 4.3 | 86.7 |

*Rows highlighted in grey with a subscript "A" are the average for the treatment type.*

In the remaining time of the experiment, plant growth was continuous, and faster on soils amended with spent grain. Pots with S+Pj treatment also appeared to have a slightly higher growth rate than the control, yet slower than the other two treatments. Figures 8.9 and 8.10 are shown as examples at two different time points.


*Figure 8.9. Examples of soils 34 days after seed sowing. (a) Soil control; (b) S+Pj treatment; (c) S+SG+Pj treatment.*

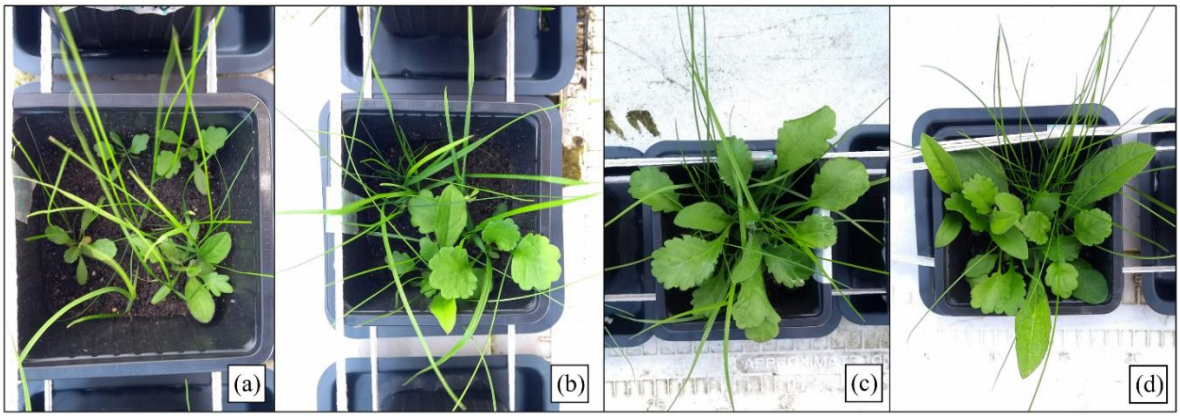


*Figure 8.10. Examples of soils 48 days after seed sowing. (a) Soil control; (b) S+Pj treatment; (c) S+SG treatment; (d) S+SG+Pj treatment.*

On day 56 wildflower plants and grass abruptly had begun to wither, though more obviously in pots with larger plants, across all treatment types (Figure 8.11). Six days with no registered precipitation and an average maximum temperature of 21.7°C (highest maximum temperature of 28.0°C on 26/08/2019) (190) followed by 2 days of intense precipitation (43.4 mm over the 29/08/2019 and 30/08/2019 up until 9:00 am, averaged value from Dalmarnock and Killermont Rainguage station, SEPA online database) may have resulted in the withering primarily of some of the wildflower plants. For this reason, the decision was made to harvest above ground biomass on day 56, to avoid any loss due to decomposition of dry above ground biomass.

*Figure 8.11. Examples of soils 48 days after seed sowing. (a) Soil control; (b) S+Pj treatment; (c) S+SG treatment; (d) S+SG+Pj treatment.*

### 8.4.2 Above and below ground biomass

Above ground (AG) biomass and main root (MR) biomass weights for each sample are shown in Table 8.3 and Figure 8.12 in box plots per treatment type. Table 8.4 shows p values according to the t-test of all treatment types against each other, for main root biomass (MR) weight on the bottom left and above ground (AG) biomass on the top right. Anova p values are also included for the analysis of all AG and MR weights together.

*Table 8.3. Above ground (AG) biomass weights and main root biomass (MR) weights per sample.*

| | S a | S b | S c | S+Pj a | S+Pj b | S+SG a | S+SG b | S+SG c | S+SG+Pj a | S+SG+Pj b | S+SG+Pj c |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **AG (g)** | 0.1894 | 0.0986 | 0.1599 | 0.3419 | 0.3099 | 0.7618 | 0.6005 | 0.7223 | 0.5626 | 0.5263 | 0.6096 |
| **MR (g)** | 0.3489 | 0.1132 | 0.1825 | 0.2177 | 0.5202 | 0.4750 | 0.5115 | 0.5584 | 0.6107 | 0.6604 | 0.6681 |

*Table 8.4. p-values for above ground (AG) biomass weights and main root biomass (MR) weights.*

| **t-Test: Two-Sample Assuming Unequal Variances (α=0.05)** | | | | |
|---|---|---|---|---|
| | **S** | **S+Pj** | **S+SG** | **S+SG+Pj** |
| **S** | **MR\AG** | 0.01079 | 0.00224 | 0.00031 |
| **S+Pj** | 0.52491 | **MR\AG** | 0.01872 | 0.00355 |
| **S+SG** | 0.05575 | 0.51521 | **MR\AG** | 0.09800 |
| **S+SG+Pj** | 0.02688 | 0.31962 | 0.01200 | **MR\AG** |
| **Anova AG** | 3.2367E-05 | | | |
| **Anova MR** | 0.00874 | | | |

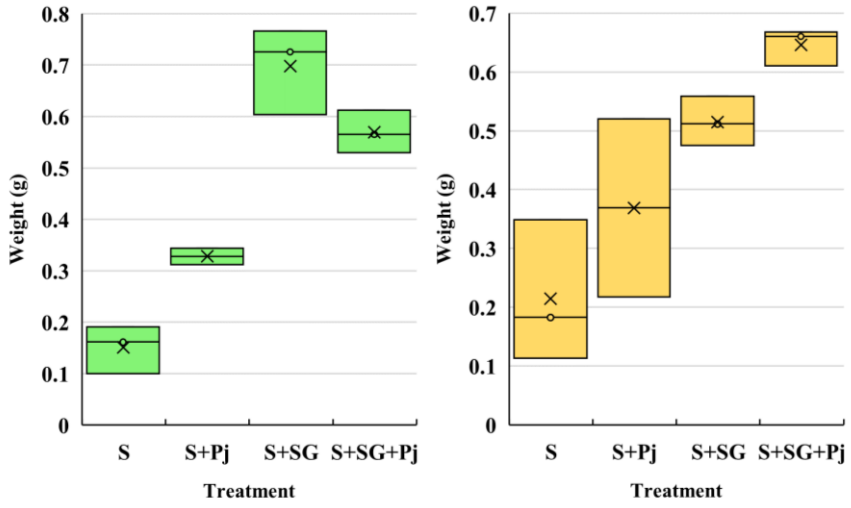



*Figure 8.12: Box plots with above ground (AG) biomass weights in green and main root biomass (MR) weights in yellow, according to soil treatment.*

Above ground biomass was highest in S+SG treatment, by over 3 times the weight of biomass in the control with only soil (S); closely followed by S+SG+Pj treatment. The only treatment pair which is not statistically different is S+SG and S+SG+Pj, both with the higher weights. S+Pj treatment also shows increased growth of above ground biomass in relation to the control.

The average value of the main root body weight, per treatment, increases in the order S < S+Pj < S+SG < S+SG+Pj. Anova analysis indicates statistically significant differences in the group as a whole, but the t-test only shows significant p values for S+SG+Pj with S (0.027) and S+SG (0.012).

From these results it is clear that the addition of spent grain as an amendment in the soil, stimulated the growth of vegetation cover, both above ground and in the main root biomass. The effect of *P.javanicus* inoculation, however, is less evident. When comparing soil with only spent grain (S+SG) and soil with inoculated spent grain (S+SG+Pj), there is not a consistent increase or decline in biomass growth, as one has slightly higher average above ground mass, and the other higher below ground mass. The treatment with *P.javanicus* inoculum and no spent grain amendment (S+Pj), has higher average above ground and main root biomass than the control (S). This could be the effect of the successful introduction of the fungus, or the nutrients the added fungal biomass (in the inoculation process) may have supplied.

### 8.4.3 Soil cultures

As a purely qualitative method, no certain conclusions can be drawn from soil culturing. However, some interesting observations can be made from the results.

Growth on MEA plates was fast relative to *P.javanicus*-only cultures. After four days all plates had well developed colonies. Though diverse organisms were visible across different plates, the colonies present in plates prepared from S+SG+Pj soils were consistent in all replicates, and distinct from those in plates prepared from other soils.

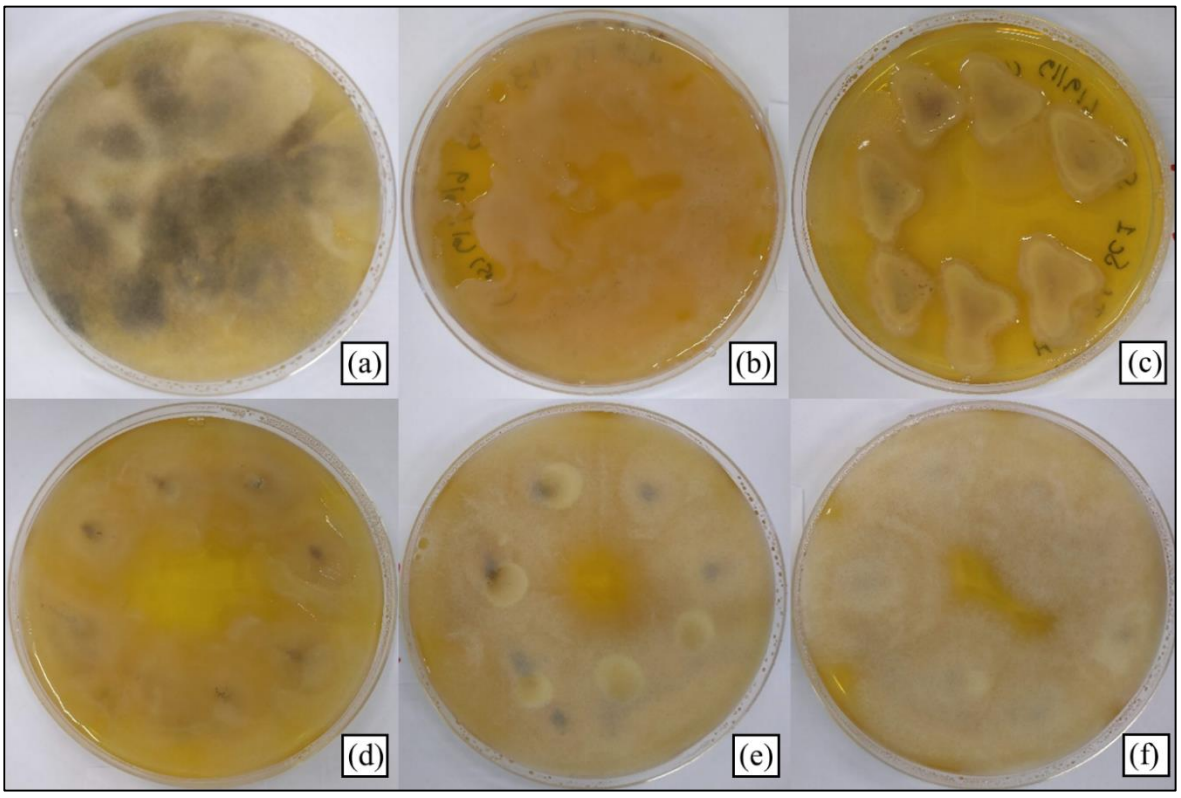



*Figure 8.13. MEA soil cultures. (a), (b) and (c) correspond to one of each soil only control pots (S); (d), (e) and (f) are replicates from both S+Pj treatment pots.*

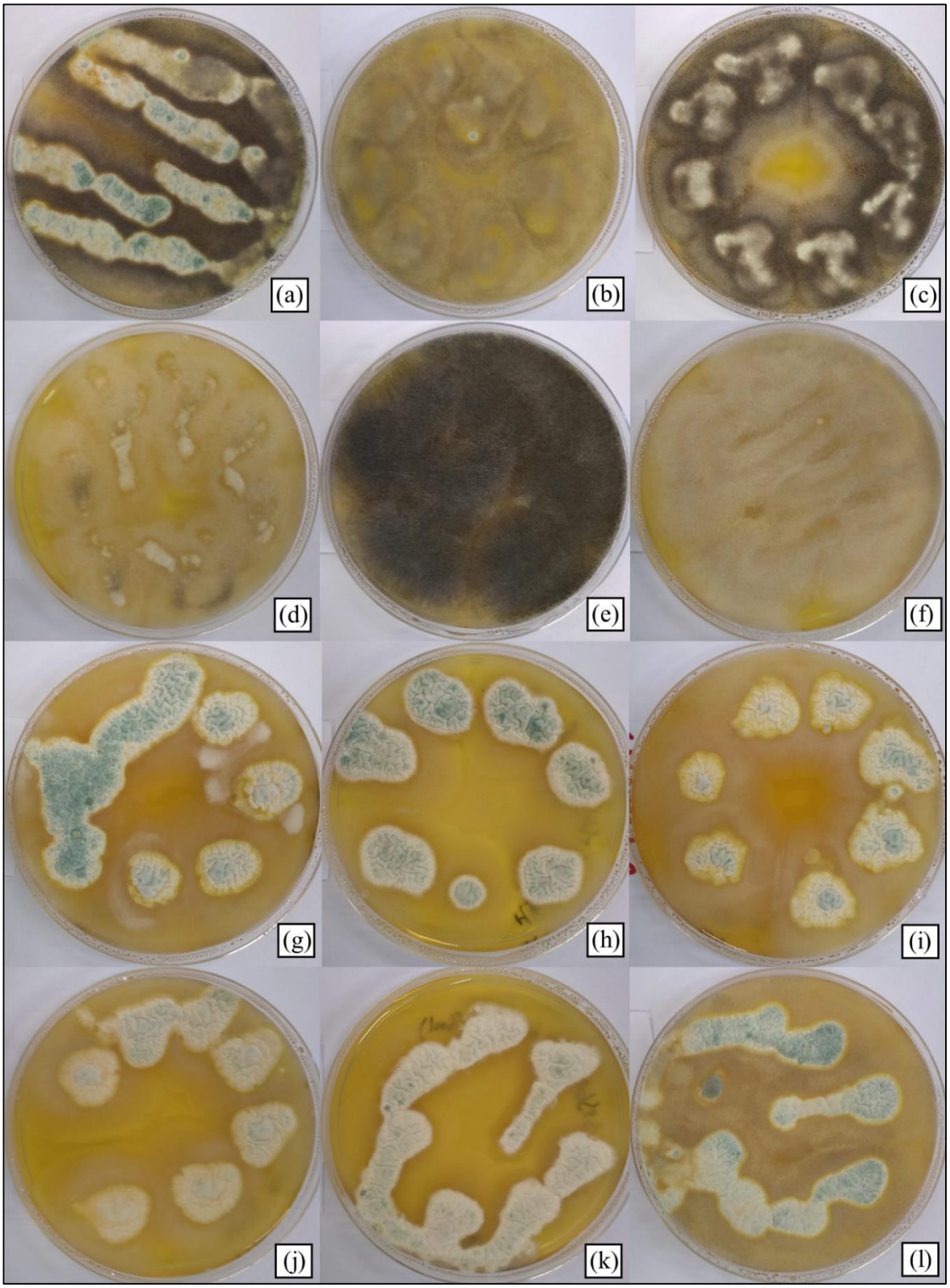



*Figure 8.14. MEA soil cultures. (a), (b), (c), (d), (e) and (f) are respectively 2 replicates from each S+SG treatment pots; (g), (h), (i), (j), (k) and (l) are respectively 2 replicates from each S+SG+Pj treatment pots.*

Plates prepared from S and S+Pj soils developed slimy non filamentous colonies with a light brown to pinkish colour and/or cottony filamentous white to grey mould colonies (Figure 8.13).

Plates prepared from S+SG soils (Figure 8.14-a to f), had a similar appearance, but with more abundance of darker grey filamentous mould, sometimes covering the whole plate (Figure 8.14-e) and displaying long hair-like filaments. Only one out of nine plates prepared from S+SG soil (Figure 8.14-a) had a similar appearance to those prepared from S+SG+Pj soils (Figure 8.14-g to l). In all nine replicates of the latter, thick irregular colonies with a raised elevation, a rugose brain-like texture and light pinkish colour, were predominant. A dark green-grey mould cover appeared to be spreading, to different extents in different plates, over the main rugose colonies.

Over the next few days of incubation, dark moulds spread further, prevailing in the majority of plates (with exception of some of those with a slimy texture) and covering the other colonies (Figure 8.15).

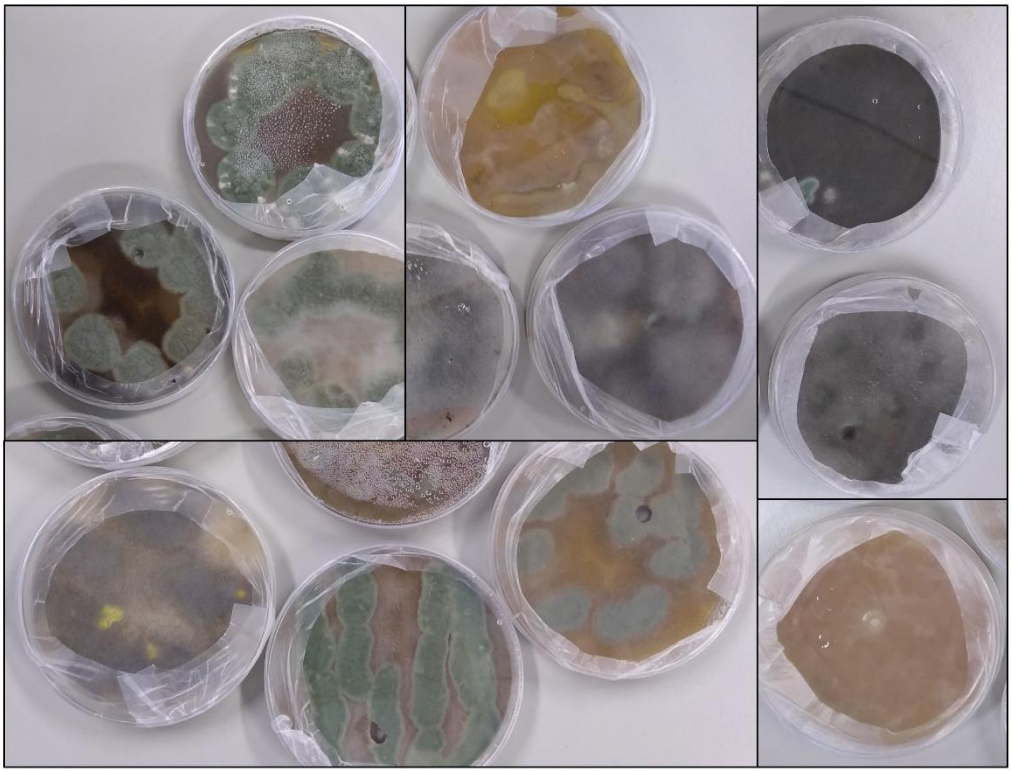


*Figure 8.15. Examples of culture plates after 11 days of incubation. Replicates from all 4 soil treatments are included.*

Although *P.javanicus* cannot be identified from the macroscopic description of colonies only, the fact that all cultures prepared from soils treated with spent grain inoculum showed a consistent and distinct population, may be indication of success in the inoculation and bioaugmentation strategy. If so, registered environmental conditions of temperature and precipitation during the time period of the experiment, would appear to be favourable for the proliferation of *P.javanicus* in soil. The inoculum grain: amendment grain: to soil volume

ratio would also have favoured the spreading of *P.javanicus* into the soil, and provided enough competitive advantage with the native microbiota, to allow its growth.

The one culture prepared from S+SG soils that showed similar colonies, could be the product of cross contamination, or of actual migration of the fungus from inoculated soil onto an adjacent pot. During the execution of the experiment, particularly before wild flowers and grass had begun to grow, individual amendment grains being transported from one pot to another by the wind had been noted, specifically through the presence of isolated grains in non-amended pots. Via this process, inoculated grain may have been transported from a S+SG+Pj pot into a S+SG pot, causing the fungi to migrate with it.

### 8.5 Key findings

- The amendment of poor quality urban soil with brewery spent grain, non-sterile and as a *P.javanicus* inoculum carrier, significantly increased above and below ground biomass of a native grass and wildflower mixture.
- Through the visual observation of MEA soil culture plates, it would appear that bioaugmentation of soil with *P.javanicus*-spent grain inoculum under real environmental temperature and precipitation conditions, produced a change in the soil microbial population.

# CHAPTER 9 Discussion

## 9.1 Pb remediation

### 9.1.1 Amendment of soil in microcosms and Pb fractionation

#### 9.1.1.1 Inefficiency of phosphate amendment of soil in microcosms

Full understanding of the mechanisms driving soil remediation is difficult to infer as a result of matrix heterogeneity and the numerous biotic and abiotic parameters that control soil functions (e.g. clay content, soil minerals, humic substances, soil colloidal materials, biogenic debris and exudates, and living organisms) (74, 75). Generally, only data of a few selected parameters from the amended soil is available (e.g. pollutant concentration in soil) allowing only for an estimation of the mitigation performances of a remediation strategy, without certainty of the reasons behind its success or failure (75).

Under laboratory conditions, pyromorphite can be easily obtained in a liquid media with soluble sources of Pb and inorganic P (65, 86, 191). For example Debela *et al*. (2010) produced the instant formation of pyromorphite by reacting 0.5 mol $l^{-1}$ $Pb(NO_3)_2$ with 0.3 mol $l^{-1}$ $H_3PO_4$ and 0.1 mol $l^{-1}$ NaCl solutions in a 4 l beaker (65).

With a P source that is not readily available, such as the organic Glycerol 2 phosphate ($C_3H_7Na_2O_6P*5H2O$) used in liquid culture experiments shown in Chapter 3, pyromorphite formation is not as straightforward. While precipitation of a solid phase occurred in abiotic controls in the presence of a P source, it was not of a crystalline nature, and the activity of *P.javanicus* was required to biomineralize pyromorphite. Failure to form pyromorphite from available Pb, even in the presence of a phosphate source, without the aid of biotic activity has previously been reported (42, 48, 50, 76, 78).

Soil conditions are often harsher and more complex compared to conditions in cultures. In soils several factors co-occur and produce complex responses (75), and differing physicochemical attributes may result in complex and differing processes in the formation of mineral species (78).

Pyromorphite formation in the natural environment depends on various chemical and biological processes and requires mobile $Pb^{+2}$, chloride ($Cl^-$) and phosphate ($PO_4^{-3}$) (46, 76, 85). $Cl^-$ deficiency in soils is rare because of the many sources of chlorine ($Cl_2$) or chloride ($Cl^-$) input from rainwater, fertilizer applications, irrigation waters, sea spray, dust and air pollution (76). In spiked soil microcosm experiments, Cl was added at an excess Pb/Cl molar ratio of 5/3.5, to ensure this was not a limiting factor.

Soil phosphorus occurs in inorganic and organic forms, and while it is abundant in the environment, most of it is in an insoluble form (42, 76, 192-194). From commonly found average total soil phosphorus concentration of 400–1200 mg $kg^{-1}$, as little as 1 mg $kg^{-1}$ can be present in an available/soluble form (194, 195). Inorganic phosphorus (mainly $H_2PO_4^-$ and $HPO_4^{-2}$) can be easily adsorbed onto soil particles and react with a variety of divalent and trivalent cations (e.g. Fe, Al and Ca) in soil, forming inorganic compounds or large insoluble complexes that reduce its bioavailability (42, 45, 47, 76, 192, 193). Organic phosphorus comprises 50–80% of the phosphorus in soil and is derived from living and dead plants and animals (76).

Soils are seldom, if ever, in equilibrium, and the assumption of equilibrium is often a convenience to simplify an experimental design (82). Immobilization of Pb as pyromorphite via soil amendments is a case-in-point of a non-equilibrated, perturbed environmental system (82) that may lead to different results under different soil conditions.

It is widely accepted that in order to produce the precipitation of pyromorphite, the dissolution of both the pre-existing Pb and P source is required (46, 47, 85, 191, 192, 196-199). In the soil environment, it is not unusual for the solubility of both Pb and P to remain low, particularly at neutral soil pH, limiting the effectiveness of P induced Pb immobilization (68, 85, 195, 199-202). Sorption of Pb to other soil phases (e.g. clay and organic matter) has been shown to increase as pH rises, because surface functional groups become negatively charged at high pH (192). Reduced soil pH (i.e. induced acidic conditions) is hence required, to facilitate the dissolution of soil Pb and P, enhancing the formation of pyromorphite (46, 47, 68, 85, 199).

For example, in a study of the stabilisation of Pb in shooting range soils, Sanderson *et al.* (2016) (199) showed that the addition of P amendment caused the transformation of Pb-carbonate minerals to iron oxide bound Pb, a greater proportion of humic acid bound Pb and a small proportion of hydroxypyromorphite, contrary to what would be expected (199).

Hashimoto *et al*. (2009) (47) studied neutral to alkaline Pb contaminated soils collected from a shooting range, separately treated with calcium phosphate, hydroxyapatite synthesized from ceramic waste, and incinerated poultry litter. EXAFS–LCF (extended X-ray absorption fine structure spectroscopy combined with linear combination fitting) analysis showed that about 70% of Pb species was not immobilized as a form of chloropyromorphite, and the additional supply of phosphate amendment scarcely promoted chloropyromorphite formation (47). No significant increase in chloropyromorphite occurred after 7 days of incubation, in an overall 380 days of experiment duration (47). Organically-complexed Pb

was persistent in all amended soils, suggesting that an enriched soil organic carbon may be an inhibitory factor for pyromorphite transformation (47, 199).

Zhang and Ryan (1998) in laboratory experiments with anglesite and apatite, identified the optimal pH for an almost complete transformation to chloropyromorphite, as pH 4 and pH 5, while at pH 6 and pH 7 only 1 and 4% of the total added P source dissolved (191). The soluble $PO_4$ concentrations, in consequence, were insufficient to saturate the solution with respect to chloropyromorphite (191). Similar optimal pH ranges have been reported elsewhere (83, 192).

It is therefore expected that higher pH, such as those measured in soils used in the microcosm experiments, reduce the effectiveness of Pb immobilization by phosphate amendments due to low P solubility (84, 85). Concentrations of P was the highest in BCR1 in extracted spiked soils, however, the pH of BCR1 solution is considerably more acidic (~ 3) than the measured pH in soil solution. Hence, though available in the BCR1 solution, P may have remained insoluble in microcosms.

Furthermore, although the literature describes brewery spent grain as an acid matrix that can reduce soil pH (146, 155), the opposite effect occurred in microcosms with spent grain (amended or as inoculum, Chapter 5) that showed pHs between 7.2 (S+SP+Pj) and 8.0 (S+SG+Pj), in comparison to pHs of 6.1 (S+SP) to 6.7 (S) in the remaining microcosms. This may have contributed to further reducing the availability of inorganic P in solution for the immobilization of Pb, in respect to microcosms with no spent grain additions, although this was not reflected in different results for Pb bioaccessibility in microcosms with and without spent grain.

Bioaugmentation with *P.javanicus,* a fungus with the ability to biomineralize pyromorphite in laboratory cultures with organic and inorganic sources of P (Chapter 3 and (42, 48, 50)), was aimed at overcoming these known limitations of Pb remediation with phosphate amendments in neutral to alkaline soils. Both inorganic (superphosphate) and organic (spent grain) sources of P were provided in microcosms, bioaugmented and non-bioaugmented with *P.javanicus*. However, similarly to Hashimoto *et al*. (2009)'s (47) experiment, in spiked soils a possible initial formation of pyromorphite, by reaction with P already present in the soil, seems to have occurred very quickly during soil ageing, but no major further increase was seen after the extended incubation time (150 days). T0 (pre-incubation) spiked soil had an average Gc-bioaccessible Pb difference of ~10% compared to the reference matrix ($Pb(NO_3)_2$), while incubated microcosms between ~12-15%. Similarly, BCR4 residual Pb did not increase significantly in incubated S+SP+SG+Pj soil (16.7 ± 1.4 %) in comparison

to T0 soil (15.6 ± 2.7 %). This shows that neither the amendments nor the bioaugmentation with *P.javanicus* appear to have greatly increased the immobilization of Pb as pyromorphite.

#### 9.1.1.2 *P.javanicus* in microcosms versus liquid cultures

It has been observed that bioaugmentation with microorganisms known to perform a particular function, does not always result in a successful remediation (74, 133). This is because these functions are highly dependent on environmental conditions (133) and soil chemistry is considerably more complicated than growing medium (200). Successful bioaugmentation that achieves the desired effect (e.g. biodegradation of organic contaminants or in this case biomineralization of Pb) depends on soil properties (e.g. porosity, pH and temperature), the characteristics of the indigenous microbial population, the stability of population density of the bioaugmented organism, amongst others (133, 137). Thus, in many cases, isolates that are efficient under laboratory conditions, are not necessarily effective in situ (133, 200).

Biomineralization of metals by fungi is species specific and dependent on the physiology of the organism, and the changes it can make to its environment (42, 67). The specific characteristics of a substrate (e.g. soil) affects the metabolism and biomineralization capacity of a microorganism (42). The nature and amount of acids excreted by fungi are influenced by the pH and buffering capacity of the environment, the carbon, phosphorus and nitrogen sources, and the presence or absence of certain metals and other trace elements and contaminants (12, 42, 67, 137).

Enzyme production by inoculated organisms can also be inhibited by competitive microorganisms under nonsterile conditions, as well as by non-biological deactivation factors, such as presence of other metals, extreme acidity/alkalinity, protease degradation, and adsorption to soil constituents (203).

Difficulties have previously been reported for mycorrhizal fungi when comparing responses to toxic metal stress in single species cultures, to those in situ in the mycorrhizal soil environment, because metal sensitivity in the mycorrhizal symbiosis depends on many other factors besides metal tolerance (67).

Templeton *et al*., (2003) (204) studied the surface complexation of Pb in the bacterial species *Burkholderia cepacia*. Results showed that varying factors such as pH and Pb concentration determined whether sorption or biomineralization as pyromorphite was predominant (204).

Wang et al. (2014) (134) carried out a study with a mutant bacterial species (*Bacillus subtilis* 38, B38) with high cadmium tolerance, showing that this microorganism was a good

biosorbent for the sorption of multiple heavy metals (Cd, Cr, Hg, and Pb) in solution (134). But when used for bioaugmentation in heavy metal contaminated soil in pot experiments, the immobilization efficiency of Cd, Cr, Hg and Pb was not as effective as expected (134).

A study of the effects of phosphate solubilizing bacteria (PSB) in the presence of phosphate rock (RP) on the immobilization of Pb, showed much more efficient reduction of ammonium nitrate extractable Pb (as a form of bioavailability measurement) in agar cultures than in soil microcosms (200). In nutrient agar experiments treated with RP and PSB inoculum, soluble Pb was reduced to 5.82 ± 2.41 mg $kg^{-1}$ in comparison to the control (12.4 ± 1.5 mg $kg^{-1}$), while in soils, ammonium nitrate extractable Pb was only reduced to 19.8 ± 0.7 mg $kg^{-1}$ in RP+PSB amended soil in comparison to the control (21.7 ± 1.0 mg $kg^{-1}$) (200).

Fomina *et al.* (2004) in a study of zinc phosphate and pyromorphite solubilization by soil plant-symbiotic fungi, noted that the production of some organic acids (e.g. oxalic acid) occurred only as a response to the presence of specific toxic metal minerals in the medium, and were not synthesized if these minerals were absent (67). Conversely, it was also found that some fungal strains significantly reduced the production of some organic acids, in some cases to undetectable levels, in the presence of other toxic metal minerals (67).

These examples support the conclusion that the excretion of oxalic acid by *P.javanicus* that lead to the initial "capture" of Pb in liquid cultures (Chapter 3), may not have occurred equivalently in the complex soil matrix utilized in microcosm experiments.

Furthermore, in liquid cultures, *P.javanicus* had clear control on the overall pH of the system (shown by pH measurements in both Pb and Phen experiments) allowing for the necessary pH modifications required for the capture and transformation of Pb-oxalate into pyromorphite. In microcosms however, other components such as the soil's own buffering capacity and spent grain amendment seemed to have had greater control of the pH of the system. Unlike growth medium, soils have a strong pH buffering capacity and therefore the pH of the soil is less easily affected by the growth of microorganisms (192, 200). The potential influence of *P.javanicus* on the pH may hence have been limited only to its very immediate microenvironment.

The success of a bioaugmentation process is also limited by the interaction of introduced microorganisms with toxic contaminants (121). Microbial surfaces provide chemically reactive sites for sorption, which can then lead to the nucleation and formation of mineral precipitates around biomass (74). In soil, as opposed to well-mixed aqueous systems, a large fraction of the inoculated microorganism can be physically separated from the target contaminant (75).

Accordingly, the *P.javanicus* biomass /soil ratio and in consequence *P.javanicus* biomass/Pb ratio was visibly lower in microcosms than the *P.javanicus* biomass/Pb in liquid cultures. This may have reduced the potential for physical contact and interaction between the fungus and target contaminant (Pb), in comparison to the significantly more readily achieved contact/interaction of *P.javanicus* with spiked Pb in sterile liquid culture media. Furthermore, organic acids secreted by the fungus (e.g. oxalic acid), may not have had contact with a significant portion of the Pb, ultimately being unable to influence change in Pb-speciation in the system as a whole.

In microcosm P.jav2 (preliminary soil experiment) however, *P.javanicus* inoculated in a sterile soil-spent grain mixture had profuse visible growth, notably higher than in all other microcosms (of both experiments), significantly enhancing the potential for fungus-Pb contact and interaction. Despite this, no increase in BCR4 residual Pb or significant decrease in UBM Gc-bioaccessible Pb was observed. In fact, BCR4 Pb appeared to be reduced, and hence Pb mobilized in favour of BCR3 (organic matter-bound target phase). Although P concentrations were lower in this microcosm, in comparison to spiked soils, BCR results showed that the overall (and BCR1, BCR2 and BCR4) P/Pb molar ratio was sufficient for pyromorphite formation (stoichiometric P/Pb molar ratio of 3/5). Furthermore, the spent grain amendment also provided an additional source of P, as shown by the chemical analysis of amendment and fresh spent grain (section 5.4.2.2).

This evidence would suggest that the apparent failure to biomineralize Pb as pyromorphite in microcosm P.jav2, was more likely due to a different metabolic response of *P.javanicus* in the soil system and/or an overriding control of other soil components in the general physicochemical conditions, rather than caused by a physical separation from the target contaminant or a limited supply of P.

Rhee *et al*. (2016) (78), in a later experiment, using fungal pellets of *P.javanicus* under the same experimental conditions as previously published research with the fungus, detected the formation of Pb-carbonates and oxalate in cultures, but not pyromorphite as in all other published studies. The absence of pyromorphite in that case, was attributed to differences in the microenvironment such as pH, organic acid concentration, substrate availabilities and insufficient transfer of oxygen (78). The growth of the fungus in pellet form, was also considered as a possible cause (78), as it may have produced differing physicochemical and biotic conditions inside or at the surface layers of the fungal colony, which may not have promoted pyromorphite formation (78).

### 9.1.2 Spent grain amendment and sorption of heavy metals

Sorption of Pb by organic and inorganic soil amendments is considered a desirable outcome, particularly when aiming for reduced environmental availability to ground water and plants (134, 205).

Spent mushroom compost, for example, has been used for the stabilization of mine sites, as it has vast sorption capacity for Pb, Cd and Cr, and provides nutrients for vegetation cover (205). Other studies have used organic fertilizers as an agricultural soil amendment, due to their immobilization efficiency of Cd, Cr, Hg, and Pb through sorption(134). Wang *et al*. (2014) using a registered organic fertilizer (NovoGro) concluded that the amendment significantly inhibited the adverse effect of heavy metal contamination on plants and soil microbes, enhancing the proliferation of both exotic and native microbes (134).

By increasing adsorption strength of Pb and aiding the establishment of vegetation that can reduce soil transfer to receptors, amendment with biosolids could potentially reduce human bioavailability (53, 55, 59). However, the success of reducing Pb bioaccessibility has proven variable and case specific, in previous research (46, 53, 55, 57-60).

Besides, the quoted benefits towards environmental availability, overall soil quality and vegetation establishment, the fate of biosolid-sorbed metals in the long term, as organic matter decomposes, would require attention (53, 55, 56, 58, 60, 198). When considering human receptors, mineralogical changes such as pyromorphite formation, are likely to have more longevity with respect to Pb-bioavailability than biosorption on organic materials (55), and would hence be more desirable in an urban environment.

However, Kuhad *et al*. (2011) (73) in a review publication on phosphate-solubilizing microorganisms, proposed that the eventual decomposition of biosolid amendments may aid in the solubility of P, through the production of humic acids which chelate $Ca^{2+}$ and release $H^{+}$, and can also form stable complex with Fe and Al, liberating P (73). This could potentially aid in making P more available to react with Pb to form pyromorphite.

### 9.1.3 Lessons on bioaugmentation with *P.javanicus*, spent grain and superphosphate

The success of a bioaugmentation process depends on the ability of the strains to survive, colonize and exert the desired effect on the contaminants in soils (e.g. degrade or biomineralize) (121, 137). The interaction of introduced microorganisms with toxic contaminants and the competition between allochthonous and autochthonous microbial communities for space and nutrients, are both limiting factors (121).

Failure of bioaugmentation with inoculated microbial strains has been reported, due to their rapid decline and early disappearance from the natural environment (118, 136). Therefore,

an important challenge with bioaugmentation approaches, is to ensure that the inoculation strategy allows microorganisms to survive under harsh soil conditions, long enough to perform their intended clean-up task (118).

Microbial selection for bioaugmentation must also consider performances toward pollutants (i.e., tolerance of high concentrations of contaminants, high degradation rate or change in chemical forms for metals) in a wide range of environmental conditions (75). In other words, although adequate starter conditions and the inoculation strategy are a crucial step for the success of bioaugmentation, selected microorganisms must be versatile and adaptable, producing their desired function in varying conditions (118, 136).

While the preliminary soil experiment and pot experiment seem to indicate that the bioaugmentation strategy with *P.javanicus* and spent grain may have been successful, in terms of the effective introduction of *P.javanicus* in the soil system (less so in spiked soil experiment, where successful inoculation was only evident at the beginning), the desired effect of reducing Pb bioaccessibility though *P.javanicus*-induced biomineralization of pyromorphite was not achieved. The biomineralizing function of *P.javanicus*, observed in the controlled conditions of liquid cultures, was hence not easily transferred to the soil matrix.

Further optimization of the desired process (e.g biomineralization of Pb) could be achieved by altering the physico-chemical and nutritional conditions of the contaminated area (74).

The particular physical, chemical and nutritional conditions in microcosm experiments may not have favoured the occurrence of *P.javanicus*-induced pyromorphite biomineralization, and variations of these conditions could be studied to determine a specific set of soil parameters that lead to the effective immobilization of Pb as pyromorphite with *P.javanicus* bioaugmentation. However, the application of this as a bioremediation strategy *in situ* in real contaminated top soil would be challenging, if a controlled set of specific conditions is required. A nature-based approach to controlling those conditions, such as the combination with phyto/rhizo-stabilization could be considered, and is discussed later in section 9.3.

The potential for other, more adaptable, microorganism could also be studied for the enhancement of pyromorphite biomineralization, with the same bioaugmentation approach, using spent grain as a carrier and alternative P source.

## 9.2 PAH remediation

### 9.2.1 Biostimulation with spent grain

#### 9.2.1.1 Potential of adapted native microbiota for degradation: spiked soils vs real aged contaminated soils

Pre-exposure of a microbial community to hydrocarbons, increases its hydrocarbon oxidizing potential through adaptation (8, 100, 103). Although the use of artificially contaminated or spiked soil is quite common in PAH biodegradation studies (75, 133), the disadvantage of doing so is that the native microbiota of the utilized soil may not be adapted to the spiked contamination, as in true aged contaminated soils. Hence, hydrocarbon-degrading species or activity might not be easily promoted in spiked soil, even if artificial aging is carried out.

The difference between a long-term contaminated soil and a spiked unpolluted soil has been shown in biostimulation studies (75, 126).

Hamdi *et al.* (2007) (in (133)) studied the enhanced biodegradation of anthracene (ANT), pyrene (PYR) and B(a)p in spiked soil. In this case, previously treated aged PAH-contaminated soil containing PYR and B(a)p degraders was mixed with the experimental PAH-spiked soil (133) (133). In the control samples (only spiked soil), the PAH removal was the lowest, revealing ANT, PYR and B(a)p dissipation rates of 63, 33 and 35%, respectively, after 120 days (133). In turn, in mixed samples, the final degradation rates were higher, above 96% for ANT and PYR and 60% for B(a)p (133).

Da Silva *et al.* (2016) showed that biostimulation with peanut cake in soil contaminated with pesticide (paclobutrazol, a recalcitrant organic contaminant as are PAHs), produced double the degradation, after 50 days, when in spiked soil combined with historically contaminated soil, compared to spiked soil alone, indicating that the historically contaminated soil was a more appropriate source of microorganisms adapted to the degradation of paclobutrazol (126).

In the microcosm experiment, this may have prevented further B(a)p and Phen degradation through biostimulation with spent grain, which could have otherwise been more efficient in real aged contaminated soils. Despite there being evidence of PAH contamination in the Meat market site, provided by the preliminary chemical data, collected samples had no detectable PAH concentrations by ASE extraction and GC MS analysis (section 4.4.3). The potential presence of a pre-conditioned native microbiota, that encourage the presence of PAH-degrading types (100, 103), was hence presumed to be limited in these samples, leading to the use of spiked soil in microcosm experiments.

#### 9.2.1.2 Effects of nutrient balance

Microorganisms require a source of carbon to provide energy and multiplication of new cells, as well as a nitrogen source for building cell proteins (131). Nitrogen is also crucial because it triggers enzyme production by fungi and thus affects PAH degradation rates (132, 206). In addition, phosphorus and other trace minerals are required by microorganisms for optimum activity (131).

Special care is required when balancing the carbon and nitrogen ratio in the substrates, which has a significant influence on the degrading performance of microorganisms (137).

Various ratios have been proposed as optimal for enhancing hydrocarbon degradation and production of ligninolytic enzymes by fungi (75, 131, 133, 137, 207). After comparing various sources of nutrients with different C:N:P ratios for enhancing biodegradation of oil hydrocarbons in soil, Zawierucha & Malina (2011) (133) concluded that the highest enhanced biodegradation rates were observed at the C:N:P ratio of 100:10:5.

In the context of soil fertility, a soil C:N ratio of 25 to 35:1 has been proposed as optimum (131). Ratios similar to those of microorganisms themselves, have alternatively been proposed as desirable (20, 200, and 300 for C:N, C:S and C:P respectively) (75).

In experiments using the fungus *Trametes versicolor* (137) to degrade soil pollutants, aided by several processed and unprocessed low-cost lignocellulosic substrates as inoculum carriers, growth and activity yielded better results in the substrates containing lower C/N ratios, in contrast to previous reports of enzymatic production by white rot fungi (137).

Most lignocellulosic residues have wide C:N ratios varying from 35 to 325:1 (131). Brewery spent grain in particular, is considered to be rich in nitrogen (N) owing to its high protein content, with recorded C:N ratios of 8–25 (146). P is also found in relatively high concentrations (120, 143).

Though reduction of the soil C:N ratio with the addition of N rich amendments can increase microbial growth, it can also change the structure of the community (206). Hence excess fertilization can have detrimental effects on microbial processes (206).

Spiked soils used in microcosms (commercial topsoil), had a relatively high organic matter content, providing good water holding capacity and ample nutrients for plant growth. From an average total air dried sample weight of 4.63 g, spiked soils contained 49.8 ± 2.3 % of moisture content, 14.7 ± 2.5 % organic matter based on loss on ignition (LOI) and 35.5 ± 3.7 % of residual mineral fraction (29.3 ± 5.4 % of LOI and 70.7 ± 5.4 % mineral residue, if moisture content is excluded). Further addition of nutrient rich spent grain may have produced an imbalance in nutrient.

In real urban brownfield type contaminated soils, however, nutrient content is likely to be much lower. Air dried soil from the CP08 homogenized- Meat market sample had an average moisture content of 8.1 ± 2.2 %, 11.5 ± 3.7 % of organic matter by LOI and 80.0 ± 3.5 % of residual mineral fraction (13.0 ± 1.9 % of LOI and 87.0 ± 1.9 % of residual mineral fraction if excluding moisture content). Hence, the nutrient contribution from the spent grain amendment would most likely be beneficial for microorganisms in this type of soil.

#### 9.2.1.3 Effects of oxygenation

Aerobic respiration involves the use of oxygen as an electron acceptor by microorganisms to oxidize sources of carbon, including contaminants (75, 102, 208). During the process of aerobic respiration, oxygen is reduced, resulting in the formation of water (208). Thus, a drop in oxygen concentration occurs when aerobic microbes are active (208). For this reason, continuous oxygen supply in soil is necessary to stimulate microbial activity and enhance aerobic biodegradation (102, 133, 206).

Commonly used oxygen supply techniques include tilling, forced aeration and chemical methods (133). Tilling is recommended as a physical method to accelerate biodegradation in top soils, particularly if combined with the addition of nutrients (107, 133).

Previously published laboratory microcosms studies have included regular mixing/stirring overtime, as a form of tilling to increase oxygenation in microcosm experiments (113, 114, 127, 136).

In soil with high water contents (near field capacity or over saturation) pores are filled with water, limiting the oxygen transfer that determines the activity of aerobic microorganisms and hampering biodegradation of contaminants (75, 133). Sustaining accurate soil drainage is hence favourable for biodegradation in top soil, with attention to the frequency of irrigation and length of the intervals between irrigation periods (75).

Microcosm experiments (Chapter 5) were designed as a closed system, to reduce possible external contamination or inputs, in order to have more certainty if the resulting effects were attributable to the treatment additions, namely *P.javanicus* inoculum and spent grain amendment. In this sense, although some interventions were carried out over the experiment time, conditions were always kept sterile, and continuous “tilling”/ mechanical aeration was avoided.

Further sterile water additions, in order to maintain enough moisture for fungal growth (without drainage) may also have influenced soil oxygenation. Rotating conditions (150 rpm) in the incubator where intended to increase aeriation and mobilise the water-air

interface, but the effect may not have been sufficient and comparable to mechanical mixing/stirring.

A more open system where there is provision of water, moisture and also drainage, such as pot experiments, may allow improved oxygenation, compared to the laboratory microcosm system, further enhancing degradation.

#### 9.2.1.4 Effects of pH

Soil pH is an important parameter when it comes to biodegradation, since it has direct influence on the organic molecule (126, 206). pH influences the availability and absorption of the compound and the metabolic activity of microorganisms also depend on pH conditions (126, 206). Optimum pH values for pollutant-degrading microorganisms are said to range from 6.5 to 7.5 (132, 207, 208).

Research with the white rot fungus *T. versicolor* showed that fungal growth was favoured at pH values in the acidic range and maximum laccase activity occurred at pH around 5, while pH values over 7.4 may cause inactivation of white rot fungi enzymes (137).

On the contrary, enhanced counts of hydrocarbon utilizing bacteria (HUB) have been attributed to increased soil pH (8.27) caused by amendment with banana skin (BS), suggesting that slightly alkaline pH in soil encourages the growth of hydrocarbon utilizing bacteria (120). A follow up study showed that in comparison to the BS, the rate of oil breakdown in soil amended with spent mushroom compost was lower, as was the soil pH, concluding that low pH conditions affect the growth and biodegradative activities of HUB in soil (114).

The slightly alkaline soil pH ranges found in microcosm soils with added spent grain (~ 7.2-8.0) should therefore be beneficial for the activity of hydrocarbon-degrading microorganisms.

#### 9.2.1.5 Spent grain amendment and sorption of PAHs

It is generally considered that sorption of PAHs onto soil phases, particularly organic matter can be detrimental to- and prevent biodegradation, as PAHs would be more available to microbes (particularly bacteria) when in the dissolved phase (75, 99, 100, 103, 124). However, in the case of sorption to organic and lignocellulosic soil amendments, such as spent grain, there may be some advantages.

It has been observed that some microorganisms can access compounds by direct adhesion, thanks to biofilm formation (75, 124). Microorganisms that form biofilms can be especially

well suited for the treatment of recalcitrant or slow-degrading compounds, because of their high microbial biomass and ability to immobilize compounds by biosorption, bioaccumulation, and biodegradation (75, 124).

Biosorbents have been recently gaining importance, with an increasing number of publications on their environmental applications (136). Sorption of hydrophobic organic contaminants (e.g. PAHs) to organic amendments, can reduce the toxicity of the environment to microorganisms, hence aiding in an increase in the population, as well as inhibiting the movement of contaminants to other compartments, like groundwater (106, 128, 186) Sorption of both organic compound and microbes to biosolid surfaces can give rise to a greater concentration of the contaminant close to the colonizing microorganism cells, and therefore may increase the rate of biodegradation of these compounds (124).

Lignin is a biopolymer with the ability to scavenge free radicals, chelate metal ions and donate hydrogen atoms or electrons (122). Lignocellulosic amendments, while offering sites for contaminant sorption, can maintain aerobic conditions, improve structure, and regulate moisture in the soil, promoting to a greater extent all microbially-mediated processes and consequently contaminant degradation efficiency (121, 128, 130, 136). Furthermore, compounds associated with the composition of these natural biosorbents enhance and stimulate a greater enzymatic microbial activity, associated to PAH breakdown (112, 121, 128, 130, 136).

Researchers have found that the addition of lignocellulosic co-substrates promotes higher performance of microbial consortia, by contributing to improved access to hydrophobic molecules and facilitating a longer contact time between the consortia and the contaminant (101).

For example, a study using cork waste for biostimulation, showed that sorption of pesticides by the cork mitigated the toxicity to microorganisms in the bio-mixture, especially in the early stages, improving the feasibility of the native soil microflora to remain metabolically active and degrade pesticides (136). As the increased microbial population began to degrade available compounds, reducing concentration in soil, sorption of pesticides to the cork material became reversible, allowing for further degradation of initially sorbed compounds, due to the concentration gradient reversal (136). It was concluded, that to achieve efficient biodegradation with the use of a biomixture (soil plus a lignocellulosic amendment), a good adsorption capacity is desirable, to retain organic compounds for degradation by an active microbial biomass (136).

In conclusion, while sorption of PAHs may hinder their immediate degradation (187-189), in the longer term, sorption of PAHs to spent grain may favour their degradation, by reducing their availability and hence toxicity to potential receptors through volatilization (187), or ingestion/inhalation of fine contaminated soil particles (187).

### 9.2.2 Lessons on bioaugmentation with *P.javanicus* and spent grain (PAHs)

Pollutant degraders are often present in historically polluted sites, but their numbers and metabolic activities may be depleted in response to pollutant concentration and general poor soil quality (110, 112). The use of agroindustrial wastes with a rich nutrient composition, such as spent grain, helps solve the challenge of nutrient limitation in polluted sites, encouraging biodegradation (101, 106, 110, 112, 136). It can also encourage an active microbial biomass that is genotypically and phenotypically versatile, for the degradation of different residues even at high concentrations (136).

Previous research has shown that the addition of co-substrates to stimulate the bioremediation process can enable almost complete removal of the polycyclic aromatic hydrocarbons (PAH) in soil (101)

Absorption of organic pollutants, such as PAHs, present in soil, to lignocellulosic residues may result in inhibition of the movement of organic pollutants while stimulating their biodegradation through enzymatic co-degradation (101, 128, 130, 136).

Physically, agroindustrial residues can also improve soil properties, in terms of aeration, moisture, nutrition and structural properties, all of which may lead to the acceleration of hydrocarbon bioremediation (101, 128, 130, 136). Finally, agroindustrial residues also have their own associated microbial community which may contribute to the degradation of hydrocarbons (128, 130). Studies have shown that, although biostimulation with sterile agroindustrial amendments alone was effective in enhancing the development of indigenous microbiota and consequently PAH degradation, the bioaugmentation effect of using non-sterile amendments can have better PAH removal efficiency (106).

While bioaugmentation with *P.javanicus* did not appear to contribute to the degradation of PAHs, the bioaugmentation strategy, with spent grain as an inoculum carrier showed promise, and could be applied with other known degrader organisms. Furthermore, the microbial population naturally present in non-sterile spent grain may result in a positive bioaugmentation input, due to their potential for biodegrading lignocellulosic substrates.

The nutritional content and lignocellulosic co-substrate potential of spent grain, is likely to be beneficial for biostimulation in historically contaminated soil, with an adapted native

micro-biota but poor soil quality, enhancing degradation of PAHs, particularly those of a more recalcitrant nature, with higher molecular weights and hydrophobicity, such as B(a)p.

Furthermore, overtime as the spent grain decomposes it may continue to offer a slow and extended provision of co-substrate (e.g. lignin) for the co-degradation of PAHs, while improving soil quality and increasing fertility.

While in microcosms experiments, the rate of reduction of PAH concentrations was not high, a system with historically contaminated soil and enhanced aeration, may produce a more efficient biodegradation. Combining the bioaugmentation/biostimulation of soil with spent grain, with vegetation cover may be an appropriate way of enhancing soil oxygenation and biodegradation efficiency (discussed later in section 9.3).

An important concern regarding bioremediation efficiency, is to monitor the mineralization of the targeted pollutants, as intermediate metabolites can have a higher toxicity (75, 189).

Although the identification of metabolites in GC-MS chromatograms, both in the liquid culture and spiked soil microcosm experiments, was planned as part of this project, the execution of such analysis was impossible due to the COVID-19 worldwide lockdown, contingent to the final stage of this project. It is recommended, however, that in future studies stemming from this project, emphasis is placed on a detailed monitoring of PAHs degradation pathway, allowing for a more certain assessment of bioremediation efficiency.

## 9.3 Recommendations for future research

### 9.3.1 Combination with phyto/rhizo-remediation

The pot experiment described in Chapter 8 showed that the amendment of poor quality urban soil with both *P,javanicus*-carrier spent grain and non-sterile spent grain, significantly increased the growth of a native grass and wildflower mixture. Below ground biomass was also significantly increased by the amendments (Figure 9.1), indicating a positive effect towards the development of an active rhizosphere in poor quality soil.

While effects on soil contamination were not measured in this experiment, the potential that an enhanced rhizosphere development, combined with the studied soil biostimulation/ bioaugmentation strategy, for the co-remediation of Pb-PAHs contaminated urban soil, is recognized and recommended for study in future research.


*Figure 9.1. Root development in harvested pots, from the pot experiment (Chapter 9), comparing control unamended soil (a and b) to spent grain-amended soil (c and d).*

#### 9.3.1.1 Potential for the enhancement of PAHs biodegradation

Future prospects for *in situ* bioaugmentation will benefit from the ability to exert control over systems with variable environment conditions (75). The latter can be achieved with nature-based engineering, particularly with the combination of plants with microorganisms, which has shown promise (75, 102). Plants are relevant means of controlling environmental conditions surrounding microorganisms used in pollutant degradation (75, 102). Furthermore, physicochemical conditions in the rhizosphere are less susceptible to change in the course of time, than in bulk soil (75).

Plants can aid in the settlement, survival, higher growth rate and stability of the microbial population irrespective of the environmental conditions (75, 100, 102, 103, 128, 157). In contrast to bulk soils characterized by its poor nutrient conditions, rhizosphere soil is well known as a site of elevated microbial numbers and activity (5 to 100 times more organisms per unit volume), as root exudates supply nutrients ensuring a continuous flux of substrates to microorganisms (8, 75, 102, 107, 117, 157, 209, 210). This in turn can enhance pollutant-degrading enzymes production (75, 100, 102, 103, 128, 157).

Plant roots are also able to improve soil aeration, thus increasing activity of aerobic microorganisms, acting as a replacement to other oxygenation methods like tilling (75, 107, 209). Roots are able to transport oxygen in soil, maintaining oxic conditions and providing oxygen necessary for biodegradative pathways (75, 117, 209).

The contribution of the rhizosphere, and its inhabiting microbial population, referred to as rhizoremediation, has shown promise in soils contaminated with organic pollutants (75, 102, 157).

Greater reduction in organic contaminant concentrations can be achieved when biostimulation is supplemented with rhizoremediation, as opposed to the use of only biostimulation or rhizoremediation (107). The addition of exogenous substrates increases initial microbial concentrations and reduces the toxic effect of pollutants, encouraging plant growth (75, 110, 117, 122) and accelerating the rate of bioremediation (110, 122).

Bioaugmentation can also contribute to plant and root growth (75, 102, 110). Microbes and plants develop a symbiotic relation, for example hyphae of mycorrhiza forming fungi increase the volume of the root-soil colonization, while also promoting colonization by bacteria, even in deeper horizons (75, 102, 117).

Grass (*Poaceae* or *Gramineae*) plants have been shown to accelerate remediation of organic pollutants by enriching the microbial population in soil adjacent to plant roots (102). Furthermore they are effective for stabilisation of soils and, therefore, can result in excellent restoration effects in degraded land (107).

By combining biostimulation with spent grain, or bioaugmentation using spent grain as a carrier, with the establishment of native grass and wildflower species, not only an enhanced biodegradation of PAHs could be achieved, but the general quality of an otherwise degraded soil would be enhanced in the long term, promoting the ecological rehabilitation of contaminated urban derelict land.

#### 9.3.1.2 Potential for Pb immobilization as pyromorphite

Immobilization of metals with soil amendments, combined with the activity of plants can be undertaken with phytostabilization (75, 196, 201, 211, 212), as plant roots and their free-living and symbiotic microbial populations can significantly alter the physicochemical characteristics of the rhizosphere by metabolic activities (65, 67, 198, 212-215).

The rhizosphere plays an important role in movement and redistribution of soil metals (74, 192, 196, 215). Plant growth promoting mechanisms involve the mobilisation of unavailable

nutrients in the rhizosphere, such as phosphorus (74, 102, 216). Many rhizosphere inhabiting phosphate solubilizing bacteria and fungi can make insoluble soil phosphate bioavailable, as well as release phosphate from organic sources (42, 66, 73, 194, 210, 217). The latter is achieved by the production of phosphatases for organic sources and organic acids or chelators (e.g. citrate, oxalic acid and lactate) for inorganic sources (42, 65, 74, 76, 130, 192, 194, 198, 213, 216, 217). Release of $CO_2$ by rhizosphere microorganisms, forming carbonic acid ($H_2CO_3$), can also help in P solubilisation, through pH reduction (67, 73). The importance of mycorrhizal fungi in plant phosphorus nutrition has been appreciated for a long time, and their ability to dissolve and transform calcium containing insoluble phosphates in pure culture and in mycorrhizal association, has been widely studied (67, 73, 74, 209, 216). Released phosphate can then precipitate with metals like Pb forming insoluble metal phosphates (42, 74, 76, 192, 195, 198, 216, 217).

Furthermore, pH changes in the rhizosphere compared to bulk soil, can affect the transformation rate of Pb from pre-existing phases into pyromorphite in P-amended soil (73, 191).

Several studies have shown that P additions combined with microorganisms can reduce metal uptake by plants, through immobilization around plant roots (74, 83, 192, 196, 198, 200-202, 212, 218-220). Using analytical methods such as SEM-EDX as well as sequential extraction and bioaccessibility assays, an increase in residual Pb and formation of pyromorphite has been identified in P amended rhizosphere soils, in comparison with amended bulk soils (45, 68, 196, 198, 201, 218, 219).

Austruy *et al*. (2014) studied the mechanisms involved in metal-phosphates formation in P-amended rhizosphere soils of pea and tomato (201). Results showed that P amendments had no effect on metal-P complex formation in the absence of plants, but in the presence of plants, P amendments caused Pb and Zn immobilization by forming metal-P complexes (201).

A recent field study combining triple superphosphate amendment and biosolids amendment with growth of *Miscanthus* plants, showed that a one-time addition of soil amendments to Pb-contaminated soil, supports establishing and stabilizing *Miscanthus*, increasing biomass yield as well as reducing phytoavailability and bioaccessibility of Pb (196). Plots amended with biosolids had significantly less total Pb uptake, plant tissue Pb concentration, and Pb bioaccessibility, and more soil enzyme activities, organic carbon, and microbial biomass (196).

Indigenous grass species are proposed to be the best plants for phytostabilization, due to their tolerance to metals and capacity to develop a dense and strong root system (202, 221, 222). Grass species are also advantageous as a phytostabilization plant because they tend to accumulate smaller amounts of metals in aboveground tissues compared to other species (202).

Concern has been voiced on the long-term stability of pyromorphite in the rhizosphere, due to the activity of microbes and root exudates, particularly low molecular weight organic acids (LMWOA) such as oxalic acid (65-67). However, Sayer *et al*. (1999) in a study with fungal cultures, showed that only one of the tested fungal species (*Aspergillus niger*) was able to partially dissolve pyromorphite, transforming it to Pb-oxalate, but only when pyromorphite was the sole source of P (66). Debela, *et al*. (2010) in a year-long laboratory batch dissolution experiment with LMWOA showed no changes to the morphological and surficial properties of added pyromorphite crystals (65). Uptake of P and Pb from pyromorphite by rye grass (*Lolium perenne*) was also shown only in cases where pyromorphite was the sole source of P (66). The dissolution of pyromorphite by root activity was significantly lower compared to other sources of P such as apatite, demonstrating the relative difficulty in extracting phosphorus from pyromorphite (66). With a slow and constant alternative provision of P through amendments such as spent grain and superphosphate, the preferential dissolution of pyromorphite would hence be unlikely.

While in microcosm experiments the P source appears to have remained insoluble despite the addition of amendments, if combined with sowing of a grass and wildflower mixture, P from the spent grain amended and/or from a traditional P amendment like superphosphate, could be solubilized in the developing rhizosphere, and become available for precipitation with Pb as pyromorphite.

Conditions in the rhizosphere may also be more suitable for bioaugmentation with microorganisms such as *P.javanicus*, providing a more controlled environment where both growth and the biomineralization of pyromorphite is favoured, producing positive effects in the reduction of both human and environmental bioavailability of Pb.

The production of oxalic acid by *P.javanicus* in liquid cultures appears to have been of relevance in the immobilisation of Pb as pyromorphite. Pb-oxalate was the main mineral form present in fungal hyphae at 10 days of cultivation and was later replaced by pyromorphite in pseudomorphism. In soil microcosms the capture of Pb through the excretion of oxalic acid may not have occurred, but as oxalic acid is a well-known product of rhizosphere inhabiting organisms (67), the growth of plants in amended soil may aid in

achieving this initial immobilization of Pb as oxalate. The enhanced P solubilisation in the rhizosphere could then allow the later transformation of Pb-oxalate to biomineralized pyromorphite by *P.javanicus*.

### 9.4 Considerations for sites resembling the Old Glasgow Meat market

The Meat market site, as is the case for many other urban brownfield sites, did not possess an active or historical critical spot source of contamination (e.g fuelling stations, oil or metal manufacturers, shooting ranges, mine, etc. (223)). Rather, it consisted in heterogeneous made ground, with poor soil quality and discrete, moderate contamination. Pb contamination was only detected in high concentrations, notably higher than the Glasgow urban background, in one out of four of our sample collection points and 16.7 % of samples from the preliminary chemical data, provided by Glasgow City Council. PAHs, though locally identified in the preliminary chemical data (11.1% of samples showed B(a)p above the Category 4 Screening Level for residential use with home-grown produce of 5 mg $kg^{-1}$(9)) were not detectable in the samples collected for this project.

As such, the risk for human receptors was not high, and simple non-invasive approaches may be sufficient to control such risks.

The ultimate objective of a remediation process is not only to remove/immobilise pollutants from the soil but, most importantly, to sustain soil health, i.e. the continued capacity of soil to function as a vital living system, sustaining biological productivity, promoting the quality of air and water, and maintaining plant, animal, and human health (213, 223, 224). By taking all these factors in consideration, contaminated soils are no longer treated as waste to be dealt with, but as a valuable resource to be cleaned and reused (223).

Furthermore, in the context of brownfield sites, even if nature-based remediation approaches do not completely resolve all pollution issues, they may still be acceptable best practice if there is no evidence to suggest exacerbation of risk (27). Because they benefit soil health in the long term, while providing green infrastructure and ecosystem services (20), which have added socio-economic benefits.

The re-use of agroindustrial wastes also enhances the sustainability of the soil restoration process, via the implementation of a circular-economy (20).

For this reason, the use of the approach proposed in this PhD project, namely the amendment of soil with superphosphate and spent grain, and bioaugmentation with potentially beneficial microorganisms such as *P.javanicus*, combined with the establishment of native grass and wildflower meadows, is recommended.

## 9.5 Summary of key points

- The ability of *P.javanicus* to biomineralize pyromorphite in a liquid culture spiked with Pb and an organic P source was corroborated.
- *P.javanicus* was tolerant to the presence of selected PAHs in the growth medium, being able to grow in the presence of both B(a)p and Phen.
- Sorption by fungal biomass appeared to be the main process responsible for the removal of Phen from solution in spiked *P.javanicus* liquid cultures.
- Samples collected from the Old Glasgow Meat market had concentration of Pb above selected reference guideline values in one out of 4 sampling points and no detectable PAH concentrations.
- Brewery spent grain proved to be a good matrix for use as a carrier for the inoculation of microorganism in a soil matrix.
- The ability of *P.javanicus* to biomineralize pyromorphite was not easily transferred to a soil matrix, and the limitations of P amendments in soils with neutral to alkaline pH were not overcome with bioaugmentation in microcosm experiments.
- No significant changes in Pb bioaccessibility were caused by the amendment of Pb-spiked soils with spent grain, superphosphate or *P.javanicus* inoculum.
- The desired increase in the BCR-residual Pb fraction was not achieved by amending Pb-spiked soils with spent grain, superphosphate or *P.javanicus* inoculum.
- The inoculation of *P.javanicus* in Phen and B(a)p spiked soil did not appear to have an effect on their concentration overtime.
- The amendment of Phen and B(a)p spiked soil with sterile or non-sterile spent grain produced a significant decrease in B(a)p concentration after 150 days of incubation.
- The amendment of poor quality urban soil with brewery spent grain, non-sterile and as a *P.javanicus* inoculum carrier, significantly increased above and below ground biomass of a native grass and wildflower mixture.
- A combination of the proposed amendments with the growth of native grass and wildflower species is recommended for future studies on the co-remediation of Pb and PAHs in urban brownfield soils.

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# Appendices

## CH3 A1 Examples of recovery and absolute Phen calculations

Example of Phen concentration adjustment with the surrogate recovery, using sample 10 day1-1 as an example.

- FPhen: final Phen concentration (mg $kg^{-1}$) in a sample 10 day1-1.
- ES: expected surrogate (d-10 Anthracene in this case) concentration = 260.87 µg $ml^{-1}$
- Sx: obtained surrogate (d-10 Anthracene in this case) concentration = 185.44 µg $ml^{-1}$
- Phen x: obtained Phen concentration = 269.86 mg $kg^{-1}$

$$\text{FPhen} = \frac{260.87\ \mu g\ ml^{-1}}{185.44\ \mu g\ ml^{-1}} * 269.86\ mg\ kg^{-1} = 379.63\ mg\ kg^{-1}$$

Example of total Phen content (µg) in harvested fungal biomass, using culture 10 days-1 and sample 10 day1-1 as an example.

- APhen: total Phen content (µg) in harvested fungal biomass from a culture 10 days-1, represented by sample 10 day1-1
- FPhen: final Phen concentration in sample 10 day1-1 = 379.63 mg $kg^{-1}$ (or µg $g^{-1}$)
- Wx: total fungal biomass dry weight (g) harvested from culture 10 days-1 = 0.1684 g

$$\text{APhen} = 379.63\ \mu g\ g^{-1} * 0.1684\ g = 63.93\ \mu g$$

## CH4 A1 Validation of pseudo-total metal aqua regia digestion of soil

A certified reference material (Sandy Clay 1 CRM049 Lot 010248 Sigma-Aldrich, Laramie, Wyoming 82070, USA) was digested with both the USEPA 3050B method (Acid digestion of sediments, sludges and soils) and a simplified in-house aqua regia digestion method.

Both methods were carried out with the use of DigiTUBEs® and a DigiPREP Jr.® digestion block (SCP Science, Quebec H9X 4B6 Canada).

**In house aqua regia digestion method**

~1.0 g of the CRM was weighed in a DigiTUBE®. 10 ml of aqua regia (1:1 HCl 37%: HNO3 69%, both ARISTAR®, VWR chemicals, Leuven, Belgium) was added and the tube placed in the digestion block at 95°C with watch glasses on, for 4 hours. Watch glasses were then removed, and the solution allowed to reduce to ~2.0 ml. The remaining solution was transferred by filtering (Whatman Nº42 filter paper) to 100 ml volumetric flasks. To make sure transference was complete, 5% $HNO_3$ was added repeatedly to the tubes, and filtered into the volumetric flask, until the volume was made up to a 100 ml. Solutions were then transferred into Nalgene® plastic bottles for storage.

**USEPA method 3050B**

~1.0 g of the CRM was weighed in a DigiTUBE®. 10 mL of 1:1 $HNO_3$ was added to the tube, subsequently covered with a watch glass. Tubes were placed in the digestion block at 95°C and refluxed for 15 minutes. After the tubes were cooled to room temperature, 5 mL of concentrated $HNO_3$ (69%) was added, tubes re-covered with watch glasses and refluxed for 30 min at 95°C. The last step was repeated until no brown fumes were generated. Covered watch glasses tubes were refluxed at 95°C for two hours. Maintain a covering of solution over the bottom of the vessel at all times. After tubes had cooled to room temperature, 2 ml of Milli-Q water and 3 ml of 30% $H_2O_2$ was added. Covered with watch glasses, tubes were placed in the digestion block and heated in increments of 10°C from 40°C to 80°C, until effervescence subsided, after which tubes were allowed to cool. This step was repeated 8 more times with 1 ml aliquots of 30% $H_2O_2$. After the las $H_2O_2$ addition tubes were heated with watch glasses on at 95°C for two hours. 10 ml of HCl (37%) was added, samples refluxed at 95°C for 15 minutes, and the solution volume allowed to reduce to ~ 5 ml. The remaining solution was transferred by filtering (Whatman Nº42 filter paper) to 100 ml volumetric flasks. To make sure transference was complete, 5% HNO3 was added repeatedly to the tubes, and filtered into the volumetric flask, until the volume was made up to a 100 ml. Solutions were then transferred into Nalgene® plastic bottles for storage.

## Validation

Two validation batches were extracted on separate occasions. The first batch consisted of five ~1.0 g replicates of the CRM which were digested using the USEPA 3050B method and a single ~1.0g replicate of the CRM which was simultaneously digested with the in-house aqua regia digestion method. The second batch consisted of five replicates of the CRM digested with the in-house aqua regia method at later date.

Batch 1 extracts were analysed with ICP-OES (Perkin Elmer 5300DV ICP-OES) at the Scottish Universities Environmental Research Centre (SUERC, East Kilbride G75 0QF) whereas Batch 2 extracts were analysed with ICP-OES at an external laboratory (Concept Life Science laboratory, G75 0YF, East Kilbride), due to malfunction of the in-house machine.

## Results

A summary of the results is shown in Table 1.

*Table 1: Summary of results of the digestion of a CRM with USEPA 3050B method compared to in-house aqua regia digestion.*

| Element (wavelength in nm for SUERC analysis) | USEPA 3050B | | Aqua regia digestion | | | Certified concentrations | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | SUERC analysis (mg kg$^{-1}$) | External analysis | | CI (mg kg$^{-1}$) | | PI (mg kg$^{-1}$) | |
| | Average (mg kg$^{-1}$) | error (mg kg$^{-1}$) | | Average (mg kg$^{-1}$) | error (mg kg$^{-1}$) | min | max | min | max |
| **Al (396.153)** | 634.0 | *19.88* | 602.9 | | | 537.0 | 584.0 | 394.0 | 726.0 |
| **Ba (233.527)** | 13.01 | *0.196* | 13.45 | | | 12.40 | 13.00 | 10.60 | 14.80 |
| **Ca (317.933)** | 4241 | *81.77* | 4380 | | | 4680 | 4910 | 4020 | 5570 |
| **Cd (228.802)** | 78.66 | *1.393* | 81.75 | 91.03 | *0.990* | 78.90 | 81.00 | 71.50 | 88.40 |
| **Co (228.616)** | 78.08 | *1.194* | 80.16 | 91.23 | *1.002* | 82.80 | 85.20 | 75.30 | 92.70 |
| **Cr (267.716)** | 342.1 | *5.091* | 349.3 | 392.2 | *4.614* | 350.0 | 360.0 | 314.0 | 396.0 |
| **Cu (327.393)** | 83.49 | *1.142* | 85.32 | 96.82 | *1.032* | 87.10 | 89.80 | 77.80 | 99.10 |
| **Fe (238.204)** | 8443 | *124.3* | 8659 | 8085 | *577.0* | 8950 | 9380 | 7630 | 10700 |
| **K (766.490)** | 2745 | *38.11* | 2853 | | | 2960 | 3080 | 2600 | 3440 |
| **Mg (285.213)** | 832.5 | *12.05* | 842.8 | | | 881.0 | 916.0 | 777.0 | 1020 |
| **Mn (257.610)** | 603.8 | *10.96* | 621.1 | | | 625.0 | 646.0 | 561.0 | 710.0 |
| **Na (589.592)** | 456.8 | *8.141* | 460.8 | | | 645.0 | 685.0 | 536.0 | 795.0 |
| **Ni (231.604)** | 325.6 | *3.901* | 332.9 | 388.0 | *4.044* | 339.0 | 349.0 | 304.0 | 383.0 |
| **Pb (220.353)** | 109.4 | *1.451* | 111.4 | 121.1 | *1.100* | 109.0 | 112.0 | 97.50 | 124.0 |
| **P (178.221)** | 709.2 | *16.54* | 8.820 | | | | | | |
| **Sn (235.485)** | 2912 | *43.02* | 3039 | | | 230.0 | 241.0 | 203.0 | 269.0 |
| **Sr (460.733)** | 9.092 | *0.126* | 9.387 | | | 8.380 | 8.870 | 7.280 | 9.970 |
| **Zn (206.200)** | 576.4 | *40.23* | 536.4 | 646.5 | *6.468* | 534.0 | 549.0 | 485.0 | 599.0 |

**Column two shows average concentration per element in replicates digested with USEPA method 3050B (Batch 1). Column four shows concentrations in the single replicate digested with aqua regia, simultaneously to those in column two (Batch 1). Column five shows average concentrations in the further 5 CRM replicates digested with the in-house aqua regia method (Batch2). Certified confidence interval (CI) and prediction interval (PI) for the CRM are shown in the last four columns. Concentrations that fall within the CI are highlighted with blue. Concentrations that fall within the PI are highlighted with light orange when below the CI and with bright orange when above the CI. Concentration that are lower than the PI are highlighted in grey and those higher than the PI in yellow. The element column shows the wavelength in nm used in the ICP-OES analysis of Batch1 performed at SUERC.*

With both methodologies, the majority of elements fell within the prediction interval, though concentrations tended to be higher in the in-house aqua regia digested replicates.

The average Pb concentration fell within the confidence interval for replicates digested with the USEPA 3050B method, as did the value for the initial in-house aqua regia digested replicate (Batch 1). The average Pb concentrations for replicates from Batch 2, digested with the in-house aqua regia method, were at the higher end of the prediction interval.

With recoveries being similar using both methods and the in-house aqua regia digestion method being significantly more time efficient than USEPA 3050B method, the in-house aqua regia digestion was selected as the method for determining pseudo-total metal concentrations in soil samples, for the remainder of the project.

## CH4 A2 Comparison of ashed versus air dried soil for the aqua regia digestion of pseudo-total metal concentrations.

In order to assess the effect of the inclusion of an ashing step previous to the aqua regia digestion of samples, the following procedure was carried out using a certified reference material (Sandy Clay 1 CRM049 Lot 010248 Sigma-Aldrich, Laramie, Wyoming 82070, USA).

A sample of ~ 3.0 g of the CRM was accurately weighed in a crucible. After recording the weight, the crucible was placed in an oven at 105°C for 8 hours. The crucible was removed from the oven, and once at room temperature, its weight recorded. The crucible was then placed in a muffle furnace for ashing at 450°C for 8 hours, removed and the weight again recorded once at room temperature.

Air dried (AD), oven dried (OD) and dry ash (DA) sample weight was calculated by subtracting the previously recorded empty crucible weight to the initial "empty crucible + sample" weight; after 8 hrs at 105 °C "empty crucible + sample" weight; and after 8 hrs at 450 °C "empty crucible + sample" weight, respectively (Table 1).

*Table 1: Weights recorded for air dried, oven dried and ashed CRM.*

| AD weight (g) | OD weight (g) | DA weight (g) | Moisture (%) | LOI (%) |
|---|---|---|---|---|
| 3.0206 | 2.9305 | 2.8704 | 2.983 | 1.990 |

Two ~ 1.0 g replicates of the ashed CRM and two ~ 1.0 g replicates of the CRM direct from the storage container were digested with aqua regia as described in section 4.3.2.3.1 and analysed with ICP-OES as described in section 4.3.2.3.2.

Final element concentrations in the ashed replicates were corrected with the initial and final (ashed) sample weights as follows:

$$\mathrm{Fxy} = \mathrm{Dxy} * \frac{\mathrm{DA}}{\mathrm{AD}}$$

With:

- Fxy: final concentration in mg $kg^{-1}$ of an element x in sample replicate y.
- Dxy: direct concentration in mg $kg^{-1}$ obtained from the ICP-OES analysis of an element x in sample replicate y
- AD: initial weight of a sample in g. Air dried (AD) weight for a soil sample and direct from the storage container for a CRM.

- DA: dry ash weight in g of the same sample, after 8 hours at 450°C in a muffle furnace.

Example using Pb concentration in replicate Dry ash 1

- Dxy: 115.6 mg $kg^{-1}$ of Pb
- AD (initial weight of CRM): 3.0206 g
- DA (weight of CRM after 8 hrs at 450°C in the muffle furnace): 2.8704

$$\text{Fxy} = 115.6\ \text{mg kg}^{-1} * \frac{2.8704\ \text{g}}{3.0206\ \text{g}} = 109.9\ \text{mg kg}^{-1}$$

So the final concentration of Pb in replicate Dry ash 1 is 109.9 mg $kg^{-1}$.

**Results**

Resulting element concentration (after correction in ashed replicates) are shown in Table 2.

*Table 2: Element concentrations of ashed CRM replicates (Dry ash 1 and Dry ash 2) and CRM replicates direct from the storage container (Air dried 1 and Air dried 2).*

| | CRM Sandy Clay 2010 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Dry ash 1 (mg $kg^{-1}$) | Dry ash 2 (mg $kg^{-1}$) | Air dried 1 (mg $kg^{-1}$) | Air dried 2 (mg $kg^{-1}$) | CI (mg $kg^{-1}$) | | PI (mg $kg^{-1}$) | |
| **Al** | 921.2 | 943.3 | 711.1 | 725.2 | 537.0 | 584.0 | 394.0 | 726.0 |
| **Ba** | 12.30 | 12.47 | 12.18 | 12.19 | 12.40 | 13.00 | 10.60 | 14.80 |
| **Be** | 55.95 | 56.51 | 55.37 | 56.45 | 59.40 | 61.50 | 52.70 | 68.30 |
| **Ca** | 4234 | 4275 | 4252 | 4284 | 4680 | 4910 | 4020 | 5570 |
| **Cd** | 71.91 | 72.72 | 72.11 | 72.84 | 78.90 | 81.00 | 71.50 | 88.40 |
| **Co** | 74.34 | 75.55 | 74.85 | 75.46 | 82.80 | 85.20 | 75.30 | 92.70 |
| **Cr** | 323.5 | 324.5 | 322.0 | 328.8 | 350.0 | 360.0 | 314.0 | 396.0 |
| **Cu** | 77.42 | 77.84 | 77.29 | 78.58 | 87.10 | 89.80 | 77.80 | 99.10 |
| **Fe** | 8162 | 8186 | 8138.3 | 8218 | 8950 | 9380 | 7630 | 10700 |
| **K** | 2949 | 2979 | 2930 | 2977 | 2960 | 3080 | 2600 | 3440 |
| **Mg** | 811.3 | 817.1 | 799.2 | 815.2 | 881.0 | 916.0 | 777.0 | 1020 |
| **Mn** | 578.6 | 585.8 | 570.8 | 582.0 | 625.0 | 646.0 | 561.0 | 710.0 |
| **Ni** | 314.7 | 321.2 | 317.0 | 319.7 | 339.0 | 349.0 | 304.0 | 383.0 |
| **Pb** | 109.9 | 112.9 | 114.0 | 112.3 | 109.0 | 112.0 | 97.50 | 124.0 |
| **Ti** | 43.74 | 44.26 | 42.61 | 43.60 | 46.10 | 48.00 | 41.80 | 52.40 |
| **Zn** | 485.4 | 489.3 | 486.8 | 491.1 | 534.0 | 549.0 | 485.0 | 599.0 |

**Certified confidence interval (CI) and prediction interval (PI) for the CRM are shown in the last four columns. Concentrations that fall within the CI are highlighted with blue. Concentrations that fall within the PI are highlighted with light orange when below the CI and with bright orange when above the CI. Concentration that are lower than the PI are highlighted in grey and those higher than the PI in yellow.*

Concentrations were very similar for ashed and non-ashed CRM replicates, falling within the prediction interval in most elements. The only element that showed a substantial difference was Al, which had higher concentrations in ashed replicates, by approximately

200 mg $kg^{-1}$, well above the prediction interval, while non-ashed replicates were at the upper end of the prediction interval.

As the addition of an ashing step did not significantly improve the recovery of pseudo-total metal concentrations by digestion with aqua regia, it was not included when obtaining pseudo-total metal concentrations for the remainder of the project.

## CH4 A3 Examples of soil Moisture and LOI calculation

Soil moisture calculation using replicate FSCP14(0.7m).1-4D.1 as an example:

- AD: air dried sample weight = 5.0124 g
- OD: oven dried sample weight = 4.8961 g

$$\mathrm{M\%} = \frac{(5.0124\ \mathrm{g} - 4.8961\ \mathrm{g}) * 100}{5.0124\ \mathrm{g}} = 2.320\ \%$$

Loss on ignition (LOI) calculation using replicate FSCP14(0.7m).1-4D.1 as an example:

- AD: air dried sample weight = 5.0124 g
- OD: oven dried sample weight = 4.8961 g
- DA: dry ash sample weight = 4.6319 g

$$\mathrm{LOI} = \frac{(4.8961\ \mathrm{g} - 4.6319\ \mathrm{g}) * 100}{5.0124\ \mathrm{g}} = 5.271\ \%$$

## CH5 A1 Spent grain calculation examples

**1.** Example of calculation of a spent grain sorbed element (SR) using Cu in microcosm P.jav1:

- ASGxn: average concentration of Cu in the amendment spent grain of microcosm P.jav1 = 74.67 mg $kg^{-1}$
- FSGx: average concentration of Cu in the fresh spent grain = 6.985 mg $kg^{-1}$
- 0.01375: theoretical total weight in kg of amendment spent grain in microcosm P.jav1
- 0.055: theoretical total weight in kg of soil in microcosm P.jav1
- SR: sorbed Cu in spent grain of microcosm P.jav1

$$\mathrm{SR} = \frac{(74.67\ \mathrm{mg\ kg^{-1}} - 6.985\ \mathrm{mg\ kg^{-1}}) * 0.01375\ \mathrm{kg}}{0.055\ \mathrm{kg}} = 16.92\ \mathrm{mg\ kg^{-1}soil}$$

That is, in microcosm P.jav1 16.92 mg of Cu per kg of soil were sorbed by the amendment spent grain.

In percentage (% of SG+S)

- Sbxn: concentration of Cu sorbed in amendment spent grain from microcosm P.jav1 = 16.92 mg $kg^{-1}$
- Xn: average concentration of Cu in the soil of microcosm P.jav1 = 119.8 mg $kg^{-1}$
- % of SG+S: percentage of sorbed Cu in P.jav1

$$\%\ \mathrm{of\ SG+S} = \frac{16.92\ \mathrm{mg\ kg^{-1}} * 100}{16.92\ \mathrm{mg\ kg^{-1}} + 119.8\ \mathrm{mg\ kg^{-1}}} = \frac{16.92\ \mathrm{mg\ kg^{-1}} * 100}{136.7\ \mathrm{mg\ kg^{-1}}} = 12.38\ \%$$

That is, from a theoretical initial concentration of 136.7 mg $kg^{-1}$ Cu in P.jav1 soil, 12.38% was sorbed by the amendment spent grain, leaving 119.8 mg $kg^{-1}$.

**2.** Example of calculation of a spent grain released element (SR) using P in microcosm P.jav1:

- ASGxn: average concentration of P in the amendment spent grain of microcosm P.jav1 = 2444 mg $kg^{-1}$
- FSGx: average concentration of P in the fresh spent grain = 4551 mg $kg^{-1}$
- 0.01375: theoretical total weight in kg of amendment spent grain in microcosm P.jav1
- 0.055: theoretical total weight in kg of soil in microcosm P.jav1

- SR: sorbed P in spent grain of microcosm P.jav1

$$\mathrm{SR} = \frac{(2444\ \mathrm{mg\ kg^{-1}} - 4551\ \mathrm{mg\ kg^{-1}}) * 0.01375\ \mathrm{kg}}{0.055\ \mathrm{kg}} = -526.8\ \mathrm{mg\ kg^{-1}soil}$$

That is, in microcosm P.jav1 526.8 mg of P per kg of soil were released by the amendment spent grain.

In percentage (% of SG+S)

- Rlxn: concentration of P released by amendment spent grain from microcosm P.jav1 = 526.8 mg $kg^{-1}$
- Xn: average concentration of P in the soil of microcosm P.jav1 = 935.7 mg $kg^{-1}$
- % of SG+S: percentage of released P in P.jav1

$$\%\ \mathrm{of\ SG+S} = \frac{-526.8\ \mathrm{mg\ kg^{-1}} * 100}{935.7\ \mathrm{mg\ kg^{-1}}} = -56.30\ \%$$

That is, from the final soil concentration of 935.7 mg $kg^{-1}$ Cu in P.jav1 soil, 56.30% was released by the amendment spent grain, leaving 119.8 mg $kg^{-1}$. This would imply an initial theoretical soil concentration of:

$$935.7\mathrm{mg\ kg^{-1}} - 526.8\ \mathrm{mg\ kg^{-1}} = 408.9\ \mathrm{mg\ kg^{-1}\ P}$$

## CH5 A2 Pseudo-total metal concentrations in preliminary soil experiment soils

Aqua regia digestion was carried out in ~1.0 g duplicates as described in section 4.3.2.3.1. ICP-OES analysis was performed externally by Concept Life Science laboratory (G75 0YF, East Kilbride). Results are shown in the following table. Recoveries in the CRM digested alongside samples are shown in section 4.4.4.

| | Control (mg kg$^{-1}$) | | | | P.jav1 (mg kg$^{-1}$) | | | | P.jav2 (mg kg$^{-1}$) | | | | P.jav3 (mg kg$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | Average | SD | 1 | 2 | Average | SD | 1 | 2 | Average | SD | 1 | 2 | Average | SD |
| **Al** | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | |
| **Ba** | 218.7 | 228.8 | 223.8 | *5.017* | 232.8 | 190.1 | 211.4 | *21.35* | 212.3 | 217.6 | 214.9 | *2.672* | 223.9 | 208.3 | 216.1 | *7.773* |
| **Be** | 1.699 | 1.669 | 1.684 | *0.015* | 1.599 | 1.596 | 1.598 | *0.001* | 1.800 | 1.580 | 1.690 | *0.110* | 1.779 | 1.898 | 1.839 | *0.060* |
| **Ca** | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | |
| **Cd** | 0.400 | 0.491 | 0.445 | *0.046* | 0.500 | 0.399 | 0.449 | *0.050* | 0.500 | 0.494 | 0.497 | *0.003* | 0.395 | 0.600 | 0.497 | *0.102* |
| **Co** | 14.60 | 15.91 | 15.25 | *0.655* | 14.19 | 13.17 | 13.68 | *0.508* | 16.30 | 13.53 | 14.91 | *1.382* | 15.22 | 15.39 | 15.30 | *0.083* |
| **Cr** | 35.19 | 35.15 | 35.17 | *0.020* | 32.77 | 30.83 | 31.80 | *0.969* | 40.29 | 31.81 | 36.05 | *4.243* | 34.89 | 36.27 | 35.58 | *0.690* |
| **Cu** | 130.0 | 127.6 | 128.8 | *1.161* | 129.9 | 109.8 | 119.8 | *10.062* | 130.0 | 108.7 | 119.3 | *10.661* | 138.4 | 499.6 | 319.0 | *180.6* |
| **Fe** | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | | > LOD | > LOD | > LOD | |
| **K** | 1705 | 1722 | 1713 | *8.231* | 1498 | 1677 | 1587 | *89.83* | 1401 | 1491 | 1446 | *44.95* | 1590 | 1502 | 1546 | *43.80* |
| **Mg** | 3776 | 3942 | 3859 | *82.90* | 4065 | 4155 | 4110 | *44.76* | 3955 | 4228 | 4091 | *136.5* | 3960 | 4274 | 4117 | *157.0* |
| **Mn** | 1015 | 1168 | 1091 | *76.65* | 900.1 | 821.7 | 860.9 | *39.20* | 1214 | 886 | 1050 | *163.6* | 862.5 | 1130 | 996.2 | *133.7* |
| **Ni** | 44.99 | 54.59 | 49.79 | *4.802* | 42.76 | 39.81 | 41.29 | *1.475* | 46.39 | 40.00 | 43.20 | *3.193* | 45.76 | 48.56 | 47.16 | *1.398* |
| **P** | 877.5 | 880.5 | 879.0 | *1.487* | 910.5 | 961.0 | 935.7 | *25.25* | 827.9 | 776.4 | 802.2 | *25.78* | 1115 | 1027 | 1071 | *44.33* |
| **Pb** | 333.2 | 334.4 | 333.8 | *0.607* | 384.2 | 311.8 | 348.0 | *36.17* | 372.3 | 341.9 | 357.1 | *15.23* | 461.9 | 378.3 | 420.1 | *41.80* |
| **Ti** | 454.5 | 408.2 | 431.4 | *23.11* | 509.2 | 509.9 | 509.6 | *0.318* | 504.6 | 507.8 | 506.2 | *1.602* | 452.5 | 414.0 | 433.2 | *19.27* |
| **Zn** | 240.5 | 231.9 | 236.2 | *4.309* | 223.6 | 207.6 | 215.6 | *7.978* | 237.1 | 220.3 | 228.7 | *8.392* | 276.6 | 255.8 | 266.2 | *10.38* |

## CH5 A3 Spent grain: sorbed/released concentrations per element compared to soil concentrations

*Table 1. Summary table with sorbed/released concentrations per element compared to soil concentrations. Sorbed/released percentages are also included.*

| | Pjav1 | | | | | P.jav3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sorbed (+)/released (-) by spent grain | | | Remaining in soil | | Sorbed (+)/released (-) by spent grain | | | Remaining in soil | |
| | mg kg$^{-1}$ soil | SD | % of SG+S | mg kg$^{-1}$ soil | SD | mg kg$^{-1}$ soil | SD | % of SG+S | mg kg$^{-1}$ soil | SD |
| **Cd** | -0.052 | *0.318* | -11.50 | 0.449 | *0.050* | -0.080 | *0.382* | -16.00 | 0.497 | *0.102* |
| **Co** | 1.016 | *0.297* | 6.900 | 13.68 | *0.508* | 0.840 | *0.102* | 5.200 | 15.31 | *0.083* |
| **Cr** | 2.754 | *0.238* | 8.000 | 31.80 | *0.969* | 2.962 | *0.154* | 7.700 | 35.58 | *0.690* |
| **Cu** | 16.92 | *0.229* | 12.40 | 119.8 | *10.06* | 16.74 | *0.915* | 5.000 | 319.0 | *180.6* |
| **Fe** | 2854 | *145.8* | | | | 2685 | *201.3* | | | |
| **Mg** | 37.59 | *22.99* | 0.900 | 4110 | *44.76* | 13.50 | *55.33* | 0.300 | 4117 | *157.0* |
| **Mn** | 101.3 | *7.740* | 10.50 | 860.9 | *39.20* | 134.6 | *19.23* | 11.90 | 996.2 | *133.7* |
| **Ni** | 5.742 | *0.360* | 12.20 | 41.29 | *1.475* | 5.801 | *0.889* | 11.00 | 47.16 | *1.398* |
| **P** | -526.8 | *56.09* | -56.30 | 935.7 | *25.25* | -567.2 | *105.7* | -53.00 | 1071 | *44.33* |
| **Pb** | 55.47 | *1.375* | 13.70 | 348.0 | *36.17* | 57.02 | *1.436* | 12.00 | 420.1 | *41.80* |
| **Ti** | 18.31 | *2.455* | 3.500 | 509.6 | *0.318* | 20.44 | *2.321* | 4.500 | 433.2 | *19.27* |
| **Zn** | 25.92 | *3.006* | 10.70 | 215.6 | *7.978* | 30.11 | *2.019* | 10.20 | 266.2 | *10.38* |
| | **P.jav2** | | | | | **Control** | | | | |
| | Sorbed (+)/released (-) by spent grain | | | Remaining in soil | | Sorbed (+)/released (-) by spent grain | | | Remaining in soil | |
| | mg kg$^{-1}$ soil | SD | % of SG+S | mg kg$^{-1}$ soil | SD | mg kg$^{-1}$ soil | SD | % of SG+S | mg kg$^{-1}$ soil | SD |
| **Cd** | -0.003 | *0.481* | -0.700 | 0.497 | *0.003* | 0.100 | *0.236* | 18.30 | 0.445 | *0.046* |
| **Co** | 0.489 | *0.312* | 3.200 | 14.91 | *1.382* | 1.387 | *0.180* | 8.300 | 15.25 | *0.655* |
| **Cr** | 3.169 | *0.472* | 8.100 | 36.05 | *4.243* | 3.498 | *0.120* | 9.000 | 35.17 | *0.020* |
| **Cu** | 24.48 | *0.786* | 17.00 | 119.3 | *10.66* | 26.47 | *0.333* | 17.00 | 128.8 | *1.161* |
| **Fe** | 1991 | *156.5* | 6.500 | 28817 | *2725* | 2261 | *133.9* | 7.700 | 27211 | *5646* |
| **Mg** | 38.66 | *40.05* | 0.900 | 4091 | *136.5* | 72.84 | *28.02* | 1.900 | 3859 | *82.90* |
| **Mn** | 73.37 | *3.093* | 6.500 | 1050 | *163.6* | 91.30 | *2.872* | 7.700 | 1091 | *76.65* |
| **Ni** | 4.756 | *0.887* | 9.900 | 43.20 | *3.193* | 5.187 | *0.447* | 9.400 | 49.79 | *4.802* |
| **P** | -449.1 | *54.41* | -56.00 | 802.2 | *25.78* | -715.9 | *40.36* | -81.40 | 879.0 | *1.487* |
| **Pb** | 34.73 | *2.222* | 8.900 | 357.1 | *15.23* | 148.8 | *2.897* | 30.80 | 333.8 | *0.607* |
| **Ti** | 8.228 | *1.361* | 1.600 | 506.2 | *1.602* | 29.84 | *6.651* | 6.500 | 431.4 | *23.11* |
| **Zn** | 21.04 | *1.637* | 8.400 | 228.7 | *8.392* | 33.12 | *1.084* | 12.30 | 236.2 | *4.309* |

**Control = MMS+SG; P.jav1 and P.jav3 = MMS+SG+Pj; P.jav2 = SMMS+SSG+Pj.*

## CH6 A1 Example of %BCR calculation

Example of %BCR calculation using Pb in BCR4 of replicate T0c1, with:

- BCR1 = 18.45 mg $kg^{-1}$ Pb
- BCR2 = 557.1 mg $kg^{-1}$ Pb
- BCR3 = 484.0 mg $kg^{-1}$ Pb
- BCR4 = 216.2 mg $kg^{-1}$ Pb
- ∑BCR = 18.45 + 557.1 + 484.0 + 216.2 = 1276 mg $kg^{-1}$

$$\%\mathrm{BCR4} = \frac{216.2\ \mathrm{mg\ kg^{-1}} * 100}{1276\ \mathrm{mg\ kg^{-1}}} = 16.95\ \%\ \mathrm{Pb}$$

## CH6 A2 Example of P-Pb molar ratio calculation

Calculation P/Pb molar ratio using sample T0c1 in the BCR1 extract as an example:

- Sample weight: 1.049g = 0.001049 kg
- Pb concentration in BCR1: 18.5 mg $kg^{-1}$ = 0.0185 g $kg^{-1}$
- Pb molar mass: 207.2 g $mol^{-1}$
- P concentration in BCR1: 1842.1 mg $kg^{-1}$ = 1.8421 g $kg^{-1}$
- P molar mass: 31.0 g $mol^{-1}$

$$\text{mol Pb} = \frac{0.0185\ \text{g kg}^{-1} * 0.001049\ \text{kg}}{207.2\ \text{g mol}^{-1}} = 9.366\ \text{E} - 8$$

$$\text{mol P} = \frac{1.8421\text{g kg}^{-1} * 0.001049\ \text{kg}}{31.0\ \text{g mol}^{-1}} = 6.233\ \text{E} - 5$$

$$^{\text{P}}/_{\text{Pb}}\ \text{molar ratio} = \frac{\text{mol P}}{\text{mol Pb}} = \frac{6.233\ \text{E} - 5}{9.366\ \text{E} - 8} = 665.5$$

## CH6 A3 Example of Gc% Ist% and RMIst% calculation

Gc available Pb (%) using sample S1aGc as an example:

- Pb concentration in the Gc phase: 946.1 mg $kg^{-1}$
- Pb concentration in the sample residue: 103.6 mg $kg^{-1}$

$$\text{Gc\%} = \frac{946.1\ \text{mg kg}^{-1} * 100}{946.1\ \text{mg kg}^{-1} + 103.6\ \text{mg kg}^{-1}} = 90.13\%\ \text{Pb}$$

Ist available Pb (%) using sample S1aIst as an example:

- Pb concentration in the Ist phase: 183.5 mg $kg^{-1}$
- Pb concentration in the sample residue: 899.3 mg $kg^{-1}$

$$\text{Ist\%} = \frac{183.5\ \text{mg kg}^{-1} * 100}{183.5\ \text{mg kg}^{-1} + 899.3\ \text{mg kg}^{-1}} = 16.95\%\ \text{Pb}$$

Normalizing Ist% to the reference matrix, using sample S1aIst as an example:

- Pb concentration in the Ist phase: 183.5 mg $kg^{-1}$
- Pb concentration in the sample residue: 899.3 mg $kg^{-1}$
- Average Ist% of the reference matrix (RM Ist%): 41.95 %

$$\text{RM normalized Ist\%} = \frac{183.5\ \text{mg kg}^{-1} * 100}{(183.5\ \text{mg kg}^{-1} + 899.3\ \text{mg kg}^{-1}) * (\frac{41.95\%}{100})} = 40.40\%\ \text{Pb}$$

# CH6 A4 UBM reagent brands

*Table 1. Reagents used for the UBM procedure and their respective brand.*

| **Reagent name** | | **Brand** |
|---|---|---|
| Potassium chloride | KCl | Fisher Scientific, Loughborough, UK |
| Monosodium phosphate | $NaH_2PO_4$ | Sigma-Aldrich, St. Louis, MO, USA |
| Potassium thiocyanate | KSCN | Sigma-Aldrich, St. Louis, MO, USA |
| Sodium sulphate | $Na_2SO_4$ | Fisons Scientific Equipment, Loughborough, UK |
| Sodium chloride | NaCl | Merck KGaA, Darmstadt, Germany |
| Calcium chloride | $CaCl_2*2H_2O$ | Sigma-Aldrich, St. Louis, MO, USA |
| Ammonium chloride | $NH_4Cl$ | Fisher Scientific, Loughborough, UK |
| Sodium bicarbonate | $NaHCO_3$ | Johnson Matthey, Materials Technology, Royston, UK |
| Potassium dihydrogen phosphate | $KH_2PO_4$ | Sigma-Aldrich, St. Louis, MO, USA |
| Magnesium chloride | $MgCl_2*6H_2O$ | VWR chemicals, Leicestershire, UK |
| Sodium hydroxide | NaOH | BDH Laboratory Supplies, Poole, UK |
| Hydrochloric acid | HCl | ARISTAR®, VWR chemicals, Leuven, Belgium |
| Urea | | Merck KGaA, Darmstadt, Germany |
| D-(+)-Glucose | | Sigma-Aldrich, St. Louis, MO, USA |
| D-Glucuronic acid | | Sigma-Aldrich, St. Louis, MO, USA |
| Glucosamine hydrochloride | | Sigma-Aldrich, St. Louis, MO, USA |
| Alpha amylase (from Bacillus sp) | | Sigma-Aldrich, St. Louis, MO, USA |
| Mucin (from porcine stomach) | | Sigma-Aldrich, St. Louis, MO, USA |
| Uric acid | | Sigma-Aldrich, St. Louis, MO, USA |
| Bovine serum albumin (Fraction V) | | Carl Roth GmbH, Karlsruhe, Germany |
| Pepsin (from porcine gastric mucosa) | | Merck KGaA, Darmstadt, Germany |
| Pancreatin (from porcine pancreas) | | Merck KGaA, Darmstadt, Germany |
| Lipase (from porcine pancreas) | | Sigma-Aldrich, St. Louis, MO, USA |
| Bile (bovine) | | Sigma-Aldrich, St. Louis, MO, USA |

## CH6 A5 P concentrations in BCR blanks

Blanks from discarded BCR rounds (due to omission of pH adjustments in BCR2 and BCR3) are shown under the "Discarded BCR" header. The two Blank replicates corresponding to the data shown in section 6.4.1, as well as the average concentration for the round. BCR3 concentrations are highlighted in grey, and consistently show a P background.

| BCR stage | Discarded BCR | | | | | | Corrected BCR | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Round 1 (mg $kg^{-1}$) | | Round 2 (mg $kg^{-1}$) | | Round 3 (mg $kg^{-1}$) | | Round 1 (mg $kg^{-1}$) | | Average (mg $kg^{-1}$) | SD |
| **BCR1** | -0.142 | -0.072 | -0.176 | -0.026 | -0.122 | -0.050 | 0.035 | 0.084 | 0.060 | 0.025 |
| **BCR2** | -0.013 | -0.068 | -0.058 | -0.019 | -0.090 | -0.045 | -0.050 | 0.169 | 0.059 | 0.110 |
| **BCR3** | 8.489 | 4.852 | 9.952 | 8.239 | 14.89 | 14.72 | 15.56 | 14.87 | 15.21 | 0.343 |
| **BCR4** | -0.064 | -0.093 | 0.001 | -0.004 | -0.083 | -0.039 | 0.085 | 0.185 | 0.135 | 0.050 |
| **Total** | 8.270 | 4.619 | 9.719 | 8.190 | 14.59 | 14.59 | 15.63 | 15.31 | 15.47 | 0.159 |

## CH6 A6 Example of adjustment of P concentration in BCR3 extract with the average Blank P background

Example of adjustment of P concentration in BCR3 extract with the average Blank P background, using replicate T0c1.

- Sample weight: 1.049 g
- Final volume of sample: 41 ml ≈ 41g
- P concentration in the BCR3 extract: 649.4 mg $kg^{-1}$
- Average P concentration in BCR3 Blank extracts: 15.21 mg $kg^{-1}$
- aBCR3P: adjusted P concentration in BCR3

$$\mathrm{aBCR3P} = 649.4\ \mathrm{mg\ kg^{-1}} - \left(15.21\ \mathrm{mg\ kg^{-1}} * \frac{41\ \mathrm{g}}{1.049\ \mathrm{g}}\right) = 54.92\ \mathrm{mg\ kg^{-1}}$$

Hence, the final adjusted P concentration in the BCR3 extract of replicate T0c1 is 54.92 mg $kg^{-1}$.

## CH6 A7 Correlations: calculation example

Using "Table 6.7. Correlation coefficients for BCR results of the spiked soil experiment", the following is an example of how correlations were obtained:

All correlations were calculated using the "Correlations" tool in the "Data analysis" package in Microsoft Excel (2016). For this purpose, all parameters to be correlated were ordered in columns, so that the value for each parameter belonging to the same sample or replicate were positioned next to each other in rows, as in the following table:

| | BCR1 Pb | BCR2 Pb | BCR3 Pb | BCR4 Pb | Total Pb | BCR1 P | BCR2 P | BCR3 P | BCR4 P | Total P | BCR1 P/Pb | BCR2 P/Pb | BCR3 P/Pb | BCR4 P/Pb | Total P/Pb | Pj |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T0c1 | 1.446 | 43.67 | 37.94 | 16.95 | 1276 | 56.43 | 28.25 | 1.680 | 13.63 | 3264 | 667.8 | 11.08 | 0.758 | 13.77 | 20.24 | -1 |
| T0c2 | 1.473 | 41.46 | 41.23 | 15.84 | 1323 | 56.71 | 28.02 | 2.049 | 13.23 | 3376 | 657.2 | 11.54 | 0.848 | 14.26 | 20.14 | -1 |
| T0c3 | 1.715 | 42.41 | 41.83 | 14.05 | 1284 | 58.07 | 28.21 | 3.004 | 10.71 | 3600 | 635.2 | 12.48 | 1.347 | 14.30 | 22.01 | -1 |
| S+SP+SG+Pj22a | 1.201 | 38.12 | 44.64 | 16.04 | 1221 | 62.54 | 23.49 | 3.320 | 10.65 | 3904 | 1113 | 13.18 | 1.590 | 14.20 | 24.72 | 1 |
| S+SP+SG+Pj22b | 1.234 | 39.76 | 41.35 | 17.66 | 1182 | 62.74 | 23.74 | 2.206 | 11.32 | 3788 | 1090 | 12.80 | 1.144 | 13.74 | 24.90 | 1 |
| S+SP+SG+Pj23a | 1.925 | 38.87 | 41.67 | 17.53 | 1097 | 62.42 | 24.56 | 1.352 | 11.67 | 3675 | 726.7 | 14.16 | 0.727 | 14.92 | 26.04 | 1 |
| S+SP+SG+Pj23b | 1.655 | 36.26 | 44.72 | 17.36 | 1171 | 63.15 | 23.71 | 1.970 | 11.17 | 4024 | 877.1 | 15.03 | 1.012 | 14.80 | 26.46 | 1 |
| S+SP+SG+Pj24a | 1.678 | 37.29 | 46.40 | 14.63 | 1218 | 64.95 | 23.01 | 2.012 | 10.03 | 4003 | 851.1 | 13.57 | 0.953 | 15.07 | 25.41 | 1 |

In the table, for a given sample (column 1, e.g. T0c1) the following parameters were included next to each other, in their respective columns: the % of Pb corresponding to each BCR stage of the sequential extraction procedure (column 2-BCR1 Pb, column 3-BCR2 Pb, column 4-BCR3 Pb and column 5-BCR4 Pb); the total Pb concentration in mg $kg^{-1}$, resulting from the addition of the concentration in each BCR stage (column 6: Total Pb); the % of P corresponding to each BCR stage of the sequential extraction procedure (column 7-BCR1 P, column 8-BCR2 P, column 9-BCR3 P and column 10-BCR4 P); the total P concentration in mg $kg^{-1}$, resulting from the addition of the concentration in each BCR stage (column 11: Total P); the P/Pb molar ratio corresponding to each BCR stage of the sequential extraction procedure (column 12-BCR1 P/Pb, column 13-BCR2 P/Pb, column 14-BCR3 P/Pb

and column 15-BCR4 P/Pb); the overall P/Pb molar ratio (column 16: Total P/Pb); finally Pj (column 17) corresponds to the presence or absence of soil amendments with *P.javanicus* inoculum, as a qualitative parameter a value of "1" was assigned when the sample belonged to microcosms with soil amendments and *P.javanicus* inoculum (S+SP+SG+Pj22a, S+SP+SG+Pj22b, S+SP+SG+Pj23a, S+SP+SG+Pj23b and S+SP+SG+Pj24a) and a value of "-1" when the samples belonged to microcosms without the latter (T0c1, T0c2 and T0c3).

Once all the data was ordered, columns 2 to 17 were selected and the "Correlations" tool applied. A table with the correlation coefficients for every possible pair of parameters then appeared automatically in a separate sheet:

| | BCR1 Pb | BCR2 Pb | BCR3 Pb | BCR4 Pb | Total Pb | BCR1 P | BCR2 P | BCR3 P | BCR4 P | Total P | BCR1 P/Pb | BCR2 P/Pb | BCR3 P/Pb | BCR4 P/Pb | Total P/Pb | Pj |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BCR1 Pb | 1 | | | | | | | | | | | | | | | |
| BCR2 Pb | -0.120 | 1 | | | | | | | | | | | | | | |
| BCR3 Pb | 0.107 | -0.869 | 1 | | | | | | | | | | | | | |
| BCR4 Pb | -0.162 | -0.182 | -0.320 | 1 | | | | | | | | | | | | |
| Total Pb | -0.342 | 0.643 | -0.292 | -0.588 | 1 | | | | | | | | | | | |
| BCR1 P | 0.086 | -0.907 | 0.793 | 0.160 | -0.742 | 1 | | | | | | | | | | |
| BCR2 P | 0.087 | 0.910 | -0.749 | -0.282 | 0.722 | -0.976 | 1 | | | | | | | | | |
| BCR3 P | -0.493 | 0.002 | 0.317 | -0.533 | 0.375 | -0.002 | -0.020 | 1 | | | | | | | | |
| BCR4 P | -0.129 | 0.663 | -0.831 | 0.386 | 0.389 | -0.781 | 0.686 | -0.474 | 1 | | | | | | | |
| Total P | 0.037 | -0.926 | 0.903 | -0.011 | -0.565 | 0.931 | -0.902 | 0.244 | -0.868 | 1 | | | | | | |
| BCR1 P/Pb | -0.645 | -0.588 | 0.469 | 0.319 | -0.396 | 0.678 | -0.790 | 0.395 | -0.498 | 0.670 | 1 | | | | | |
| BCR2 P/Pb | 0.415 | -0.895 | 0.712 | 0.234 | -0.793 | 0.825 | -0.771 | -0.094 | -0.659 | 0.850 | 0.373 | 1 | | | | |
| BCR3 P/Pb | -0.500 | -0.171 | 0.401 | -0.367 | 0.138 | 0.204 | -0.233 | 0.965 | -0.596 | 0.421 | 0.577 | 0.099 | 1 | | | |
| BCR4 P/Pb | 0.753 | -0.671 | 0.693 | -0.215 | -0.453 | 0.565 | -0.444 | -0.269 | -0.502 | 0.555 | -0.156 | 0.726 | -0.224 | 1 | | |
| Total P/Pb | 0.226 | -0.896 | 0.693 | 0.309 | -0.875 | 0.947 | -0.916 | -0.069 | -0.719 | 0.893 | 0.593 | 0.937 | 0.161 | 0.588 | 1 | |
| Pj | -0.013 | -0.889 | 0.665 | 0.393 | -0.831 | 0.960 | -0.983 | -0.057 | -0.637 | 0.858 | 0.754 | 0.812 | 0.174 | 0.446 | 0.951 | 1 |

The resulting table with correlation coefficients was then studied and formatted as presented in the text.

In cases other than “Table 6.7. Correlation coefficients for BCR results of the spiked soil experiment”, the procedure was carried out analogously, utilising assigned values of “1” (presence) and “-1” (absence) for qualitative parameters. In some cases, when only some of the paired parameters (e.g. coefficient for BCR1 Pb paired with Total Pb) were considered relevant, the correlation coefficients were extracted from the automatically produced table and presented in a more compact table (e.g. Table 6.13. Correlation coefficients for results of the UBM bioaccessibility assay).