Bioremediation of Glyphosate by Lignin-Modifying Fungi *Pleurotus oestreatus*

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Abstract

This study analyzes the potential of the fungi *Pleurotus ostreatus* as a bioremedial solution for glyphosate contamination, and highlights why these practices are of growing importance. The experiment showed *P. ostreatus* was not able to mineralize phosphorus from glyphosate, indicating no degrading abilities. In fact, fungistatic effects of glyphosate were found in the range of 0.01 to 0.04 mg mL^{-1} and fungicidal effects were observed for concentrations 0.6 mg mL^{-1} and higher. Based on literature, degradation potential of alternative organisms are evaluated in regard to glyphosate. The most promising strain for mycoremediating glyphosate was *Aspergillus oryazae*, though bacterial remediation and even phytobial remediation seemed possible as well. Finally, future glyphosate use and bioremediation is contextualized with social and political considerations. This study emphasizes that bioremediation of glyphosate should be considered a priority, however *P. ostreatus* does not seem to be a viable organism for this role.

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Contents

1 Introduction

1.1 Glyphosate Popularity

Glyphosate, an organophosphorus compound, is the main active ingredient in many non-selective, systemic herbicides, gaining popularity in the 1990s with the rise of genetically modified, herbicide resistant crops [Koroleva et al., 2015]. These herbicides are formulated for a wide range of both private and commercial applications, and respectively, usage has increased more than 5 and 14-fold from 1994 to 2014 globally[Benbrook, 2016]. Application on both resistant and non-resistant crops occurs [Tkacz et al., 2006].
Glyphosate is unique in it's ability to disrupt aromatic amino acid production in plants by targeting the enyzme 5-enolpyruvylshikimate-3-phosphate synthase in the shikimate pathway [Zhan et al., 2018]. It acts as an analogue to phosphophenolpyruvate (PEP), binding to EPSP synthase, however this competition is not observed for other PEP reactions [Boocock and Coggins, 1983], likely the reason is not generally considered toxic to mammals.

1.2 Glyphosate Concerns

The most common metabolite from glyphosate degradation is aminomethylphosphonic acid (AMPA), via the pathway shown in figure 1. Degradation for AMPA is slower than for the parent compound and due to documented accumulation, it is considered persistent and resilient to degradation [Grandcoin et al., 2017].
A comprehensive U.S field study spanning 38 states found glyphosate and AMPA in soils, sediments, streams, rivers, ponds, lakes, wetlands, ditches and drains and the groundwater, proposing both urban and agricultural origins of the contaminants [Battaglin et al., 2014]. Furthermore, both compounds have also been detected in air and rain, attributed to spray application techniques and wind erosion [Battaglin et al., 2014, Chang et al., 2011]. Over the years, both laboratory and field studies have observed negative impacts on growth, mobility and reproduction in both terrestrial and aquatic animal and non-target plant species resulting from exposure to glyphosate or glyphosate herbicides [Baker et al., 2014, Helander et al., 2018, Mateos-Naranjo and Perez-Martin, 2013, Hansen and Roslev, 2016, Lopes et al., 2014, Martínez et al., 2018]. Some studies have highlighted how the toxicity of herbicide formulations can be significantly greater than just glyphosate alone [Peixoto, 2005], and genotoxicity of AMPA was found to be comparable to glyphosate in fish [Guilherme et al., 2014]. Glyphosate has been detected in urine samples of both children and adults [Knudsen et al., 2017] and since 2015 glyphosate has been classified as *probably carcinogenic to humans* by the WHO [International Agency for Research on Cancer, 2015], referring to non-Hodgkin lymphoma, specifically. An early 2019 meta study further reported a 45% relative risk increase of non-Hodgkin lymphoma in humans exposed to glyphosate, supported by experimental animal trials [Zhang et al., 2019]. Since August 2018, US juries have ruled on two occasions that a cancer illness in private individuals was caused by the Monsanto glyphosate formulation, RoundUp [Bellon and Schuetze, 2019] and in April 2019, a french court found Monsanto liable for a farmers illness, caused by glyphosate inhalation [Carraud and Lagrange, 2019]. In March 2019, Vietnam announced a ban on glyphosate, to take effect by the summer [Polansek and Vu, 2019], following the example of Indian states, Punjab and Kerala [Special Correspondent, 2018, TNM Staff, 2019]. In December 2017, the European Union renewed the approval of glyphosate for five years [Commission Européenne, 2017].

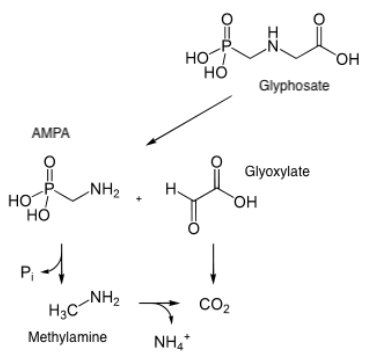

Figure 1: *The main degradation pathway for glyphosate ($C_3H_8NO_5P$), where glyoxylate and AMPA are the initial degradation products. The latter evetually degrades to methylamine and ammonium. (Modified from [Giesy et al., 2000])*

1.3 Mycoremediation for Glyphosate Contamination

Bacteria have been the main subject of exploration for glyphosate remediation so far, producing several metabolites via different degradation pathways. In many cases, the primary metabolite is AMPA and to date, this is the only pathway found in fungi [Zhan et al., 2018, Sviridov et al., 2015, Pizzul et al., 2009] Examples of further degradation to the secondary metabolite methylamine are very limited to just one fungal strain, Aspergillus oryzae, isolated from a pesticide factory [Fu et al., 2017]. In recent years, the value of fungal remediation has become increasingly evident. Researchers and companies have investigated various multi-step detoxification practices of contaminated agricultural soil, including their in-situ potential. Studies have shown the benefits of using matrix-based-technologies inoculated with fungi or other microbes that can produce effective, ecological and low-cost alternatives to common expensive and synthetic decontamination practices [Koroleva et al., 2015]. However, most studies focus on specific pollutants and fail to investigate any real life possibilities for fungal use in bio-remediation. This is largely due to the fact the contaminated soils contain a range of complex chemicals that can often inhibit fungal growth and reduce their enzymatic activity [Loffredo et al., 2014].

Pleurotus ostreatus (oyster mushroom) is a popular edible fungus worldwide and was chosen as the subject of this project due to being readily available and fast growing. It is grouped with white rot fungi, that decay wood with extra-cellular, lignin-modifying enzymes and can cleave cellulose, hemicellulose, lignin and their derived metabolites. [Koroleva et al., 2015, Bellettini et al., 2016, Mohamed and Farghaly, 2014].

Pleurotus spp. are already used commercially in biofuel production [Lundell et al., 2010] and mycoremediation, especially for bio and agro waste management [Bellettini et al., 2016]. Essential to the lignin-modifying system in *P. ostreatus* are laccases, manganese peroxidases and versatile peroxidases, that act in combination with other fungal secretions, such as organic acids and metal ions [Hatakka, 1994, Lundell et al., 2010].

While *P. ostreatus* has previously demonstrated a comparatively limited ability to degrade certain herbicides [Bending et al., 2002], pure fungal laccase and manganese peroxidase have been able to degraded glyphosate at up 90 and 100% in 24h, supported by different mediators. Manganese peroxidase also degraded Monstanto's RoundUp formulation, although at a slower rate, however neither enzyme was ables to degrade AMPA [Pizzul et al., 2009].

1.4 Expectations and Hypotheses

Given the ubiquitous nature of glyphosate and AMPA contamination, due to agricultural practices, the aim of this study is to explore the value of *P. ostreatus* for potential bioremediation solutions, because the abilities of purified lignin-modifying enzymes seem promising in short term studies. The long term capabilities of an in vivo lignin-modifying system desire further exploration and to address all potential ecotoxicological concerns, degradation past the primary metabolite AMPA is also necessary.

In this study, degradation of both compounds is provoked by limiting the amount of readily available phosphorus in the growth medium. Despite degradation abilities, growth will likely decrease with higher glyphosate concentrations, as accessible phosphorus becomes increasingly limited. The experiment was based on the following hypotheses: (1) *P. ostreatus can grow under glyphosate exposure, but biomass will decline with increased glyphosate level*, (2) *Growth of P. ostreatus in medium with 100% phosphorus from glyphosate will be comparable to the medium without any phosphorus*, and these questions guided the research: *To what extent is the growth and development of P. ostreatus affected by glyphosate?*, *Can glyphosate act as a sole source of phosphorus for P. ostreatus?*.

A similar second experiment was carried out as well, further framed by these hypotheses: (1) *Growth inhibition will be avoided below* $0.04\,\mathrm{mg\,mL^{-1}}$ (2) *P. ostreatus will be more resilient to glyphosate after 3 additional weeks of cultivation*.

2 Materials and Methods

This project includes two experiments trials, that examine growth of *P. ostreatus* under exposure to glyphosate. The first trial was initiated in week 11 (15/03) and due to no visible

growth in the glyphosate containing samples by the time of harvest, a similar second experiment was set up in week 15 (10/04).

2.1 P. ostreatus Cultivation and Sampling

35 g Potato Glucose Agar (PGA) powder was added to 1000 mL de-ionized water, boiled until clear and homogeneous and then sterilized in an autoclave (120 °C). The PGA liquid was set in 40 petri dishes in a Laminar Air Flow bench (LAF bench), to insure no foreign organisms were introduced. The center of a fully grown fruit body stem was used to cultivate the fungi. Special care was given to not contaminate this part with bacteria from the mushrooms surface. To get enough material to cultivate five plates without risking bacterial contamination, two separate fruit bodies were needed.
After a week of cultivation at 23°C in a climate chamber, two plates were chosen to cultivate 10 further plates each. As these two plates could stem from different fruit bodies, the two sets of plates were labelled strain A and B, and the individual plates were assigned a number from 1 to 10. This way, deviations of growth in individual plates could be monitored. These plates were left to grow for another week.
5mm agar plugs were used to inoculate the samples, from 5 plates in the first trial and 3 plates in the second. They were cut from the periphery of growth with an agar plug cutter, to ensure consistency in size, age and residual agar as much as possible.

2.2 Growth Medium

1000 mL Highley medium stock solution was prepared without any phosphorus. Because the amounts of $MnCl_2 \cdot 4\,H_2O$, $ZnSO_4 \cdot 7\,H_2O$, $CuSO_4 \cdot 5\,H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ and $FeSO_4 \cdot 7\,H_2O$ are so small, $100\times$ the required mass of these compounds was dissolved in 100 mL deionized water. 1 mL of this solution was added to a 1000 mL volumetric flask to achieve the desired concentrations. The final pH of this solution was 4.48. Glyphosate salt and KH_2PO_4 were used to make two additional stock solutions and the compositions are shown in table 1. Since glyphosate adheres to glass, plastic materials were used for the majority of this experiment.

Stock Solutions		
	g/L	*mg/L*
Modified Highley Medium		
Glucose	5,365	
NH_4NO_3	2,0	
$MgSO_4$	2,44	
$CaCl_2 \cdot 2\,H_2O$	0,1	
H_3BO_4		0,57
$MnCl_2 \cdot 4\,H_2O$		0,036
$ZnSO_4 \cdot 7\,H_2O$		0,31
$CuSO_4 \cdot 5\,H_2O$		0,039
$(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$		0,018
$FeSO_4 \cdot 7\,H_2O$		0,015
	mL	*mg*
Glyphosate stock solution		
Highley Medium	140	
Glyphosate		705.8
Phosphorus stock solution		
Highley medium	20	
KH_2PO_4		840

Table 1: *Composition of the three stock solutions used to construct the growth medium in the samples.*

2.3 Experimental Set-Up: First Trial

Each experimental sample contained 0.309 mmol of phosphorus provided by glyphosate and/or KH_2PO_4. The ratio varied, with 25 %, 50 %, 75 % and 100 % phosphorus added as glyphosate, shown in table 2, and illustrated in figure 2. Three replicates were made for each experimental level.

Level	Glyphosate	mg/mL
No Phosphorus	0 mg	0.0
0 % *No Glyphosate*	0 mg	0.0
25 % *Low*	13 mg	0.62
50 % *Medium*	26.1 mg	1.24
75 % *High*	39.1 mg	1.86
100 % *Extreme*	52.2 mg	2.49

Table 2: *Phosphorus composition at each level.*

In addition to the control (*No Glyphosate*), one series of four samples was assembled without any phosphorus component in the medium (*No Phosphorus*) to control for phosphorus in the residual agar. The fungi was allowed to grow for 21 days weeks in the same conditions as the PGA plates.

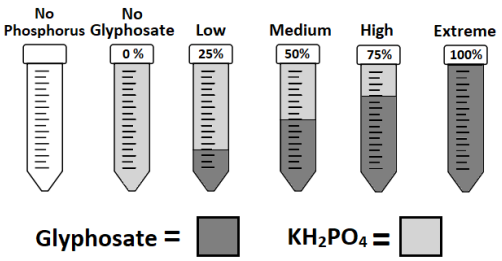

Figure 2: *Visual presentation of the sample compositions in the first trial. The percentages and indications are in relation to phosphorus only. The total volume of growth medium is* 21 mL *in each sample.*

2.4 Experimental Set-Up: Second Trial

12 samples were created at a smaller volume (10 mL) and with varying concentrations of glyphosate, shown in table 3. Each level of exposure was tested in triplicate and the control contains neither glyphosate, nor added phosphorus.

Level	Glyphosate Content
Control	0 mg
Low	0.1 mg
Medium	0.2 mg
High	0.4 mg

Table 3: *Glyphosate content at each level in the second trial.*

In order to achieve the low amounts of glyphosate, the stock solution was diluted to a tenth. Glyphosate concentrations were restricted to *Low*: 0.01 mg mL^{-1}, *Medium*: 0.02 mg mL^{-1}, and *High*: 0.04 mg mL^{-1}. The growth period was 14 days, in the same conditions as the first trial.

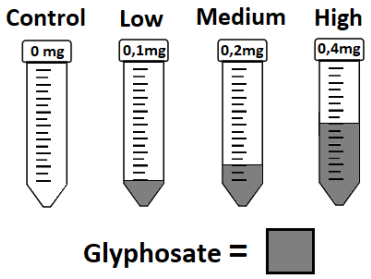

Figure 3: *Visual presentation of sample compositions in the second trial. The mass indications are in relation to phosphorus only. The total volume of growth medium is* 10 mL *in each sample.*

2.5 Separation of Mycelium and Medium

After the exposure period, the mycelium was separated from the medium by filtration with Munktell Swedish Filter Paper (00M cellulose filter paper, 7 cm diameter and 10 µm pore size), weighed before use, in the LAF bench. The filters were folded and placed, in plastic funnels over sterile Falcon tubes, and the contents of the sample was poured through the filter, ensuring the mycelium was suspended in the medium.
After the medium was separated from the mycelium, the sample tubes were rinsed with deionized water, and poured over the filters again to ensure no mycelium was left behind. Afterwards the filters were rinsed gently with deionized water to remove remaining medium in the filter pores. For the first trial there was no set rinsing volume, however in the second trial 60 mL deionized water was used to rinse each filter. The medium and mycelium were frozen to inhibit any potential microbial activity, and the mycelium was freeze-dried with the filter.

2.6 ICP-MS Analysis

In preparation for ICP-MS analysis, the mycelium was homogenized by dissolving it with the filter in 65% nitric acid, suspending the phosphorus in an ionic state. The samples where left to dissolve overnight on a orbital rotary shaker. The concentration of nitric acid was lowered to 8%, by diluting the sample to 50 mL with milli-Q water based on the indications of the Falcon tubes.
Nitric acid was also added to the medium to break down the organophosphorus compounds, glyphosate and AMPA. 6.15 mL of 65% nitric acid was added to the medium, the samples were left overnight on a orbital rotary shaker and then diluted the same way as the filters.

2.7 Biomass Assessment

To determine the fungal growth, hereafter called biomass, the filters were weighed before filtration and after freeze drying. To account for the loss of moisture in just the filters while freeze drying, a relative moisture content was determined by freeze drying two clean filters, weighing them before and after. Since the filters used in the experiment were cut down in size freehand, a relative moisture content

was needed, as the varying sizes did not allow for an absolute value. The mass of the freeze dried filter could then be subtracted from the total filter mass after freeze drying.

3 Results and Analysis

Fungal biomass and phosphorus content was analyzed via one-way, in between subjects ANOVA and KruskalWallis one-way analysis of variance. These statistical tests were used to determine a p-value for the comparisons made later in this section. The p-value represents the percentile risk of concluding a difference where none exists. Significance was set at $p = 0.05$ or a 5 % risk, meaning $p < 0.05$ would be a significant difference. Biomass was compared and the phosphorus contents of the fungi and the filtrate is analyze below. The uncertainties that need to be considered for these results are presented in section 3.3.

3.1 First Trial

No fungal growth was visually observed in the first trial after 21 days for any of the glyphosate containing samples. Samples are referred to as *No Phosphorus, No Glyphosate, Low, Medium, High* and *Extreme* as in table 2.

3.1.1 Fungal Biomass

The average biomass of the fungi after glyphosate exposure was compared in all levels. Glyphosate did not have a significant effect on biomass with $p = 0.213$. The absence of any correlation between glyphosate concentration and biomass is further indicated by the error bars shown in figure 4, page 7. Biomass of *No Phosphorus* was comparable to *Low* and *Extreme* and the remaining three samples only deviate insignificantly. The irregular pattern does not follow the expected trend of decreased growth with increased glyphosate concentration.

3.1.2 Phosphorus Content

Average phosphorus content in the biomass after glyphosate exposure was compared at all levels, shown in figure 5, page 7. Glyphosate was found to have a significant effect on phosphorus content with $p < 0.01$. No significant difference was found among the five concentrations *No Glyphosate, Low, Medium, High, Extreme* with a certainty of $p = 0.31$.
A significant difference in phosphorus content under increasing glyphosate concentration in the medium was found among the five concentrations with a certainty of $p = 0.011$.

Mainly, the first trial suggested an inhibiting dose of glyphosate for *P. ostreatus*, based on observations during the exposure period. However, since the statistical analysis of the biomass does not support this assessment, the contribution of the remaining agar plug is explored in section 3.3.

3.2 Second Trial

Fungal growth was observed at all glyphosate concentrations in the second trial and visually, growth was seemed to be most inhibited at the highest concentration. *High* samples grew patchy and irregularly, whereas growth was more uniform in the other samples.

3.2.1 Fungal Biomass

The average biomass of the fungi after glyphosate exposure was compared in all levels, shown in figure 6, page 8 . No significant effect of glyphosate exposure to the biomass was found with $p = 0.161$. Average biomass was however greater for the control than for samples exposed to glyphosate. There was a similar growth between *Low*, *Medium*, and *High* levels of glyphosate, indicating inhibition of growth even at very low levels of glyphosate.

3.2.2 Phosphorus content

Average phosphorus content in the medium samples was compared at all levels and a significant difference was found with $p = 0.01$, as expected, due to sample composition. It should be noted, that no phosphorous was added to the *Control* medium, though some was detected. However, no significant difference was found for the phosphorus concentration in the fungi at $p = 0.89$, as shown in figure 6.

Altogether, the second trial indicated a lack of glyphosate degradation, and subsequent phosphorus assimilation, by *P. ostreatus* based on phosphorus content in the medium. Furthermore, growth inhibition in response to glyphosate was also noticeable.

3.3 Potential Uncertainties

3.3.1 Deviations in Fungal Strains

Statistical analysis was also performed between fungal strains from different PGA plates and a significant deviation was found in the second trial. Average phosphorus content was compared for the three fungal strains after glyphosate exposure and a significant difference was found at $p = 0.048$, however not for the biomass, $p = 0.543$. Figure 8 in appendix C shows that phosphorus content in Strain 2 is noticeably lower than for the other two, and that biomass does not follow the same pattern.

3.3.2 Agar Composition

As a result of the inoculation process chosen for this project, all samples contained a PGA plug, that has affected the biomass measurements made above.
Since the plug may have been degraded differently in individual samples and because there were indications that it was slightly dissolved after the exposure, the contribution to the biomass is unclear. To get an indication this input, both clean plugs and plugs fully covered in mycelium were freeze dried and weighed, seen in table 4. Biomass contribution of the agar content of plug was greater in first trial than in the second trial, as the majority of the plugs mass stems from undegraded agar, and the PGA plates were under continued degradation.

Agar	Avg. weight (n=5)
Clean Agar	2.82 mg
Degraded Agar	1.94 mg

Table 4: *Average weight of freeze dried agar plugs, clean or covered with mycelium. These plugs were taken in week 14, explaining the lower biomass compared to the second trial.*

To test both the effectiveness of dissolving cellulose filters in nitric acid and find the range of detectable phosphorus, a series of test samples was constructed using both clean filters and filters spiked with a phosphorus standard or the glyphosate stock solution. The result of these test samples are included appendix A.
Clean agar plugs were analyzed with ICP-MS, and were found to contain 6.5 µg of phosphor. It is therefore unlikely that the agar plugs have contributed substantially the phosphorus contents of the samples.

3.3.3 Filter Retention

Due to unexpected phosphorus measurements on the ICP-MS of the filters in the first trial, adsorption of the filters was tested separately. Four filters were spiked with either KH_2PO_4 and glypohsate stock solutions and rinsed with deionized water. The rinsing volumes were 30 mL and 60 mL, mimicking the first and second trial respectively. Shown in table 5, phosphorus from glyphosate is retained at significantly higher levels at a low rinsing volume.

Stock Solution	30 mL	60 mL
KH_2PO_4	2.1 µg	3.3 µg
Glyphosate	20.9 µg	7.7 µg

Table 5: *Mass of retained phosphorus in the filter, in relation to phosphorus compound and volume of rinsing liquid.*

3.3.4 Sample Construction

All measured concentrations of phosphorus in medium samples were greater than expected, based on equations (1) and (2) in appendix B. Based on propagation of uncertainties in the experiment, also shown in appendix B, ranges of uncertainty for phosphorous content in the medium are given in table 6.

Sample	P [mg]	δP	Avg. [mg]	W/I δP
First Trial				
No G	9.6	28.0 %	11.4	YES
Low	9.5	45.9 %	11.7	YES
Medium	9.4	46.7 %	11.5	YES
High	9.3	46.7 %	10.7	YES
Extreme	9.2	28.9 %	10.2	YES
Second Trial				
Low	0.0185	10.5 %	0.0197	YES
Medium	0.0369	10.5 %	0.0422	YES
High	0.0739	10.3 %	0.0899	NO

Table 6: *The input phosphorus concentration of every sample composition and their corresponding uncertainties. Samples that did not contain phosphorus were not included.*
P [mg] denotes theoretical mass of phosphorus in each sample. δP denotes the corresponding uncertainty. Avg. [mg] denotes measured phosphorus, and W/I δP states if it is within the expected range.

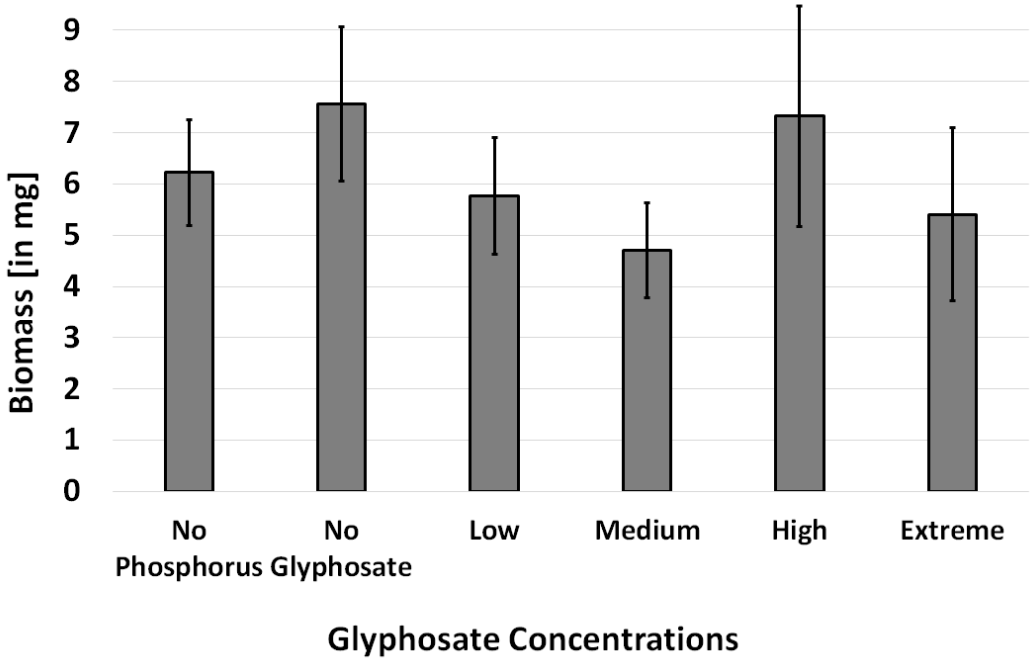

Figure 4: *First Trial: Freeze dried average fungal biomass for every sample composition after the exposure period. (n=3)*

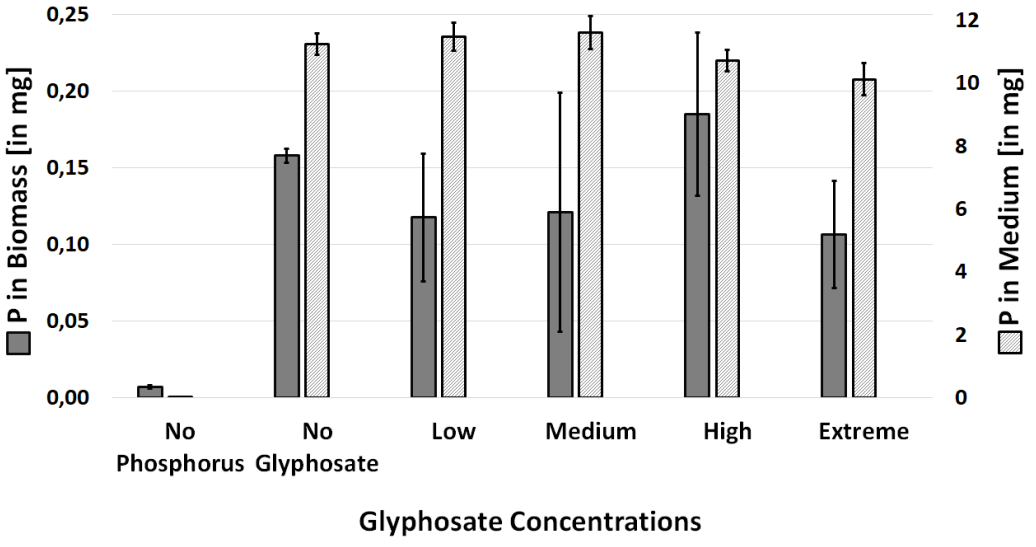

Figure 5: *First Trial: Phosphorus content in the fungal biomass and in the medium for every sample composition after the exposure period (n=3).*

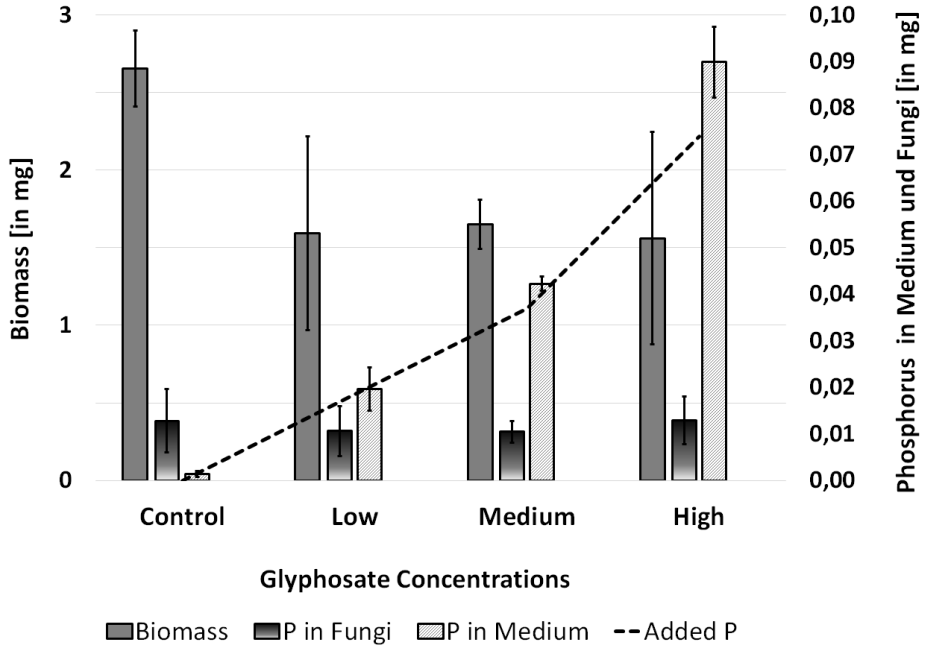

Figure 6: *Second Trial: Average fungal biomass of each sample composition and average phosphorus content in all medium and biomass samples after the exposure period (C: n=2, L/M/H: n=3). The dotted line marks the theoretical input of phosphorus (Added P).*

4 Experimental Findings

4.1 Phosphorus Limitation

The experiment was designed to limit phosphorus and it was assumed *No Phosphorous* would yield a biomass comparable to the degraded agar shown in table 4.
The sample *No Phosphorus* in the first trial should have been phosphorus limited however, the biomass of the sample was considerably greater than expected and it was insignificantly different from the biomass of the *No Glyphosate* sample. The phosphorus contents of the biomass of *No Phosphorus* was 6.8 µg, comparable to the 6.5 µg of phosphorus in a clean agar plug. This seems to indicate that the phosphorus in the agar was sufficient to sustain growth of the fungi for the experimental period, and that phosphorus limitation was not achieved. The phosphorus added to the medium, was based on the Highley medium [Highley, 1973], designed to contain all the needed nutrients required for fungal growth. Table 6 shows that the residual phosphorus in the *No Glyphosate* sample is comparable to the initial phosphorus input, further indicating that there was no limitation of phosphorus, since KH_2PO_4 is degradable by *P. Ostreatus*.
If the experimental period was extended, it is possible that phosphorus limitation might be achieved for the *No Phosphorus* sample. Considering the fungal growth in *No Phosphorus* in relation to the phosphorus available to it in the first trial, too much phosphorus was present in the agar to see clear signs of mineralization in the medium.

4.2 Phosphorus Mineralization

Figure 5 shows the phosphorus contents of the biomass in the first trial and based soley on this, it could be deduced that the fungi indeed mineralized glyphosate and absorbed the phosphorus. However, as shown in table 5, particularly glyphosate was retained in the filter if rinsing was insufficient and this effect is probably amplified in the presence of mycelium in the filter.
This could be confirmed if a different detection method was chosen. The ICP-MS analysis detected all phosphorus, despite in which compound it was bound, and therefore not possible to determine if the origin of measured phosphorus insufficient rinsing or adsorption.
Another possible source of phosphorus in the

biomass is diffusion. Agar is known to absorb some of the medium in which it is submerged. Glyphosate and KH_2PO_4 could then enter the agar plug, elevating the phosphorus content of the biomass. Since diffusion is based on concentration, it is then likely phosphorus content due to diffusion would differ in relation to the concentrations of different components in the samples, see figure 2. It further explains the lower phosphorus content measured in the *Extreme* sample, see figure 5, as the plug broke in two in all replicates for this sample.
The significant difference in phosphorus contents in the medium in the first trial, is likely due to filter retention. Glyphosate exhibits a higher affinity to the filters and it seems highly unlikely that mineralization would have occurred in any sample containing glyphosate, as all growth was visibly inhibited. It follows that greater glyphosate retention would lead to increased amounts of phosphorus in the biomass, however no significant increase was found for the glyphosate containing samples. It is possible the *No Glyphosate* assimilated phosphorus from the medium, as that sample visibly grew.

4.3 Growth Inhibition

According to [Larson et al., 2006] the inhibiting dose of glyphosate for certain soil fungal is $0.4\,\mathrm{mg\,mL^{-1}}$. Glyphosate concentrations exceeded this level in all exposed samples containing in the first trial. The biomass in figure 4 was expected to reflect this, meaning biomass was expected to be similar for all inhibited growth. However, even when accounting for the weight of the agar and adsorbed phosphorus chemicals, the biomass of the glyphosate exposed samples still exceed expectations based on visual observations. The toxic effects of glyphosate for fungi will be discussed further below.

4.4 Revisions to the Second Trial

During the first trial, it became increasingly apparent that fungal growth was inhibited and the three necessary alterations for the second trial were; (1) The lowest glyphosate concentration in the first trial was $0.6\,\mathrm{mg\,mL^{-1}}$. Glyphosate concentration was adjusted to a tenth or less of the reported inhibiting dose for soil fungi. Even if the fungi did not mineralize and assimilate phosphorus from glyphosate, this setup further examines the inhibition or toxicity of glyphosate. (2) Based on the uninhibited growth in the *No Phosphorus* samples, too much agar was present to limit phosphorus for *P. ostreatus*. Plugs for the second trial were taken from plates which were allowed to grow for 4 additional weeks in the climate chamber. The fungi had consumed more agar in this time, thereby lowering the amount of available phosphorus added with the agar. (3) Since the ICP-MS measures the presence of a single element and not molecules, differentiating between glyphosate and KH_2PO_4 was not possible. Therefore only glyphosate was used as a phosphorus source in the second trial, without supplementation with KH_2PO_4, clarifing the source of the phosphorus in the analytical results.

4.5 Excess Phosphorus in the Growth Medium

As seen in figure 6, the amount of glyphosate left in the medium samples is equal to or greater than the initial input. This indicates no signs of mineralization of glyphosate by *P. ostreatus* even at very low concentrations. It is further noticeable that the control medium registered a phosphorus content of $1.35\,\mu g$. No phosphorus should be present in the medium of this sample. However, as agar will dissolve in water over time, it is possible that this is the source of the phosphorus in the medium.

4.6 Biomass Measurements

Phosphorus content in the biomass is comparable for all samples, and diminished growth is most likely due to glyphosate exposure. It follows that exposure to glyphosate even in very low concentrations has a fungistatic effect on *P. ostreatus*.
Biomass for all levels of glyphosate exposure is comparable, and does not decline with increased concentration, however visually, growth the *High* sample was particularly affected. It seems that *P. ostreatus* has severe responses to glyphosate concentrations greater than $0.04\,\mathrm{mg\,mL^{-1}}$ and since the amount of phosphorus in the medium is so similar to the initial input, no indication of mineralization of glyphosate was found.

4.7 Recommended Revisions to the Set Up

In a future experimental setup, it is recommended, that the fungi be cultivated in a liquid medium. Since phosphorus limitation was not achieved, the composition of such a cultivation medium is very important. The growth medium used in this report, might not be suited for cultivation, as it evidently contains copious amounts of phosphorus for this type of set up. A liquid cultivation medium, should have enough phosphorus to secure good conditions for the fungi, but not so much, that phosphorus contamination of the growth medium is risked during inoculation. Cultivating in a liquid medium would also eliminate contribution from the agar to the biomass.
Uncertainties mostly stem from imprecise volumes. If autoclaved tips for step pipettes were used this would be reduced. Furthermore, if a set volume was extracted after filtering, it could then be diluted more precisely than by depending on the Falcon tubes indications.
Lastly, it is recommended that alternative or supplementing analytical method are used. By only using the ICP-MS to analyze for phosphorus, some vital information is lost. Seeing as molecular retention in the filters was greater than expected, identification of retained molecules could provide valuable insight. Identification of molecular composition after filtering could also show how exposure affected the physiology of a fungi compared to the control.

4.8 Glyphosate Resistance for Remediation

Despite the ability of lignin-modifying enzymes to both tolerate and degrade glyphosate, the in-vivo lignin-modifying system in *P. ostreatus* did not demonstrate the same potential. Inhibited growth in both trials suggests that glyphosate or AMPA either have a fungistatic effect on *P. ostreatus* and the phosphorus content in the growth medium gives no indication of glyphosate degradation. Shikimic acid is can be found in *P. ostreatus*[Kojima et al., 2015], so it can be assumed that this species synthesizes essential compounds via this pathway, and that growth inhibition is due to expression of glyphosate sensitive 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase). Fungi usually display a more complex arrangement [Liu and Cao, 2018], however this is does not seem to result in higher likelihood of glyphosate tolerance compared to bacteria, further supported by the findings in the study.
Naturally glyphosate tolerant versions of EPSP synthase are mainly found in bacteria [Maeda and Dudareva, 2012] and glyphosate resistant crops are genetically modified to express this enzyme, usually from a bacterial gene from *Agrobacterium tumefaciens*. Although fungi have not been as extensively studied there is some evidence fungal, glyphosate resistant, EPSP synthase outperforms the bacterial counterpart [Liu and Cao, 2018].
High tolerance was also found in four out of 45 fungal strains, isolated and identified from agricultural sites, where *Aspergillus niger*, *A. flavus*, *Penecillin verruculosum* and *Alternaria tenuissima* withstood a concentration of 10 mg mL^{-1} with less than 50% growth inhibition and at 0.8 mg mL^{-1} *A. flavus* and *P. spiculisporus* showed the highest degradation rates for glyphosate [Eman et al., 2013].
Aspergillus oryzae (koji) isolated from a pesticide factory was found to degrade both glyphosate and AMPA, via the pathway depicted in figure 1, where AMPA degradation was determined to be the rate limiting step. This mold is commonly used to ferment foods, suggesting more attention could be given to glyphosate contaminated foods.

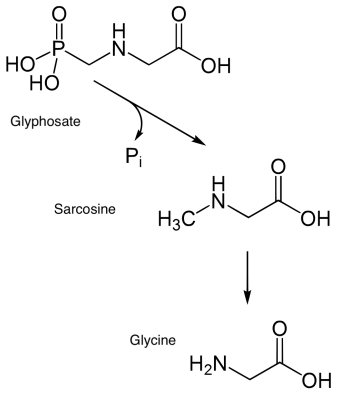

Figure 7: *An alternative degradation pathway for glyphosate, where phosphate and sarconsine are the primary metabolites. (Modified from [Giesy et al., 2000])*

If glyphosate and AMPA tolerance is a mutation produced by long term glyphosate expo-

sure, soil and waste environments likely offer the widest variety of tolerant microorganisms, however effective degradation abilities are are not necessarily implied, based on reported accumulation for AMPA in particular. Affirmatively, several strains can use glyphosate as a sole source of carbon, nitrogen or phosphorus, however when AMPA is the main metabolite, it is usually not degraded and excreted back into the environment. The other known degradation pathway for glyphosate shown in figure 7 is not considered toxic however it is seen less often [Zhan et al., 2018].

5 The Future of Glyphosate Use and the Role of Bioremediation

Finally, this study is rounded off by examining the landscape of potential bioremediation applications in agricultural practices, followed by an assessment of the future of glyphosate in an ecological and economical context and the political climate surrounding glyphosate use.

5.1 Bioremediation Potential and Practical Applications

Dig and dump methods of removing contaminated soil to licensed landfills has been a popular way of remediating soil damaged by pollution and agriculture. However, modern remediation technologies are focusing on permanent disposal solutions without exporting any secondary environmental problems. A progressive approach is microbial cultivation, by increasing the useful native microbe population of an ecosystem via forcefully aerating the soil and adding nutrients to provide an ideal growth environment [Vosátka, 2001].

5.1.1 Phytobial Remediation

An innovative approach sees potential in combining remediation strategies of multiple organisms for a more efficient yield. Phytobial remediation by plants, where roots are colonised by symbiotic microbes, create a stable community that can degrade contaminants while the macrophyte takes up and accumulates toxic materials in their harvestable tissue [Vosátka, 2001], making them unavailable to run-off into the surrounding ecosystems.

5.1.2 The Future of Bioremediation

Even though research on microbial potential is abundant, in situ bioremediation technologies remain largely unproven [Lynch and Moffat, 2005, Zhan et al., 2018]. While soil conditions need to be favourable for vegetation, microbial communities and the real life soil matrix include an abundant range of complex organic compounds that most studies can not take into account. Therefore, even though lignin-modifying fungi such as *P. oestreatus* are capable of degrading toxins in vitro and the potential of using extra-cellular enzymes to remediate polluted soil is promising [Strong and Claus, 2011], more ecological research is needed to fully understand microbial remediation abilities and how they can be applied at a large scale.

5.1.3 The Importance of Research

The need for innovative and cost effective technologies is more urgent than ever, considering that the global production of environmentally harmful compounds increased by 200 million tons annually, between 2002 and 2011 [Gavrilescu et al., 2015]. Degradation pathways continue to be a highly investigated, with little toxicological information about long-term and low-level exposure for a majority of compounds, including glyphosate. To minimize their impact on environmental and human health by way of bioremediation, the entire life-cycle and long-term impact needs to be fully understood in view of the constant production of new herbicides, especially given the accelerated use of glyphosate goes beyond current safety monitoring and risk assessment methods [Gavrilescu et al., 2015].

5.2 Economic Impact of Glyphosate

Global sales of glypohsate are approaching two billion dollars with 8.6 billion kg of glyphosate used world wide since its introduction [Benbrook, 2016]. More than 90% of US crops are treated with the herbicides [Caseley and Copping, 2000] and historically no herbicide was sprayed so widely [Benbrook, 2016].
Steady increase in agricultural application is due to its efficient non-selective properties, easy application, and an increasing price reduction due to global competition of glyphosate producers, after the patent expiration in 1999. No later introduced herbicide

has achieved this level of success [Caseley and Copping, 2000].
As the herbicide is directly associated with a growing number of tolerant weed species, corporations counteracted this by developing glyphosate resistant crops, resulting in a consequential aggravating of the so-called *transgenic treadmill* due to glyphosate overuse [Binimelis et al., 2009].

5.2.1 Alternative Herbicide Applications

Soil contamination with inorganic and organic pollutants via agricultural, industrial and urban waste has become on of the most serious issues of today. Under the pressure of a fast-growing global population the drive for *land recycling* is an essential response for a sustainable development [Lynch and Moffat, 2005].
Alternative herbicides lack glyphosates versatile, aggressive nature and are usually weed-specific, more expensive, labour-intensive and less efficient. The apparent infallibility of glyphosates weed-controlling abilities discourages the use of biological alternatives such as integrated weed management [Koon-Hui, 2007]), bio-herbicides [Miri and Armin, 2013] or traditional, physical and rotational practices, such as tilling and ploughing.
In comparison, these organic controls cost approximately 1000$ an acre, whereas herbicides can cover the same area for 50$ and chemical herbicides have a significantly higher crop yield and give the possibility for crops to grow in higher densities [Gianessi and Reigner, 2007]. The higher production costs of organic alternatives, making up only 0.5% of US cropland [Gianessi and Reigner, 2007], is the result of labour intensive hand-weeding and tilling that provides a lower crop yield. Without herbicides, US crop production would decline by 20% and could not accommodate standards of modern civilization and an accelerating population [Gianessi and Reigner, 2007]. Considering the global food production depends on the productivity that glyphosate provides, prioritization of bioremediation is further emphasizes [Powles, 2008].

5.2.2 Political Landscape and the Role of Science

Risks concerning herbicides have been heightened by the dramatic increase in application worldwide and uncontrolled production by various manufacturing companies. In the future, increase in herbicide use and next-generation herbicide-tolerant crops will pose an increased ecological and human health risk, as well as amplified crop damage call for greater political oversight and a more sustainable food production system [Bain et al., 2017].
In the past, glyphosate was repeatedly declared as safe by global regulators, such as the US EPA and the EU, which sands in direct contrast to other alarming reports and research about glyphosate being *probably carcinogenic to humans* [International Agency for Research on Cancer, 2015].
Industrial representatives present biased laboratory findings, where research validity is often questioned due to the studies not representing realistic exposure conditions. An ongoing dispute between NGOs using scientific evidence, intellectually dishonest industries and political leaders has effectively blocked a ban or globally recognized toxicological classification for glyphosate [Bozzini, 2017].

5.2.3 Food Safety vs Food Security

Above all, governments try to accommodate both food safety, due to their duty to ensure human and environmental health, and food security, that is guaranteed by herbicide use in agriculture, in a framework that satisfies consumers, companies, investors, and researchers [Mampuys and Brom, 2015]. However, currently, global herbicide regulation, including glyphosate, vary greatly [Bozzini, 2017].

5.2.4 The Future of Glyphosate Use

Environmental concerns due to pesticides are generally linked to more complex political issues and a delicate relationship between socio-political and economic forces fueled by contrasting interpretations of research [Bain et al., 2017]. Eventually, governing bodies in the EU were able to agree on banning toxic co-formulants added to the herbicide formulations, while simultaneously renewing glyphosate approval for 5 years. These events in late 2017, triggered by a large-scale EU-debate, are an indication of how the glyphosate discussion is open ended and will continue [Bozzini, 2017].

6 Conclusion

This study showed that while *P. ostreatus* can grow under glyphosate exposure, inhibition can still be expected at low concentrations. The lack of correlation between biomass and level of exposure is most easily attributed to the uncertainties with the experimental design, that seemed to span every step of the process and ultimately undermine the validity of the results. As stated in hypotheses, biomass of the *Control* and *Extreme* samples in the first trial was comparable, although this was concluded to be coincidental. Visual observations revealed that glyphosate concentrations greater than $0.6\,\mathrm{mg\,mL^{-1}}$ had fungicidal consequences and that growth inhibition is still apparent at very low concentrations.

Based on this experiment, using *P. ostreatus* for bioremediation of glyphosate does not seem feasible, as the fungi suffers negative effects at low levels of exposure. Literature also seems to confirm that *P. ostreatus* does not have the ability to degrade AMPA. Finally, as this fungi does not usually grow in soil, successful on-location remediation is implausible, and in all cases would probably carry a high financial costs.

Despite this, bioremeditiation appears to be the most promising and integrative approach that merges all factors and more focus should be put to the inevitable accumulation of future toxins and counteracting negative consequences in an adaptive and sustainable way, promoting and utilizing natural remediation pathways, instead of banning a chemical that, despite the associated risk, currently seems to be indispensable for modern civilization.

Nevertheless, this approach should to be accompanied by a maintained effort to work *harder, not smarter*, by continually weighing the effort of developing resistant crops for food security, preserving ecosystem stability in light of both introduced and naturally occurring tolerance, protecting the health of farmers and consumers long term, and the scale at which bioremedial solutions need operate to manage contamination, against developing sustainable alternatives to glyphosate in the future.

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Appendices

A ICP-MS test samples

Sample Name	Total Dilution	Method	Conc. [g/L]
Clean Filter	50 mL	Microwave	28.6
Clean Agar	50 mL	Microwave	140.4
Agar w/ Mycelium	50 mL	Microwave	77.6
Filter w/ Glyphosate	50 mL	Microwave	1878.1
Filter w/ std	50 mL	Shaken	960.0
Clean Filter	50 mL	Shaken	<0.0
Clean Agar	50 mL	Shaken	120.2
Agar w/ Mycelium	50 mL	Shaken	86.2
Filter w/ Highley medium (no P)	50 mL	Shaken	165.4
Filter w/ Glyphosate	50 mL	Shaken	1688.0
Clean std	50 mL	Shaken	964.3
Clean Highley medium (no P)	50 mL	Shaken	13.6

Table 7: Test samples run on the ICP-MS prior to the analysis of trial 1. The methods mentioned were to either microwave the samples or leave them on a orbital rotary shaker.

B Calculating the Propagation of uncertainties in Trial 1 and 2

Phosphorus concentrations seemed to be a higher in every medium sample than calculated, in both the first and second trial. Under the assumption than no phosphorus has been assimilated by the fungi, equation (1) was used to calculate the phosphorus concentration for samples that contained one source of phosphorus and (2) was used for samples that contained two sources (source A and B).

$$C_P = \frac{W_P}{V_{stock}} \frac{V_{Psample}}{V_{samplesize}} \frac{V_{filtrate}}{V_{dilution}} \tag{1}$$

where,
C_P is the concentration of phosphorus in the sample going in to the ICP-MS
W_P is the weight of the phosphorus component added to the phosphorus stock solution
V_{stock} is the total volume of the phosphorus stock solution
$V_{Psample}$ is the volume of the phosphorus stock solution added to the sample
$V_{samplesize}$ is total volume of the sample
$V_{filtrate}$ is the volume of the sample after filtration
$V_{dilution}$ is the volume of the sample after dilution

$$C_P = \left(\frac{W_{PA}}{V_{stockA}} V_{PsampleA} + \frac{W_{PB}}{V_{stockB}} V_{PsampleB} \right) \frac{1}{V_{samplesize}} \frac{V_{filtrate}}{V_{dilution}} \tag{2}$$

where A and B is used to denote the two different sources of phosphorus.

Using these equations the uncertainties tied to each step can be identified. The uncertainty of W_P is the uncertainty of the weight, multiplied by the ratio of the molar mass of phosphorus and the molar mass of the phosphorus compound. The uncertainty of the stock solutions was set to ± 0 mL since step pipettes were used. The uncertainties of $V_{filtrate}$ and $V_{dilution}$ were estimated

to be ± 1 mL based on the indications on the Falcon tubes. Uncertainties for $V_{samplesize}$ and $V_{Psample}$ vary, as the samples were constructed with two different pipettes. The uncertainties of the two pipettes were ± 0.1 mL and ± 0.01 mL. The propagation of the uncertainty of these values alongside the other uncertainties, is given in table 8, where m and n denotes the amount of times each pipette was used.

δW_P	$\pm 0.01\,\text{mg} \cdot \dfrac{M_P}{M_{compound}}$
δV_{stock}	± 0 mL
$\delta V_{Psample}$	$\pm m 0.1\,\text{mL} + n 0.01\,\text{mL}$
$\delta V_{samplesize}$	$\pm m 0.1\,\text{mL} + n 0.01\,\text{mL}$
$\delta V_{filtrate}$	± 1 mL
$\delta V_{dilution}$	± 1 mL

Table 8: Uncertainties of the values used to calculate the concentration of phosphorus in the experimental samples.

Given the sample compositions and the equations for propagation of uncertainties given by [Taylor, 1982], the theoretical concentrations of phosphorus and the relative uncertainties can be calculated. Results are given in table 6, and the uncertainty of the phosphorus concentration is notably high, especially for the first trial. Measures to lower this needs to be taken in future experimentation.

Sample	C_P [mg / mL]	δC_P	Experimental Averages	Within δC_P
Trial 1				
No G	0.191189	28.0 %	0.228861	YES
Low	0.189572	45.9 %	0.233100	YES
Medium	0.187955	46.7 %	0.229015	YES
High	0.186337	46.7 %	0.219342	YES
Extreme	0.184720	28.9 %	0.203878	YES
Trial 2				
Low	0.000369	10.5 %	0.000393	YES
Medium	0.000739	10.5 %	0.000845	YES
High	0.001478	10.3 %	0.001798	NO

Table 9: The input phosphorus concentration of every sample composition and their corresponding uncertainties. Samples that did not contain phosphorus were not included.
Notice: This table is the same as table 6 in the results section, however the units have been change in the report for easier reading.

C Deviations of phosphorus contents in fungal strains

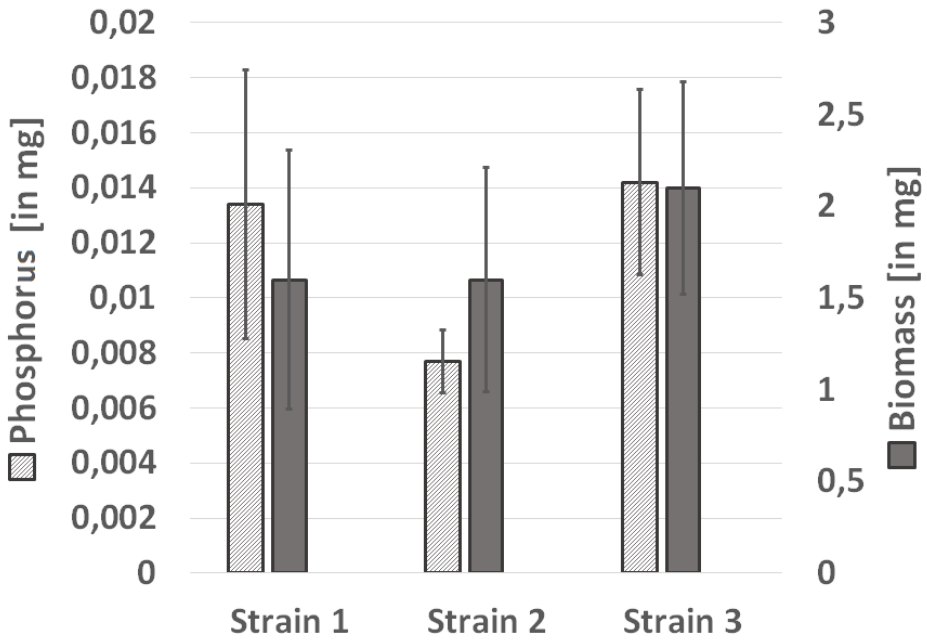

Figure 8: Trial 2: Average phosphorus mass in the three strains, with their corresponding biomass. (S2/3: n=4, S1: n=3)