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Application and Validation of a Solid Phase Bioassay to Measure Bioavailable Soil Phosphorus

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Abstract
Phosphorus (P) is an essential element needed by all living organism. Although P may be present in high concentration in soil, it is mostly not in plant available form. There is a need to measure bioavailable P. A microbial biosensor could provide an approach to determine the bioavailable P in soil. It has been shown that a P biosensor worked in aqueous systems. Yet the application and validation of a solid phase contact device to measure the response of luminescence marked *Escherichia col*i MG1655 to phosphate in a complex solid phase-like soil has not been done before. To test the applicability of such a biosensor in soil, four different pH agricultural soils were investigated chemically and with the biosensor. The expression of bioluminescence by the bioreporter (i.e. an induction by the presence of phosphate in a starvation system) enabled quantification of bioavailability in soil with increasing pH. Results from the experiments showed a correlation between the luminescence pattern and phosphate levels as determined by standard chemical procedure. It was indicated that a biological P sensor may serve as a tool for assessing phosphate bioavailability.
Keywords: Bioavailable, Bioreporter, Phosphorus, *Escherichia cola,* applicability

1. Introduction

Phosphorus (P) is an essential element needed by all living organism (Yu et al., 2014). In natural systems, it is assimilated by plant as phosphate with orthophosphate (PO43-) being the most common form. Despite its importance for plant growth and development, more than 40% of world soil are deficient in phosphorus(Vance et al., 2003; Adeloju et al., 2011). Although P may be present in high concentrations in soil, it is often not in a plant available form (Adeloju et al., 2011)

In soil, P exist in the forms of soluble P, active P and fixed P (Bushman et al.,2009). The active P is contained in the soils solid phase which accounts for most of the available phosphate easily released into the soil solution for plant biomass. The fixed pool contains phosphate that is very insoluble and resistant to mineralization by microorganism. This pool may contain phosphate for years in an unavailable form hence it is has little or no impact on soil fertility.

Soil available P has been measured using different chemical methods. Chemical extraction with weak acids (acetic acid), water, bases and salts are some of the methods used for standard nutrient diagnostic test (Preverill et al; 2001). The type of extractant used and soil type are very important in the determination of available P. Buffering of extractant and dissolution of non-labile P are some of the challenges that can occur together with the use of inadequate chemical extractant over a different soil type not designed for (Myer, et al 2005). One of the most common and simplest extractant is water (Vanderdeelen, 2002). The relatively small amount of soil P extracted with water together with difficulties related to chemical analysis makes it a poor extractant. Olsen et al, (1954) suggested the use of sodium bicarbonate (NaHCO3) as an extractant in calcareous soil. The bicarbonate helps in decreasing the Ca2+ activity in these soil by forming a complex with CaCO3. The combination of HCl and NH4 for the easy removal of acid soluble P forms largely aluminum and phosphate was suggested by Bray and Kurtz (1945). Mehlich in 1953 also introduced a combination of two acid (HCl and H2SO4) to extract P from soils in the United States later modified the test into a combination of acids (acetic and nitric), salts (ammonium fluoride and nitrate and chelating agent ethylenediaminetetraacetic acid (EDTA). The use of acetic acid as extractant is usually used in combination with other compounds. After extraction process has been completed, a calibration curve is derived resulting from colorimetric reaction and absorbance measured. However, the major limitation of chemical method in the determination of P in soil is the inability to measure bioavailable phosphorus.

Microbial biosensors have been described as an analytical device that couples biological component (Microorganism) with a detector system (transducer) enabling rapid, sensitive and accurate detection of target analytes (Yu et al; 2006).The application of bioluminescent based microbial biosensor in soil environment have been widely used for general toxicity testing (Paton et al; 1995; Trott et al; 2007; Brandt et al., 2006; George et al., 2010). They produce assays that are of rapid measured response, sensitive and highly reproducible (Brandt et al., 2008). Most bioavailability assay solely rely on extraction of soil pore water (Brandt et al.,2011) and subsequent exposure to test organism but this fails to consider the soil solid phase which is most likely to host most bioavailable phosphate . However, after the extraction of soil solution from the soil solid phase, there may

occur a shift in the physico-chemical equilibrium of the analyte speciation (Zhang et al., 1998). An advantage they have over chemical method is their ability to detect bioavailable fraction of pollutants in various environment in a relatively short time. Although there may be advantages of using microbial biosensors but there is still need to perform extraction techniques. The effectiveness of microbial biosensor application for determining bioavailable fraction of pollutants is not only dependent on the microorganism employed but also on the matrix in which it is exposed and the time of exposure. The microbial biosensor used for this study was Escherichia coli MG1655 pPHO-lux which produces light in a dose –dependent manner (Dollard and Billard, 2003) but a bit more complex that would usually assume. Using Escherichia coli MG1655 pPHO-lux, Dollard and Billard, 2003 were able to establish that luminescence patterns correlated with phosphate concentrations determined by standard chemical procedure.

This study aimed at the application and validation of a solid phase assay to measure bioavailability of phosphate in soil. The main objectives were: (1) to characterize the bioluminescent biosensor Escherichia coli MG1655 (ii) to optimize the bioassay (iii) to apply and measure the performance of an optimized assay of a phosphate specific biosensor Escherichia coli MG1655 in contact with soil solid assay/device

2. Materials and Methods

2.1 Site description and soil collection

The site from which soil was sampled is located at the Scottish Rural University Colleges Craibstone Estate, Aberdeen, UK (57 0 11' N, 2 0 12' W) at the Woodlands Field experimental facility. Since 1960, this has been an experimental site to study the relationship between an eight- stage arable rotation and soil pH value. Soil pH plots were managed using aluminium sulphate and lime to achieve a range of values between 4.0 and 7.5 at 0.5 increments.

The eight stage rotation comprise swedes (Brassica napobrassica), Spring oats (Avenasativa), Spring barley (Hordeum vulgare), Winter wheat (Triticum aestivum), Potatoe (Solanumtuberosum), Perennial rye grass (Lolium perenne) with White clover mixture (Trifolium repens) and three year grass clover ley

The soil is of sandy loam texture and was sampled between 0-15cm. In each plot, three replicate soil samples of different pH were collected using a stainless steel slide. The soil samples were stored immediately after collection. Soils were collected from plots that were sown,Grass (Trifolium repens), Swede(Brassica napobrassica) spring oat(Avena sativa) and Spring barley(Hordeum vulgare). All soils were sieved with a 2mm mesh for removal of debris and stored prior to laboratory analysis.

2.2 Chemical extraction of phosphate

Five grams (± 0.01g) of air dried and sieved soil (2mm) was weighed into a 250 ml Erlenmeyer flask. One hundred and fifty ml of a 2.5% acetic acid solution was added. The flask was capped and placed on an orbital shaker for 2 hours. The flask was removed and the solution allowed to settle. The solution was then filtered through a No 44Whatman filter paper and transferred to sterile 50ml centrifuge tube. After appropriate dilutions, analysis (triplicate) of samples and relevant controls was performed using a Flow injection analysis (FOSS, FIAstar 5000 Analyzer)

2.3 Biological assays

The phosphate specific biosensor Escherichia coli MG1655/pPHO-lux was grown on Luria-Bertani (LB) medium supplemented with Ampicillin (50µg/ml) and Kanamycin (20µg/ml). An overnight culture was prepared by growing one colony of cells in 10 ml of LB broth in a 30 ml universal bottle at 25oC and 200 rpm (Manderova et al; 2011) on an orbital shaker incubator. Batch cultures were prepared by adding 1ml of overnight culture to 3-100 ml of sterile LB media and incubation on an orbital shaker for 6 hours. The cell suspension was centrifuged at 3000g (Manderova et al; 2011) for 60 s at room temperature ,treated once with 1 ml of MOPS minimum medium prepared without phosphate and then resuspended in same medium at an OD approximating to OD550 0.9 (Dollard and Billard, 2003).

To characterise the induction bioassay, 4ml of cell suspension was added to 36ml of standard solution of $K2HPO_4$ at a range of concentration (0mM, 0.132mM, 1.32mM, 13.2mM and 132mM) to obtain a calibration. Bioluminescence was measured at 30 minutes intervals on a 4.5 hour period of incubation on a portable Jade luminometer (Labtech International ,Uckfield,UK) and Relative light unit (RLU). All measurements were performed in five replicates and a calibration response established.

2.5 Soil solid phase contact assay

Three grams (± 0.01g) of soils in triplicate were transferred to 15ml sterile corning tubes. A volume of 7.5 ml of 0.1 M potassium chloride (KCl) was added to the soil. The soil suspensions were shaken for 60 minutes on an end to end shaker at room temperature and 200 rpm. An aliquot of 1ml of cell suspension of the MOPS treated phosphate biosensor was added into the soil suspension, vigorously mixed and incubated in an orbital shaking

incubator at 25oC and 200rpm for 4.5hours. Upon incubation, the soil solid phase device (5ml Evergreen Scientific Sera –Separa serum filter) was applied by gently pressing the device downwards into the slurry as previously performed by Ma and Paton, 2011. One ml of filtrate containing cell suspension in the soil slurry was transferred into a 3ml luminometer cuvette for measurement of bioluminescence on a portable Jade luminometer (Labtech International, Uckfield, UK) and recorded in RLU.All measurement were performed in triplicates.

2.6 Statistical analysis

The biological and chemical responses were modeled using Sigma Plot, version 10.0(Systax Software, San Jose, CA, USA). Data were tested for equal variance and normality. One way Analysis of variance (ANOVA) was used to ascertain whether different concentrations of aqueous solution had an effect on biosensors. Linear regression was used to determine the relationship between bioluminescence and chemically extractable phosphate. All data were carried out using MINITAB Software 17.0 for Windows. All significant levels were quoted at the 95% confidence level ($p \leq 0.05$).

3.Results

3.1. Calibration of the biological response

The response of the biosensor to different concentrations of K2HPO4 was plotted against time at different time intervals (Fig 1). Escherichia coli MG1655/pPHO-lux responded to phosphate starvation in a dose dependent manner. There was an observable increase in bioluminescence with decrease in variable concentrations of phosphate. An increase in bioluminescence was measured after 30minutes of incubation until a time point of 270 minutes.

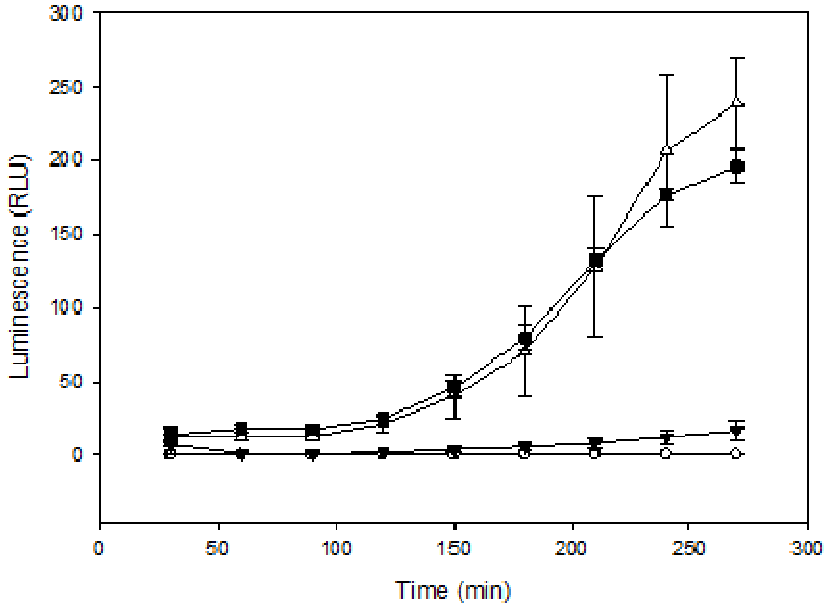

FIG. 1: Calibration response of phoA::luxCDABE to laboratory standards 132mM (▪), (◦)13.2mM, (●) 1.32mM, (△) 0.132mM and (▌) 0mM induction in Escherichia coli MG1655. Error bars represent standard deviation

While low level of bioluminescence were maintained at 132mM phosphate, lower concentration resulted in increased luminescence. The response of the biosensor to varying concentrations of phosphate differed significantly ($p<0.05$).

To test if concentration has an effect on the biosensor response in aqueous solution an ANOVA was employed. The test indicated that biosensor response in RLU units was significantly influenced by pH ($F_{4,24}=311.15$, $p<0.001$).

3.2. Chemical extraction and biological response to phosphate

There was an observable relationship between the bioluminescence (RLU) and chemically extractable phosphate (Fig 2).Results shows a positive correlation in measuring phosphate in the four soils using both methods as shown in Table 2.

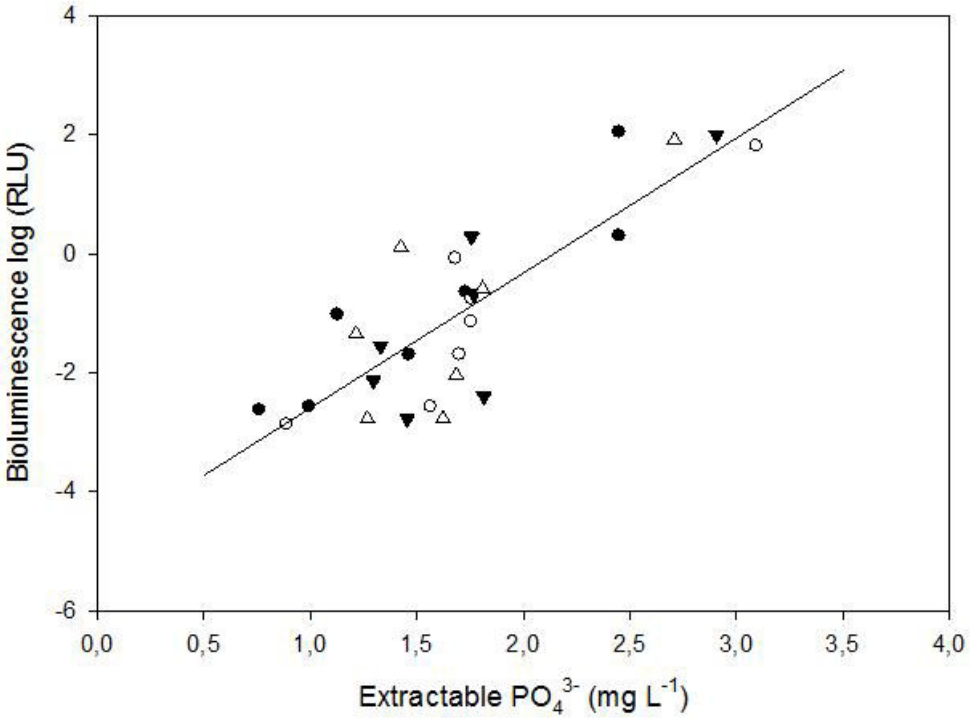

FIG. 2: Relationship between bioluminescence and extractable phosphate in four soils Grass (●) Oat (◦), Swede (△) and Spring barley (▮)

3.3 Effect of pH on bioluminescence

Bioluminescence increased with increase in soil pH (Fig 3). All soil result showed a pH gradient with highly measurable RLU at pH 7.5.

Analysis of variance showed pH had an effect on the biosensor response in four different soils. The test indicated that biosensor response in RLU units was significantly influenced with increase in pH ($F_{4,27}=78.11$, $p<0.001$)

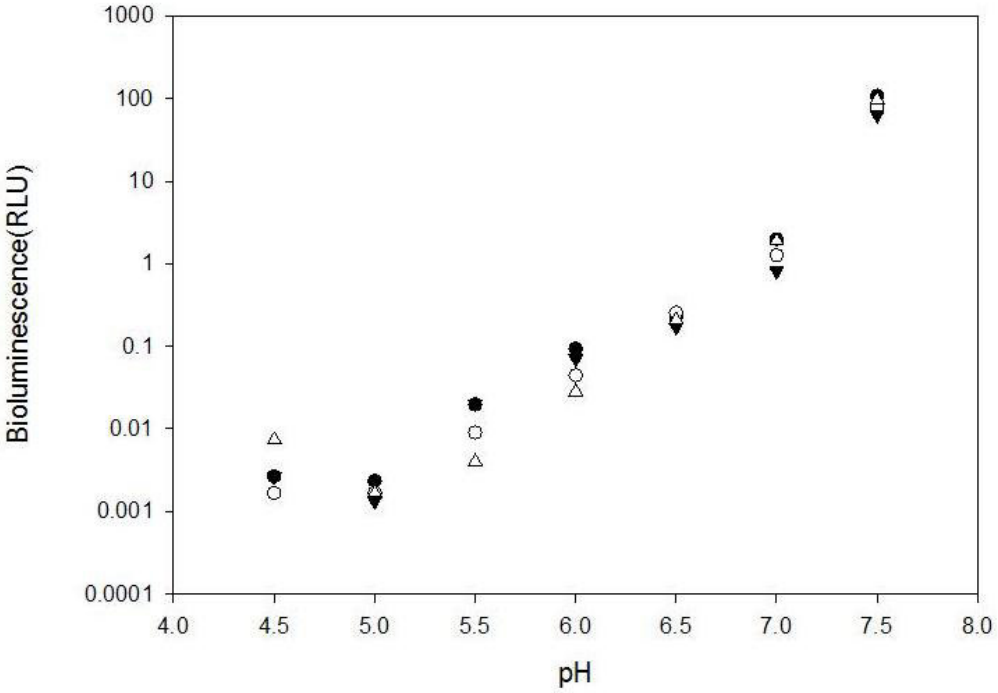

FIG. 3: Response relationship between bioluminescence of Biosensor E. coli MG1655/pPHO-lux with different pH in four soils Grass (●), Swede (①), Spring barley (Δ) and Oat (▫)

The regression analysis showed a strong correlation of RLU response from phosphate concentrations. This result was indicated for all four soils tested. Swede had the strongest relationshipof all soils while grass had the weakest relationship.

Table 1: Relationship between bioluminescence (RLU) and extractable phosphate in soils

Soil name	Regression analysis
Grass	r^2=0.688, p=0.007
Swede	r^2=0.812, p=0.043
Spring barley	r^2=0.531, p=0.025
Oat	r^2=0.833, p=0.011

The r2 values characterise linear regression between bioluminescence and chemically extractable, p value describes statistical significance of the linear regression.

Phosphate concentration in all four soils increased with increasing pH. The strongest increase was in cultivars of spring barley, while the weakest increase was in grass.

Table 2: Concentration of PO43- chemically extracted from all four soils

pH	Soil			
Grass (mg/kg)	Swede (mg/kg)	Spring oat(mg/kg)	Spring barley(mg/kg)	
4.5	29.88	38.00	46.98	38.89
5.0	22.88	48.66	26.68	43.63
5.5	43.95	50.52	51.06	54.44
6.0	33.88	36.43	52.72	39.88
6.5	51.88	54.24	52.75	52.95
7.0	73.50	42.71	50.46	52.69
7.5	73.51	81.26	92.68	87.19
pH	Soil			
Grass (mg/kg)	Swede (mg/kg)	Spring oat(mg/kg)	Spring barley(mg/kg)	
4.5	29.88	38.00	46.98	38.89
5.0	22.88	48.66	26.68	43.63
5.5	43.95	50.52	51.06	54.44
6.0	33.88	36.43	52.72	39.88
6.5	51.88	54.24	52.75	52.95
7.0	73.50	42.71	50.46	52.69
7.5	73.51	81.26	92.68	87.19

4. Discussion

The application of the biosensor Escherichia coli MG1655/pPHO-lux has been very confined to testing bioavailability in water sample as previously reported by Dollard and Billard (2003).This fusion becomes inducible with increasing amount of phosphate starvation in a dose dependant manner by producing light via the lux operon. The detectable concentration for the starvation period was not determined. Further experiments are

needed to determine the least observable effective concentration (LOEC). An LOEC of 0mM was reported by Cardemil et al., (2010); Dollard and Billard, (2003).To determine the maximal amount of phosphate at which there is no bioluminescence would require a regression analysis to infer on the maximal concentration. Bioluminescence inhibition would be a result of excess phosphate in the medium, hence the cells were unable to grow resulting in cell death. A study reported a high bioluminescence at log phase (>1000 RLU) at concentration of 1mM in relatively short time as compared to this study. The high increase in RLU may have been as a result of the kind of instrument used for measuring light output or treatment of the sensor twice with one volume of MOPS minimal media. Statistical comparison between the varying phosphate concentration values with reference to luminescence provided a clearer picture of significant difference ($p \leq 0.00$).

The biosensor response to the four soil used for this study showed a gradient in pH from the plotting and interpretation of data (Fig 3). but was further statistically tested. Higher relative light units (RLU) were measured with increase in pH ($p<0.001$).The biosensor used consist of plasmid with an inducible promoter of the alkaline phosphatase gene (phoA) hence could be the reason for the high RLU measured at pH 7.5. Dollard and Billard, (2003) reported that other phosphate ligands served to supply the biosensor with environmental phosphorus source, which could probably be through the breakdown of periplasmic phosphatases and thus repress the expression of the phoA::luxCDABE fusion. This could also be an explanation as to why the starvation system didn't work at low pH because of the immobility of phosphate. Other work previously done by Dollard and Billard, (2003) mostly applied the biosensor to detect the presence of low concentration of phosphate in waste water samples but failed to mention the pH at which the assay was done. Hence this study shows that pH is a necessary factor for any starvation assay with regards to measuring nutrients in soil.

The chemical measurement of available phosphate of all soil showed an increase in concentrations (Table 2). Measureable RLU values of 1 was detected in all soil with pH 7 but a surprising increase of 100 fold occurred at pH 7.5 which may not only be as a result of only just a starvation system but also a possibility that the biosensor works better at an alkaline pH.

Upon applying the biosensor to the different soil sample, result showed that there is astrong correlation ($p<0.05$) between the chemically extractable phosphate and the bioluminescence pattern (Fig 2).

Prior to the present study, there is no known application of the biosensor to measure phosphate in soil solid phase that adequately quantifies phosphate bioavailability. An assessment of the chemical and biological components of phosphate availability in this study linked a relationship between both methods. While this study demonstrates the usefulness of bioluminescent-based microbial assay as a technique in the context of bioavailability, it also highlights that matrix performance is not yet validated.

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Author Contributions:
Author UGO and PGI conceived and designed the experiments; Author G.F.A., U.G.O., and P.G.I. performed the experiments; Authors O.O.O. and D.O.A. analyzed the data; while all contributed other resources towards the success of the research. U.G.O. and O.O.O. wrote the paper."

Conflicts of Interest:
"The authors declare no conflict of interest."

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