# Pyroligneous acid (PA) – Evaluation of Impacts on Aquatic and Terrestrial biota

**Report prepared for Northside Industries Pty Ltd. by**

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## List of Acronyms

PA: Pyroligneous acid

CA: Chromosomal Aberrations

GCER: Global Centre for Environmental Remediation

MI: Mitotic Index

OD: Optical Density

OECD: Organisation for Economic Cooperation and Development

OTM: Olive Tail Moment

ATR-FTIR: Attenuated total reflection – Fourier-transform infrared

AGRF: Australian Genomic Research Facility

## Glossary

Aliquot: small portion.

Cladoceran: Cladocera is an order of small crustaceans commonly called water fleas

Growth inhibition: Reduction in growth caused by a (chemical) treatment.

$LC_{50}$: Lethal concentration of a compound causing 50% mortality in an organism.

$EC_{50}$: Effective concentration of a compound causing 50% inhibition in the growth of an organism

$IC_{50}$: Inhibitory concentration of a compound causing 50% inhibition in the growth of an organism

## EXECUTIVE SUMMARY

This project investigated the potential impacts of pyroligneous acid (PA) to the aquatic and terrestrial biota under laboratory conditions. Refined PA (PyroAg) was provided by "Northside Industries". The suite of tests used in this study included (a) freshwater (*Raphidocelis subcapitata*) and terrestrial (*Chlorella* sp. MM3) algal growth inhibition assays; (b) water flea (*Daphnia carinata*) acute toxicity assay; (c) earthworm (*Eisenia fetida*) acute, chronic, and genotoxicity assays; (d) phytotoxicity of duckweed (*Lemna minor*); (e) cytotoxicity/genotoxicity assay using onion (*Allium cepa*); and (f) microbial diversity in soils using pyrosequencing analysis.

The salient findings of these studies are:

- Both the algal test species representing freshwater and terrestrial environments (*Raphidocelis subcapitata* and *Chlorella* sp. MM3) exhibited similar response towards exposure to PA with $IC_{50}$ values of 0.05% and 0.06% PA, respectively.
- The toxicity of PA to Water flea (*Daphnia carinata*) was lower in natural creek water (48 h $LC_{50}$, 0.17%) compared to the cladoceran growth medium (48 h $LC_{50}$, 0.04%).
- Onion (*Allium cepa*) cytotoxicity assay showed no notable irreversible cytotoxicity in onion meristem root tip cells.
- Comet assay (a measure of DNA damage) in earthworm showed that all the concentrations of PA used in this study did not exhibit any notable DNA damage.
- The toxicity of PA to duckweed *Lemna minor* (in terms of the growth parameters such as frond number and chlorophyll content) was lower in natural creek water ($EC_{50}$, 0.07%) than the growth medium ($EC_{50}$, 0.04%).
- The earthworm acute and chronic toxicity revealed that PA at lower concentrations promoted the cocoon and juvenile production when compared to their control at a concentration up to 0.5% of PA.

- A positive effect with an increase in the microbial diversity was observed in both the soils treated with PA at lower concentrations (0.01% and 0.1%).
- The plant growth-promoting bacteria (PGPB) genera diversity was increased in both soils at lower concentrations (0.01 and 0.1%)

The environmental risk depends on the receiving environment, the presence of susceptible biota, and toxicant loading. Aquatic toxicity assays were included in this study in order to check the offsite impact of PA. The results of the present studies suggest that the PA is unlikely to pose a risk to the aquatic biota at environmentally relevant concentrations (0.001%). Whereas, in earthworms, the lower concentrations (below 0.5%) of PA promoted the cocoon and the juvenile production. The results of the pyrosequencing analysis showed a positive effect on the microbial diversity in soils treated with PA up to 1%. Also, the plant growth-promoting bacteria (PGPB) genera such as *Azospirillum*, *Acetobacter*, *Bacillus, Bradyrhizobium*, *Herbaspirillum Mesorhizobium*, *Pseudomonas,* and *Rhizobium* were observed to be increased in PA treated soils.

# 1 Introduction

## 1.1 Background

During the last decades, the use of synthetic fertilizers and pesticides have been on rising worldwide to improve crop production. This rise in the use of synthetic fertilizer, pesticide, and unsustainable farming methods affected different environmental compartments, like soil, water, and air, which in turn could lead to the major food crisis in the world. In order to, reverse the negative impact caused by the synthetic crop production systems, the attention has gone towards promoting the use of alternative environment-friendly sustainable methods. Recently, the organic by-products from agriculture, which otherwise goes as wastes in the environment are now recycled and used effectively to increase agricultural production. Likewise, in the charcoal making process, a lot of gases which otherwise emitted into the atmosphere are

being trapped to make condensate called pyroligneous acid (PA) or wood vinegar (Mathew and Zakaria, 2015). The source of PA differs from various potential plant biomass sources with different lignocellulose contents. The chemical composition of PA varies based on their type and nature of the raw material used and their heating temperature (Mathew and Zakaria, 2015). Due to the complexity of the PA composition, there has been growing interest recently in the chemical characterization of the PA obtained from different sources (Souza et al., 2012). Few studies on PA reported its positive effect in promoting soil health, crop performance, and crop tolerance to pest and disease infection (Harel et al., 2012). This improvement in the soil health and plant performance is closely related to the soil microbial community.

To attain a good knowledge of soil microbial communities, a high throughput culture-independent techniques like 16S rRNA based next-generation sequencing/ pyrosequencing that enables to analyze a vast number of the sequences to visualize and characterize microbial community was employed recently (Cole et al., 2009). Moreover, the high resolving power of this technique provides significant access to uncultured bacterial groups that are otherwise not detected by other microbial fingerprinting methods (Uroz et al., 2010). The PA contains readily degradable organic compounds which could be used by the microbes for their metabolism and thus may result in the increased beneficial microbial biomass, population growth and microbial efficiency (Steiner et al., 2008). The activity of soil enzymes such as dehydrogenase, hydrolase, urease, and phosphatase gives a broad view of soil biology and act as a good indicator of soil fertility. The enzyme activity in soil is often correlated with soil nutrition and associated biological effects.

Despite the previous reports on the beneficial uses of PA, it is essential to know the ecotoxicological effects of the PA administered in the agricultural lands. Moreover, the possibility of high priority environmental contaminant polycyclic aromatic hydrocarbons (PAHs), which are usually produced as the byproduct of pyrolysis process and incomplete combustion of fossil fuels and other organic substances may also present in PA. The USEPA highly regulates the PAHs, because some PAHs are reported to possess the carcinogenic,

mutagenic, and teratogenic properties. New biological approaches are currently being employed to monitor toxicity by measuring the biochemical and cellular responses of soil organisms (Calisi et al., 2011). As a result, several biological assays have been developed to examine the ecotoxicological effects of hydrocarbons in soils such as *A.cepa* assay to check the genotoxicity by monitoring changes in DNA (Yahaya et al., 2011), root nodulation assay in leguminous plants (Sivaram et al., 2019). Acute toxicity assay with *Lemna minor* (duckweed) and *D.carnata* (water flea) (Üçüncü et al. 2014), and algal growth inhibition assays are also widely used for assessing the toxicity of contaminated sediments and waters (Greene et al., 1988).

Earthworms have been widely used as representative organisms when analyzing soil quality and toxicity due to their direct contact with the soil through burrowing into the soil and mixing it regularly in their gut (Eijsackers et al., 2001). The earthworm species *E. fetida* is used as the standard species recommended by OECD for evaluating the ecotoxicity of xenobiotics (Yan et al., 2011; Sforzini et al., 2012). Earthworms are rich in lipid content in their body tissues and also by ingestion take up and accumulate a specific concentration of lipophilic xenobiotics in their bodily tissues (Krauss et al., 2000). The survival and reproduction rate of earthworms in contaminated soil predicts the toxicity of contaminants to higher organisms (Khan et al., 2013). Also, the single cell gel electrophoresis often referred to as the comet assay performed with various earthworm species, is regarded as a sensitive tool for examining the genotoxicity of pollutants present in the environment (Bonnard et al., 2009).

Keeping the above scientific information and the knowledge gaps on the potential impacts of PA, this project was formulated to evaluate the possible effects of PA on aquatic and terrestrial organisms and to determine the safe limits protective of these organisms.

### 1.2 Objectives

The main objectives of this investigation are to develop Australia-specific knowledge on the environmental impacts of PA, in particular, its impacts on aquatic and terrestrial environments. These environments were taken into account since the PA may be used in Australia and in agricultural fields and in close proximity to water bodies. The specific objectives of this study are:

- To determine the effect of PA on aquatic (freshwater) organisms such as alga and water-flea
- To determine the effect of PA on terrestrial alga;
- To determine the effect of PA on terrestrial biota such as earthworms (*Eisenia fetida*);
- To determine the phytotoxicity of PA to aquatic plant (*Lemna minor*);
- To determine the cytotoxicity/genotoxicity of PA to onion (*Allium cepa*) root tip meristem cells;
- To determine the impact of PA on soil health.

# 2 Materials and methods

### 2.1 Chemicals

Pyroligneous acid (PA) was provided by the Northside industries. All the reagents and solvents used in the study were analytical grade having been purchased from Sigma-Aldrich, Australia. The working solutions of PA were prepared freshly using sterile deionized water (Milli-Q, 18 $\Omega$ $cm^{-1}$, ELGA Lab Water, UK).

### 2.2 Experimental soil and water

Two soils (soil A and soil B) with no history of pesticide application collected from Lovedale, Hunter valley – soil A and Boanbong road, Palm beach – soil B, one natural - creek water from Callaghan, NSW were used in this study. The selected physicochemical properties of the soils are given in Tables 1 and 2.

### 2.3. Analysis of PA in liquid matrices

The PA spiked liquid matrices were used for quantifying acetic acid concentrations following attenuated total reflection-Fourier-transform infrared (ATR-FTIR) spectroscopy (Abinandan et al., 2019b). In brief, one milliliter of the sample was placed on a horizontal plane ATR (HATR) trough prism made of Zinc selenide (ZnSe) crystal (Refractive Index 2.4) for IR measurements (PIKE PN: 022-2010-45) along with relative control that served as blank. FTIR spectroscopy (Agilent Technologies) was used to scan mid-IR range (400–4000 $cm^{-1}$). For duplicate samples, a total of 8 scans were performed to improve the signal-noise ratio, and the mean data values were used for analysis (Abinandan et al., 2019).

### 2.4 Biological assays

Toxicity assays play an important role in the environmental risk assessment of chemicals and are included in the regulatory framework. Since no test species is consistently sensitive to all contaminants, a battery of assays comprising different organisms is required for the assessment of chemicals (Megharaj et al. 2000).

The following assays, which are routinely used for risk assessment of chemicals, were used in this study:

(a) Microalgal growth inhibition;

(b) Fresh water-flea (*Daphnia carinata*) acute toxicity;

(c) Phytotoxicity of aquatic plant (*Lemna minor*);

(d) Genotoxicity/cytotoxicity assay using onion (*Allium cepa*)

(e) Earthworm (*Eisenia fetida*) acute, reproductive, cyto- and genotoxicity assays;

#### 2.4.1 Microalgal growth inhibition assay

Algal growth inhibition tests were performed, according to Megharaj et al. (2000). *R. subcapitata* growth inhibition, an assay sensitive to a variety of chemicals, is widely used for assessing the toxicity of contaminated sediments and waters (Greene et al., 1988). Axenic culture of *R. subcapitata* was maintained in OECD-recommended growth medium (OECD, 1984a), under continuous illumination at 25 °C in an orbital shaker set at 100 rpm. Growth

inhibition of the alga was used as the endpoint in this bioassay. All assays were conducted in triplicate. Portions of sterile growth medium containing concentrations of PA ranging from 0.001 to 5% were placed in sterile culture flasks were inoculated with the exponentially-growing culture of *R. subcapitata*. Controls containing only growth medium and alga were included in the test. The test vessels were incubated in a temperature-controlled (25 °C) orbital shaker set at 100 rpm under continuous illumination [200 µE/m2/sec PPFD (Photosynthetic Photon Flux Density)] provided by cool white fluorescent lamps. At the end of 96 hours, the growth of the alga in different treatments was estimated, in terms of chlorophyll content by measuring autofluorescence (Podevin et al., 2015). In brief, 100 µL of samples were taken in 96-well microplate and measured at an excitation wavelength (440 nm) and an emission wavelength (690 nm) using fluorescence plate reader (Perkin Elmer). The data obtained as relative fluorescence units (RFUs) were used for growth inhibition measurement.

The soil algal growth inhibition assay was conducted using terrestrial alga, *Chlorella* sp. MM3 following the standard procedure. The alga was grown in Bold's basal medium. Exponentially growing *Chlorella* cells were exposed to a dilution series of PA prepared in growth medium and incubated under continuous light in a temperature-controlled illuminated incubator described as above for freshwater algal assay. The test was conducted in triplicate and untreated medium inoculated with alga served as the control. Growth inhibition was assessed in terms of chlorophyll content, as mentioned above. The test duration was 96 h.

### 2.4.2 Fresh water-flea (*Daphnia carinata*) acute toxicity

The acute toxicity was performed as per OECD guidelines for testing of chemicals (OECD, 2000). Twenty-four-hour old neonatal cladocerans (daphnids) were used as test organisms. Prior to the acute toxicity experiment, an initial range-finding test was conducted. The sensitivity of the test organism was confirmed by exposing the animals to a known toxicant (cadmium up to 1 mg $L^{-1}$) under the same experimental conditions. The nominal concentrations used in the toxicity tests ranged between 0.001 to 1%. The survival test was conducted at a temperature of 21 ± 2°C with a photoperiod of 16 h and 8 h dark cycle under

600 lux light intensity. Cladoceran growth medium without PA served as controls. Toxicity tests were conducted in triplicate with ten organisms per replication. Cladocerans exhibiting immobility within 15 seconds after the gentle stirring with the glass tube were considered mortal, which was supposed to be the endpoint for determining acute toxicity. Immobility (mortality) after 24 and 48 h were recorded from each treatment and control. Then the data were analyzed statistically in Minitab 18 software to determine the concentration of the test chemicals that immobilized 50% ($LC_{50}$ value) of the daphnids in each treatment.

### 2.4.3 Phytotoxicity of aquatic plant (*Lemna minor*)

#### 2.4.3.1 Cultivation and growth of *L. minor*

Wild type *L. minor* L. culture which is routinely maintained at the Global Centre for Environmental Remediation, The University of Newcastle, was used in the test. The modified Steinberg medium used for growing *L. minor* was prepared according to ISO (2007) Organisation for Economic Co-Operation and Development (OECD) protocol for toxicological testing (OECD, 2006). The Steinberg medium (pH 5.5) is composed of $KNO_3$ (3.46 mmol $L^{-1}$), $Ca(NO_3)_2.4H_2O$ (1.25 mmol $L^{-1}$), $KH_2PO_4$ (0.66 mmol $L^{-1}$), $K_2HPO_4$ (0.072 mmol $L^{-1}$), $MgSO_4.7H_2O$ (0.41 mmol $L^{-1}$), $H_3BO_3$ (1.94 µmol $L^{-1}$), $ZnSO_4.7H_2O$ (0.63 µmol $L^{-1}$), $Na_2MoO_4.2H_2O$ (0.18 µmol $L^{-1}$), $MnCl_2.4H_2O$ (0.91 µmol $L^{-1}$), $FeCl_3.6H_2O$ (2.81 µmol $L^{-1}$), and EDTA Disodium-dihydrate (4.03 µmol $L^{-1}$).

*L. minor* fronds were surface sterilized using 0.5 % (v/v) sodium hypochlorite solution for 1 minute and then rinsed with sterile deionized water. Surface sterilized fronds were grown for 14 days in Steinberg medium for acclimatization. Three to four frond colonies (5 colonies per treatment) (Naumann et al. 2007) were randomly selected and transferred to the test medium containing 0.001 to 1% concentrations of PA. The test containers were incubated at 24 ± 2 °C under continuous cool white fluorescent lighting (6500 – 10000 lux) for an exposure duration of 7 days (standard OECD requirements). All exposures were performed in triplicate.

#### 2.4.3.2 Growth parameters assay

According to OECD guidelines for whole organism measurement of plant health, a) frond number was recorded before exposure and at the end of the exposure; and c) inhibition of growth on the basis of frond number was calculated according to OECD guidelines Eq. (1).

$$\%I_r = \frac{\mu_c - \mu_t}{\mu_c} \times 100 \quad \text{..... (1)}$$

where:

$\%I_r$: Percent inhibition in average specific growth rate

$\mu_c$ : mean value for μ in the control

$\mu_t$ : mean value for μ in the treatment group

#### 2.4.3.3 Estimation of chlorophyll content and root cell viability

At the end of the test, the fronds were removed from the exposure vessels and rinsed with deionized water and dried with soft paper and weighed in pre-weighed microcentrifuge tubes. Fresh tissue (125 mg) was homogenized in 80% (w/v) ice-cold acetone using a glass homogenizer, centrifuged at 5000 g for 10 min and the absorbance of the clear extract was measured at 663, 646 and 470 nm wavelength using spectrophotometer (Synergy™ HT, Bio-Tek equipped with KC4 software). The concentration of total chlorophyll (mg $g^{-1}$ fresh weight) was calculated based on the method described in our previous study, Sivaram et al., (2018a).

For the root cell viability analysis, the duckweed was transferred from the tested solutions to vials containing 0.05% Evans Blue in distilled water. Staining was performed for 10 min on a rotary shaker at 60 rpm, followed by 3 times wash for about 5 min in distilled water to remove the unabsorbed stain. Roots were observed and photographed with a microscope.

### 2.4.4 Genotoxicity/cytotoxicity assay using onion (*Allium cepa*)

#### 2.4.4.1 Test system and treatment

Healthy onion bulbs were grown in the dark in a cylindrical glass beaker containing deionized water at room temperature (25 °C). The deionized water was renewed for every 24 h to avoid mould formation. When the roots reached a length of 2-3 cm, they were treated with PA at concentrations ranging from 0.05 to 3% for 24 h. For preparing dilutions, PA (PyroAg) provided by the Northside industries (PyroAg) was considered as 100%, and the dilutions were made in Milli-Q water (V/V). Three replicates were maintained for each concentration.

#### 2.4.4.2 Cytotoxicity assay

The roots were harvested when they were about 1.5 - 2.0 cm long during their second mitotic cycle and were used for cytological analysis. The roots were analyzed for their cyto-genotoxicity following Feulgen's squash technique. Accordingly, the harvested roots were fixed immediately in the mixture of absolute ethanol and acetic acid (3:1 ratio) (Carnoy's reagent) for 24 h at 4 °C. The roots were transferred to the tube containing distilled water. After that, the roots were hydrolyzed with 1N HCl in a water bath at 60 °C for 10 min. The hydrolyzed roots were then washed with distilled water and transferred to a tube containing 70% (v/v) ethanol and stored at 4 ˚C until further use. The roots were then stained with basic Fuchsin dye and kept in the dark for 2h (Bakare et al. 2000; Kumari et al. 2009).

#### 2.4.4.3 Microscopic examination

About 2 mm stained root tips were squashed with 45% glacial acetic acid using coverslips and observed for any chromosomal changes using an Olympus BX41 epifluorescent microscope at 10 X and 40 X magnifications. The microscopic analysis included recording the mitotic index, the number of micronuclei in the interphase cells, and aberrant cells during metaphase, anaphase, and telophase. The mitotic index was calculated as the number of the dividing cells per number of 500 observed cells per slide for each treatment and control. Five slides per sample were analyzed (Fiskesjo 1997).

### 2.4.5 Earthworm (*Eisenia fetida*) acute, chronic, cyto- and genotoxicity assays

*Eisenia fetida* was selected for the study due to its common occurrence, the ease with which it can be cultured and its widespread use in toxicity assays as a recommended organism by international agencies. The starter culture of *E. fetida* was purchased from Bunning's Warehouse, Wallsend, and NSW to establish the laboratory culture. Worms were maintained in earthworm bedding (Magic Products, Amherst Jct, WI) and fed with cow dung at 20 ± 2 °C, 72% humidity and a 16:8 light/dark cycle. Adult worms ranging from 350 to 600 mg weight and well-developed clitella were used for the experiments.

#### 2.4.5.1 Acute toxicity assay

The effect of PA to *E. fetida* was studied according to the OECD guidelines (OECD,1984b). The complete tests were conducted in a controlled condition at room temperature 20 ± 2 °C following 16 h light and 8 h dark cycles. The test concentrations of PA used in this experiment ranged from 0.01 to 2% (PA: soil; vol : weight – Eg. Addition of 10 ml PA to 500 g soil resulted in 2% PA).Tests were conducted in triplicate. Each replicate consisted of 500 g of soil that was maintained with 35% moisture. The soil without PA concentration served as controls. Worms were cleaned and placed on moist filter paper in a ventilated container for depuration (24 h). Ten adult worms of uniform size with fully developed clitellum were released into each test container. The lids of all test containers were perforated to regulate aeration. The depurated earthworms were weighed before exposure to soil and at the end of 14 days' incubation. No feeding was given during the incubation period. Mortality after 14 days was recorded from each treatment and control. The data were analyzed statistically in Minitab 18 software to determine the concentration of the test chemicals that resulted in 50% mortality ($LC_{50}$ value) of the earthworms in each treatment.

#### 2.4.5.2 Reproductive toxicity assay

The earthworm chronic toxicity assay was performed according to OECD guidelines (OECD 2004). The experimental set-up was similar to the acute toxicity except the worms were fed

with 5g of cow dung at weekly intervals. The moisture content was checked throughout the experiment and added when necessary. The reproduction test was performed after four weeks (28 days) of soil exposure by removing adult worms from the test vessels and the cocoons produced were also counted and reintroduced back to their corresponding containers and maintained in the conditions as mentioned above. After eight weeks (56 days), the number of juveniles produced was recorded, and the data analyzed to determine the significance between treatment and control.

#### 2.4.5.3 Cyto- and genotoxic effects

The cyto-genotoxic effects of PA on earthworms were determined by comet assays. The earthworms following their exposure to PA (at concentrations ranging between 0.01 to 1%) in two different soils were analyzed for cyto-genotoxicity. The earthworms were washed with deionized water and allowed to depurate for 24 h. Following depuration, the worms were used for the cyto-genotoxicity assays.

Coelomocytes from depurated worms were extracted following the protocol described by Dhawan et al. (2009) with a slight modification. Coelomocytes from the earthworms extruded using extrusion buffer (5% ethanol, 95 % saline, 2.5 mg $mL^{-1}$ Ethylene diamine tetraacetic acid) (EDTA) , and 10 mg $mL^{-1}$ glycerol ether), were washed thrice and centrifuged for 3 min at 8000 rpm and resuspended in 1X phosphate-buffered saline (PBS). About 50 µL cell suspensions were suspended in 150 µL of 0.5% low-melting (37 °C) agarose and layered onto a slide pre-coated and then allowed to solidify at 4 °C. The number of cells per sample was 100-150. Cells were lysed using freshly prepared cold (4 °C) lysing solution whereby cellular proteins were removed, and the damaged DNA was liberated.

Subsequently, DNA unwinding was done with an alkaline solution (300 mM NaOH and 1 mM EDTA, pH 12.6) for 60 min. The pH of 12.6 helped to distinguish the extended comet tails from the heads easily. Electrophoresis was carried out for 30 min using 1X TBE as a running buffer at 300 mA and 25V in an ice bath in the dark. The slides were neutralized by washing them with 0.4 M Tris buffer, pH 7.5, and then rinsed with ultrapure water. Finally, slides were stained

using SYBER green fluorescent dye. Images were analyzed using a fluorescence microscope equipped with an excitation filter of 515–650 nm and a 580-nm barrier filter. Images of 50 randomly selected cells from each treatment were examined using Comet Score™ software.

**2.5 Pyrosequencing technique for analyzing soil microbial diversity**

The soils, A and B were spiked with 5 different concentrations of PA ranging from 0.01 to 5% in triplicates and incubated for a period of 8 weeks, triplicate samples were taken from each spiked and control treatments and the soil DNA was extracted from each soil using a Powersoil DNA isolation kit (Mo Bio Labs Inc.,Carlsbad, CA) following the manufacturer's protocol. The quality of the extracted DNA was checked by running the agarose gel and quantified further using the QuantiFlour® dsDNA system (Promega). The triplicate DNA samples from each treatment and control were mixed to obtain one composite sample for each treatment and control for further analysis.

**2.5.1 Illumina Miseq analysis**

The extracted DNA composite samples from each treatment and control were sent to the Australian Genome Research Facility (AGRF), Melbourne, Australia. The extracted genomic DNA was amplified in the AGRF using the 16S 27F-519R (V1- V3) primer with the (Forward sequence: AGAGTTTGATCMTGGCTCAG, reverse sequence: GWATTACCGCGGCKGCTG). The conditions for the primary polymerase chain reaction (PCR) were as follows: 95°C for 7min, 29 cycles of 94°C for 45s, 50°C for 60s, 72°C for 60s; 72°C for 7min using AmpliTaq Gold 360 Master Mix (Life Technologies, Victoria, Australia), The primary PCR. Amplicons were purified using AMP pure XP magnetic beads (Beckman Coulter). A secondary 8-cycle PCR with Illumina Nextera XT V2 indices and High Fidelity Takara Taq served to index each amplicon. The resulting amplicons were AMPure purified and measured using fluorometry (Invitrogen PicoGreen), and the obtained data were then normalized. The equimolar pool was measured by qPCR (KAPA) on the AB QuantStudio followed by sequencing on the Illumina MiSeq with 2 × 300 bp Paired-End V3 Chemistry.

### 2.5.2 Bioinformatics analysis

The sequences from read 1 and read 2 were merged to improve the sequence quality. Following this, de-replication, clustering, removal of singletons, FASTQ filter, and chimera filtering using the reference database were done by combining the QIIME and UPARSE algorithms (Caporaso et al. 2010). The reads were annotated using Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) (Meyer et al. 2008) employing the Ribosomal Database Project (RDP) as the annotation source. The maximum E-value cutoff was set to 1e−5, the minimum percentage identity cutoff was maintained at 60%, and the set minimum alignment length cutoff was 15 bp. The MG-RAST metagenome overview was applied to examine the quality of sequences in terms of base-pair count, sequence length, sequence count, GC percentage, and identification of rRNA features. The QIIME data file in Bbiom^ format was obtained from the QIIME plugin in MG-RAST which was imported into MEGAN4 (Metagenome Analyzer) for further analysis, the aim being to obtain rarefaction curves. (Huson et al. 2011). To estimate community diversity indices (Taxa, Chao-1, dominance, Simpson, Shannon, alpha diversity and Evenness), data obtained from the MG-RAST pipeline were imported into the PAST 3.06 (Hammer et al. 2001) spreadsheet and were calculated by default settings (Bootstrap N = 9999, Bootstrap type: percentile).

# 3. Results and discussion

### 3.1 Experimental soil and water properties

The water and soil samples used in the study were analyzed for their physicochemical properties and inorganic ion composition (Table 1 and 2). The experimental water has a pH of 7.2, and the concentrations of inorganic ions were listed in Table1. The experimental soils, soil A and soil B, were identified as loamy sand with a pH of 5.9 and 6.6, respectively. The percentage of carbon (C) in soil A and soil B were 0.51 and 1.48, respectively. The FT-IR was performed to analyze the concentration of PA in liquid after spiking with different concentrations of PA, and the results were presented in Fig.1. The displayed band region of FT-IR spectra shows that the wavenumber 1280 and 1700 $cm^{-1}$ of 1% of 5% glacial acetic acid, which was previously reported as the acetic acid (Ferri et al., 1999) peak matches with the 1% concentration PA peak. In addition, with the increase in the concentration of PA, the intensity of the peak in the above-mentioned wavenumber was also increased.

### 3.2 Effect of PA on algal growth inhibition.

Algae play a crucial role in the aquatic ecosystem by serving as essential nourishment for food webs and contributing to the self-purification of polluted water bodies (Ji et al. 2011). Hence the aquatic toxicity assays utilize alga as a model organism to test several chemicals. Algae such as *Selenastrum capricornutum* and *Chlorella vulgaris* were previously reported to be sensitive to the emerging contaminants (Boudreau et al. 2003). In this study, the sensitivity of freshwater (*Raphidocelis subcapitata*) and terrestrial algae (*Chlorella* sp.MM3) to PA concentration ranging from 0.001 to 5% was examined.

*Raphidocelis subcapitata*, a freshwater unicellular green alga commonly used for toxicity testing of freshwaters was used in this assay. The effects of various concentrations of PA on the growth of *R. subcapitata* are shown in Fig. 2. The $IC_{50}$ concentrations of PA were 0.05% (Table 3). *Chlorella* sp.MM3, a unicellular green alga that commonly occurs in soils and

freshwaters, was also used in this assay. The effects of various dilutions of PA are shown in Fig.2. The $IC_{50}$ concentrations of PA to *Chlorella* was 0.06% (Table 3).

### 3.3 Effect of PA to the water flea (*Daphnia carinata*)

Daphnia (water flea) is also one of the standard model organisms for the assessment of aquatic toxicity. As a toxicity indicator organism, the physiology, ecology, culturing, and utilization of *Daphnia* sp. have been well documented (Cooman et al. 2005). Daphnia naturally thrives in rivers and freshwater, and serve as a sentinel organism in the environment (Cáceres et al. 2007). Among the Daphnia species, *D. carinata*, a native Australian species, was reported as a freshwater invertebrate model organism for toxicity tests (Phyu et al. 2004). In the study, *D. carinata* was used for the assessment of the effect of PA since toxicity testing using indigenous species provides a more realistic model by minimizing endpoint variation due to the regional differences in water quality (Harmon et al. 2003).

Acute toxicity of PA to *Daphnia carinata* was carried out in both cladoceran water and natural creek water (Table 4; Fig. 3a and 3b). At 48h, the $LC_{50}$ value of PA was found to be higher in creek water (0.17%) than cladoceran water (0.04%). In cladoceran water, the 100% mortality occurred at 0.5% (24h) and 0.1% (48h). In comparison, in natural creek water, 100% mortality was recorded at 1% (24 h) and 0.5% (48 h). The toxicity of PA was less in natural creek water, which could be due to the interaction of PA with the suspended particles and dissolved organic matter present in the natural water.

### 3.4 Effects of PA to plants

#### 3.4.1 Plant phytotoxicity – Duckweed *(Lemna minor)*

As a most prominent producer, aquatic plants form the base for aquatic food chains, thereby balancing the ecosystem by influencing the spread and proliferation of animal population (Jiang et al. 2012). *Lemna minor* (duckweed) is used as a model organism for studying the aquatic toxicity due to its high reproductive rate, smaller size, and ease in cultivation and harvesting (Üçüncü et al. 2014). In this study, the effect of PA on frond number, chlorophyll content, and root cell viability in *L. minor* were analyzed.

The Steinberg medium and the creek water were spiked with different concentrations of PA, ranging from 0.001 to 1%. Of the two experimental waters used in this study, the Steinberg medium resulted in 50% reduction in the number of fronds produced in *L. minor* when exposed to a concentration of 0.04% (Table 5), whereas, in the natural creek water the $EC_{50}$ value is 0.07% (Table 5). At the end of the experimental period (7 days), complete inhibition of frond production in Steinberg medium and creek water was observed at 0.5 and 1% of and respectively (Fig.4a and 4b).

In all the test compounds, plants exposed to higher test concentrations were characterized by having a number of small and pale single fronds. This was evident with a corresponding decrease in the frond number and dry weight compared to the control. The existence of more single fronds and small colonies is considered to indicate environmental stress (Li & Xiong 2004; Radić et al. 2010). The efficacy of *L. minor* culture used in the phytotoxicity experiment was verified by spiking the growth medium with cadmium (1 mg $L^{-1}$) as the positive control.

**3.4.2 Total chlorophyll and root cell viability**

Estimating the chlorophyll levels in *L. minor* can be used to indicate growth inhibition (Taraldsen et al. 1990). The decrease in chlorophyll content in *L. minor* can be linked to the inhibition of enzymes associated with chlorophyll biosynthesis or peroxidation processes in chloroplast membrane lipids generated by the reactive oxygen species (Sandalio et al. 2001; Van Assche & Clijsters, 1990). Earlier studies demonstrated that the chlorophyll and carotenoids concentrations in *L. minor* were found to be reduced due to herbicides toxicity (Kirby & Sheahan, 1994; Radić et al. 2010).

Leaves of *L. minor* at higher concentrations showed signs of chlorosis and necrosis (dead and white fronds) with a corresponding reduction in chlorophyll content at 0.5 and 1% of PA concentrations (Fig. 5). There is a potential for PA to be translocated into aquatic environments through surface runoff and leaching. The results presented here demonstrate that the PA concentrations are non-toxic to non-target aquatic macrophyte, *L. minor* at concentrations up

to 0.05%. The *L. minor* roots were viable up to the PA concentrations of 0.1% (Fig. 6). The root cell membrane permeability of *L. minor* was not affected up to 0.1% of PA treatment.

### 3.4.3 Plant genotoxicity – Onion *(Allium cepa)*

Generally, plant systems have been used as indicator organisms in studying the mutagenesis in higher eukaryotes due to its well-defined genetic endpoints such as alterations in ploidy, chromosomal aberrations and chromatid exchanges (Kumari et al. 2009). The onion root chromosomal aberration assay is an established plant bioassay validated by the International Programme on Chemical Safety (IPCS, WHO) and the United Nations Environment Programme (UNEP) as an efficient and standard test for the chemical screening and in situ monitoring for genotoxicity of environmental substances (Kumari et al. 2009; Sivaram et al., 2018b).

Among fundamental chromosomal aberrations, Mitotic index (MI) is measured as a critical parameter for evaluating the cytotoxicity of various chemicals (Leme & Marin-Morales 2009). The cytotoxicity of any compound can be assessed in terms of a decline or increase in the mitotic index level (Smaka-Kincl et al. 1996). The effect of the PA on cell division and chromosomal behavior of *A. cepa* is shown in Figure 7. In untreated control onion tips, chromosomal aberrations were absent with a mitotic index (MI) value of 76.2 %. However, the mitotic index was observed to be reduced from 0.1% PA concentration onwards. A decrease in this index below 22% and 50% of the control can result in sublethal and lethal outcomes, respectively, in test organisms (Antosiewicz, 1990). The cytotoxicity in terms of a decrease in the mitotic index is possibly due to the prolongation of S phase and inhibition of DNA and protein synthesis as a result of plant exposure to a higher concentration of PA (3%).

Chromosomal aberrations are modifications in chromosome structure that occur due to a break or interchange of chromosomal material (Kumari et al. 2009). Higher test concentrations (0.5, 1, 3% of PA) resulted in chromosomal aberrations such as bridges, vagrant, laggard, multipolar anaphase, improper chromatid separation and disrupted metaphase (Fig. 8 and Fig.9). These observed chromosomal aberrants in this study were usually reversed with the

cell division. The maximum chromosomal aberration percentage (5.8%) was observed in 3% of PA treatment and are statistically insignificant. Non-reversible chromosomal abnormalities such as chromosomal stickiness, which are usually formed due to the degradation or depolymerization of DNA or as a result of DNA condensation and stickiness of inter chromosome fibers and micronuclei were not observed in all the test concentration of PA. At concentrations above 3% of PA, a complete inhibition of cell division and cell wall disintegration were recorded, which could probably due to the acidic nature of the PA.

### 3.5 Effect of PA in earthworms

#### 3.5.1 Earthworm acute and chronic toxicity assays

Studies on the effect of the PA on the earthworms were not done before, and there is no information exists on terrestrial effects. For assessing terrestrial ecotoxicity, earthworms are highly preferred (Lionetto et al. 2012). In this study, earthworms were also used as a model since they are important non-target animals in soils. *E. fetida* was used in the present study since it is an internationally accepted model species for toxicity assessment with a cosmopolitan distribution (Edwards, 1984). Earthworms' interaction with the soil contaminants is predominantly through dermal contact and ingestion which makes it an ideal test candidate for assessing the terrestrial ecotoxicity (Vijver et al. 2003; Morgan et al. 2004). Recently, several acute toxicity assays have been standardized to observe environmentally induced responses (Pauwels et al. 2013). Earthworm's acute exposure causes severe weight reduction, whereas prolonged exposure tends to affect the earthworms' later generations due to a malfunction in reproduction.

In this study, the PA concentrations varying from 0.01 to 2% were used for the acute toxicity of earthworms. The earthworms exhibited avoidance at 0.5 and 1 % of PA spiked soils a few hours after release. After 1 day, the earthworms started burrowing into the soils, and at concentrations above 1%, the survival percentage was completely affected (Fig.10a and 10b). In both soils, survival in percentage terms declined when the concentration increased in both

the soils. The earthworm weight-loss percentage was found to be statistically significant at 1% of PA spiked soils (Fig. 11a and 11b).

The percent weight loss and reproductive toxicity are more sensitive than survival. This study clearly demonstrates that chronic toxicity of PA in earthworms was not observed in the lower concentrations (0.01 to 0.2% concentration of PA). Moreover, the lower PA concentrations promoted the cocoon and juvenile hatching than the control soil, whereas the higher concentrations 0.5 and 1% reduced the cocoon and juvenile hatching compared to the control soils (Figs 12a and 12b).

**3.5.2 Earthworm cytotoxicity and genotoxicity assay**

In all organisms upon exposure to environmental contaminants molecular, biochemical, and/or physiological compensatory mechanisms change or get affected. These parameters serve as biomarker/indicators in assessing the toxicity (Reinecke and Reinecke, 2004). Among the molecular components, DNA of aquatic and terrestrial organisms is the important target of environmental stress (Frenzilli et al. 2001). The loss of DNA integrity indicated by the level of strand breakage was proposed as a sensitive indicator of genotoxicity (Balpaeme et al. 1996). For detecting DNA strand breakage, alkaline comet assay was reported to be a very sensitive method (Fairbairn et al. 1995). The genotoxicity of different concentrations of PA to earthworm (*E. fetida*) was measured using the alkaline comet assay. DNA strand breakage/damage in terms of percent DNA in the comet tail, and the olive tail moment (OTM) was calculated.

The different concentrations of PA were evaluated for their potential to cause DNA damage in earthworms through alkaline comet assay. In the positive control (BaP @ 1 mg $kg^{-1}$) severe DNA damage like a comet tail was observed. However, no DNA damage was observed in all the tested concentrations of PA. Therefore, the results of the comet assay indicate that the PA is unlikely to induce genotoxicity in earthworms (Fig.13).

### 3.6 Effect of PA on the microbial diversity- 16S Pyrosequencing analysis

#### 3.6.1 Illumina MiSeq Sequence attributes

After eight weeks of incubation, 16S rRNA amplicon-based sequencing revealed a diverse group of microorganisms in 10 samples from A and B soils. The total obtained reads were assigned to classified and unclassified bacteria, Eukaryota and unclassified and unassigned sequences. The bacterial sequences were used for the sequencing analysis, and all the other unclassified sequences, unclassified bacteria, and unassigned sequences were omitted for further analysis.

#### 3.6.2 Microbial community structure

In soil A, the total bacterial community contained 21 phyla and unclassified bacterial sequences, whereas, in soil B 25, bacterial phyla and unclassified bacterial sequences were present (Fig.14a and 14b). Among the bacterial phyla, the unclassified bacterial phyla constituted the largest population with an estimated relative abundance of 49.42, 53.55, 48.15 % in soil A with PA concentrations of 0.01, 0.1% and control without PA treatments respectively. The next abundant bacterial phyla in this study are *Proteobacteria*. The phylum *Proteobacteria* is the major phylum of Gram-negative bacteria. The phylum *Proteobacteria* includes *Alphaproteobacteria*, *Betaproteobacteria,* and *Gammaproteobacteria*. Generally, the phylum *Proteobacteria* contain bacterial species that are known to thrive in low nutrient levels. In addition to that, the phylum *Proteobacteria* include agriculturally important plant growth-promoting bacterial genera like *Pseudomonas* and *Acetobacter.* In the soil spiked with 1% PA treatment, the relative abundance of phyla *Actinobacteria* (34.35%) was highest followed by unclassified bacterial phyla. The phylum *Actinobacteria* are the major group of Gram-positive bacteria. This bacterial phylum is of greater importance in agriculture and forestry because of its contribution to soil systems. The bacteria in the phylum *Actinobacteria* helps to decompose the organic matter of dead organisms, thereby facilitating the nutrient uptake by the plants.

In soil B the relative abundance of the phyla *Actinobacteria* was observed to be highest in all the PA-treated soils and control soils except the soil that received highest PA concentration of 5%, in which the relative abundance of bacterial phylum *Firmicutes* was highest (62.66%)

followed by *Actinobacteria* (30.20%). The phylum *Firmicutes* contains the bacterial genera that survive well under extreme conditions. The relative abundance of phylum *Firmicutes* in soil spiked with 5% of PA could be due to the stress created by higher PA concentration (5%). Whereas, lower concentration of PA (0.01 and 0.1 %) in soil promoted the bacterial diversity and also the bacterial phylum that is agriculturally important with major plant growth-promoting bacterial genera.

**3.6.3 Changes in Plant Growth Promoting Bacteria (PGPB) in response to PA**

The effect of PA on the selected plant growth-promoting rhizobacterial genera based on the report on the beneficial plant bacterial genus in Australia by Gupta (2012) was studied with two experimental soils A and B (Fig.15 a and 15b ). The relative abundance of plant growth-promoting bacterial genera like *Acetobacter*, *Azospirillum*. *Bacillus*, *Beijerinckia*, *Bradyrhizobium*, *Corynebacterium*, *Herbaspirillum*, *Mesorhizobium Micromonospora Pseudomonas,* and *Rhizobium* was high in the PA treated soil with a concentration of 0.01% and 0.1%. Relatively most abundant plant growth-promoting bacterial genus in soil A and Soil B belonged to *Bacillus* and *Bradyrhizobium*. The genus *Bacillus* is Gram-positive bacteria and is ubiquitous in nature.

The plant beneficial *Bacillus* species associated with roots or rhizosphere and develop biofilms to increase the plant growth by enhancing the plant-available form of nutrients in rhizosphere, control disease-causing pathogenic microbial growth and induce pest defense systems (Garica – Fraile et al., 2015; Kang et al., 2015). The next most abundant bacterial genus is *Bradyrhizobium,* which is the genus of Gram-negative soil bacteria that fixes atmospheric nitrogen and makes it available for plants. Among the plant growth-promoting bacterial genus, the percentage number of reads for the genus *Pseudomonas* was increased in 0.01% of PA treatment compared to the control. The genus *Pseudomonas* was reported to be linked to a wide range of process involving plant growth promotion, disease control, nutrient cycling, nitrogen fixation, and bioremediation (deSouza, 2002). The increase in *Pseudomonas* genus than the control at lower concentrations in both the soil A and soil B gives a positive impact on the application of PA to the agricultural lands. The other PGPB genera such as

*Azospirillum*, *Acetobacter*, *Bradyrhizobium*, *Mesorhizobium*, *Pseudomonas,* and *Rhizobium* were observed to be increased in the soil A in all spiked concentrations up to 1%. Whereas, in soil B, the PGPB genera *Acetobacter*, *Bacillus*, *Herbaspirillum,* and *Pseudomonas* were observed to be increased compared to the control in all the PA spiked concentration.

# 4. General Summary

The results of these investigations on the effect of the PA to aquatic and terrestrial organisms demonstrate that the PA is unlikely to pose a risk to beneficial non-target organisms at agriculturally/environmentally relevant concentrations. Moreover, the results of cyto-genotoxic assays with the onion meristem root tips and earthworm comet assay showed that PA concentrations used in this study were not cyto-genotoxic. The results of chronic toxicity studies with the earthworm revealed that the PA treated soils at concentrations below 0.5% increased the earthworm cocoon and the juvenile production compared to their corresponding controls without PA treatment. Also, both the experimental soils exhibited an increase in the microbial diversity with the PA addition at the concentrations 0.01 and 0.1% than their corresponding controls. Moreover, the plant growth-promoting bacterial diversity was also increased in the lower PA concentrations (0.01 and 0.1%) compared to their controls.

**Table 1** Selected physicochemical properties of the experimental water

| Physico-chemical properties | Creek water ($\mu g\ L^{-1}$) |
|---|---|
| **pH** | **7.2** |
| **Metal Ions** | |
| **Be** | 0.00 |
| **V** | 0.40 |
| **Cr** | 0.80 |
| **Mn** | 5.81 |
| **Co** | 0.03 |
| **Ni** | 3.11 |
| **Cu** | 1.53 |
| **Zn** | 5.22 |
| **As** | 1.21 |
| **Se** | 0.12 |
| **Mo** | 2.43 |
| **Ag** | 0.01 |
| **Cd** | 0.11 |
| **Sb** | 0.15 |
| **Ba** | 15.81 |
| **Pb** | 0.32 |

**Table 2** Selected physicochemical properties of the experimental soils

| Soil | pH | Electrical conductivity (µS $cm^{-1}$) | Carbon (%) | Particle size distribution | | | Textural class |
|---|---|---|---|---|---|---|---|
| | | | | Sand (%) | Silt (%) | Clay (%) | |
| **Soil A** | 5.9 | 32.07 | 0.51 | 82.37 | 17.41 | 0.21 | Loamy sand |
| **Soil B** | 6.6 | 119.95 | 1.48 | 85.39 | 14.44 | 0.17 | Loamy sand |

**Table 3** Effect of PA to algae – $IC_{50}$ values

| Algal sp. | $IC_{50}$ value (% dilution) |
|---|---|
| ***Raphidocelis subcapitata*** | 0.05 (0.04 - 0.08) |
| ***Chlorella* sp. MM3** | 0.06 (0.03 to 0.09) |

$EC_{50}$: Effective concentration of the PA, causing 50% inhibition in the chlorophyll content of the alga; values in parenthesis represent 95% confidence limits.

**Table 4** Effect of PA to *Daphnia carinata* survival

| Experimental water | *Daphnia carinata* - $LC_{50}$ value (% dilution) | |
|---|---|---|
| | 24 h | 48 h |
| **Cladoceran water** | 0.06 (0.04 – 0.09) | 0.04 (0.02 – 0.07) |
| **Creek water** | 0.20 (0.17 – 0.35) | 0.17 (0.12 – 0.32) |

$LC_{50}$: Lethal concentration of the PA causing 50% mortality in the organism; values in parenthesis represent 95% confidence limits.

**Table 5** $EC_{50}$ values of PA based on growth responses in *L. minor*

| Experimental water | $EC_{50}$ value (% dilution) |
|---|---|
| | Frond number |
| **Steinberg medium** | 0.04 (0.03 – 0.05) |
| **Creek water** | 0.07 (0.05 – 0.09) |

$EC_{50}$: Effective concentration of the PA, causing 50% inhibition in the frond number of the *L. minor* values in parenthesis represent 95% confidence limits.

**Table 6** Effect of PA on earthworm survival (*Eisenia fetida*)

| Experimental soil | Earthworm 14-days |
|---|---|
| | **$LC_{50}$ value (% dilution)*** |
| **Soil A** | Above 1% |
| **Soil B** | Above 1% |

***** LC50 value could not be determined given the earthworms survived up to 1% PA dilution and all worms died at 2% PA dilution

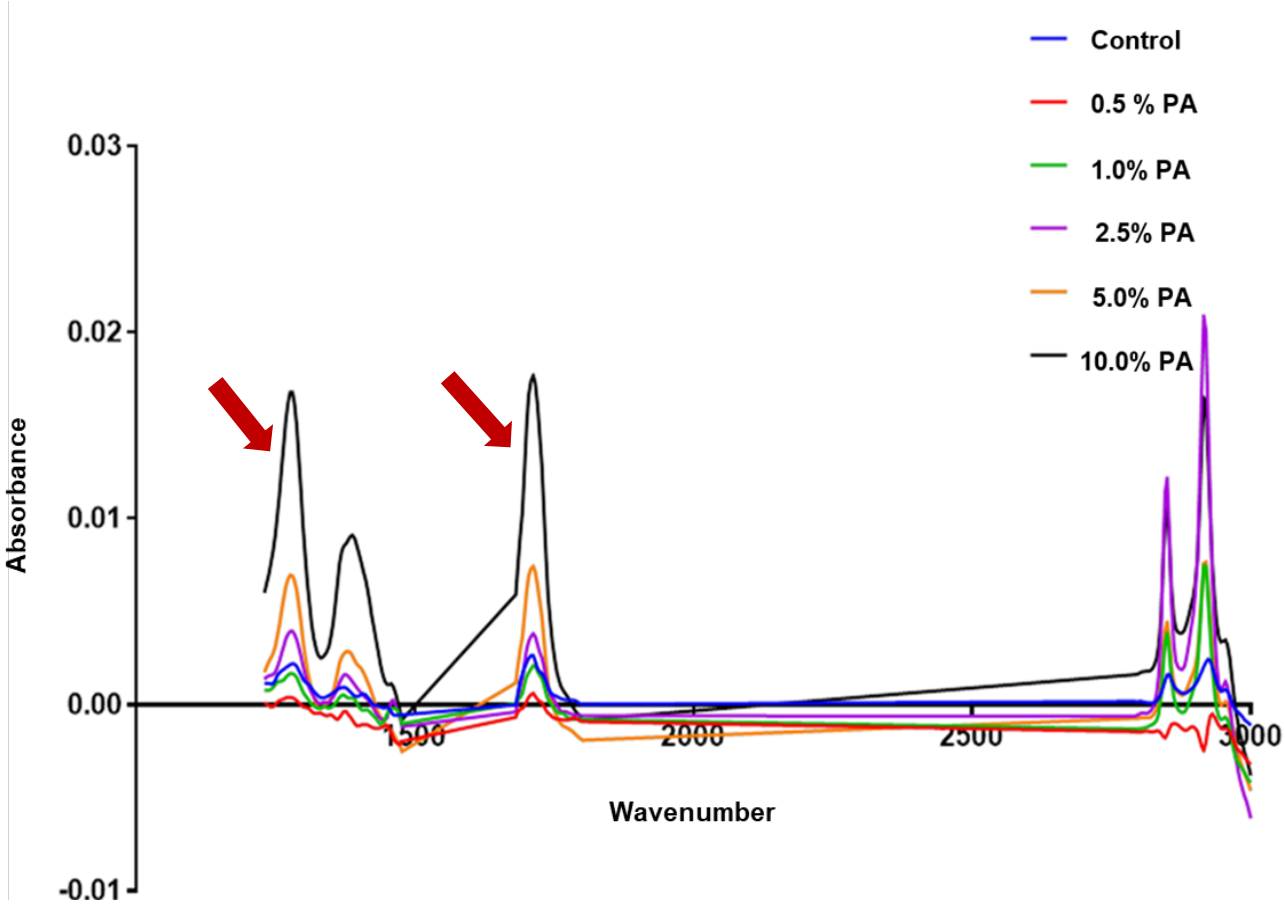
**Table 7** Estimated microbial diversity indices of Soil A and Soil B

| | **Soil A** | | | | **Soil B** | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **0.01%** | **0.1%** | **1%** | **Control** | **0.01%** | **0.1%** | **1%** | **5%** | **Control** |
| Taxa_S | 1734 | 1426 | 1375 | 1312 | 2302 | 2174 | 1929 | 1535 | 2167 |
| Dominance | 0.24 | 0.17 | 0.32 | 0.27 | 0.08 | 0.12 | 0.12 | 0.01 | 0.11 |
| Simpson | 0.76 | 0.83 | 0.68 | 0.73 | 0.92 | 0.88 | 0.88 | 0.98 | 0.88 |
| Shannon | 3.34 | 3.67 | 2.79 | 3.15 | 4.89 | 4.65 | 4.58 | 5.17 | 4.66 |
| Evenness | 0.02 | 0.03 | 0.01 | 0.02 | 0.06 | 0.05 | 0.05 | 0.11 | 0.05 |
| Alpha diversity | 299.4 | 235.91 | 253.51 | 259.00 | 426.20 | 423.50 | 392.00 | 263.50 | 404.9 |
| Chao-1 | 2349 | 2085 | 1947 | 1945 | 3151 | 2938 | 2629 | 2225 | 2933 |

**Table 8** Summary of toxicity results. (NOEC: No Observable Effective Concentration)

| Test species | PA concentration (% dilution) | NOEC (% dilution) |
|---|---|---|
| **Algae -** $IC_{50}$ (%) | | |
| *R. subcapitata* | 0.05 (0.04 - 0.08) | - |
| *Chlorella.* sp MM3 | 0.06 (0.03 - 0.09) | - |
| **Water flea** (*Daphnia carinata*) **–** $LC_{50}$ (%) - 48h | | |
| Cladoceran water | 0.04 (0.02 - 0.07) | 0.01 |
| Creek water | 0.17 (0.12 - 0.32) | 0.05 |
| **Duckweed -** $EC_{50}$ (%), Frond number | | |
| Steinberg medium | 0.04 (0.03 - 0.05) | 0.002 |
| Creek water | 0.07 (0.05 - 0.09) | 0.005 |
| **Onion (*Allium cepa*) -** $EC_{50}$ (%) | | |
| Mitotic Index | 1.20 (1.18 -1.22) | 0.05 |
| **Earthworm (*Eisenia fetida*)**<br>**Acute toxicity** – $LC_{50}$ (%) | | |
| Soil A | Above 1% | Upto 1% |
| Soil B | Above 1% | Upto 1% |

**Fig.1** FT-IR results of different concentrations of PA in water




Arrows are representing absorbance peak @ wavenumber ($cm^{-1}$) 1280 and 1700.

Control: 1% of 5% glacial acetic acid

**Fig 2.** Effect of PA on the chlorophyll fluorescence of *R. subcapitata and Chlorella* sp.MM3

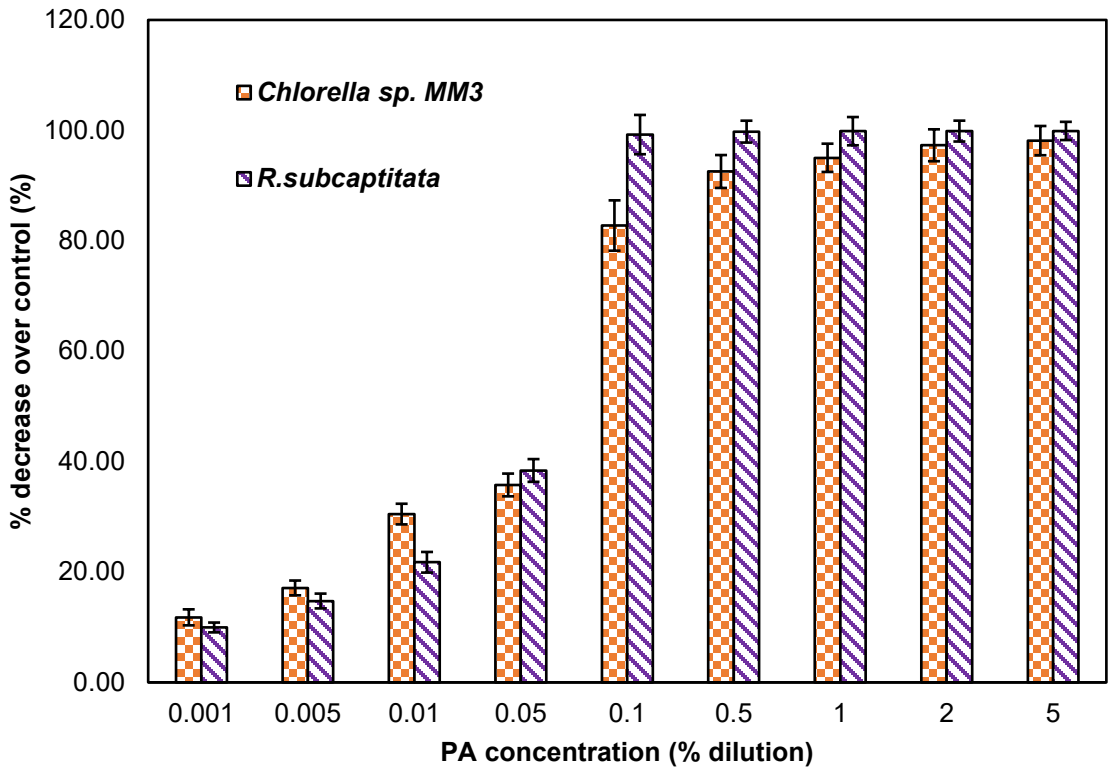


**Fig.3a** Effect of PA on the survival of *D. carinata* (24 and 48 h) in Cladoceran water

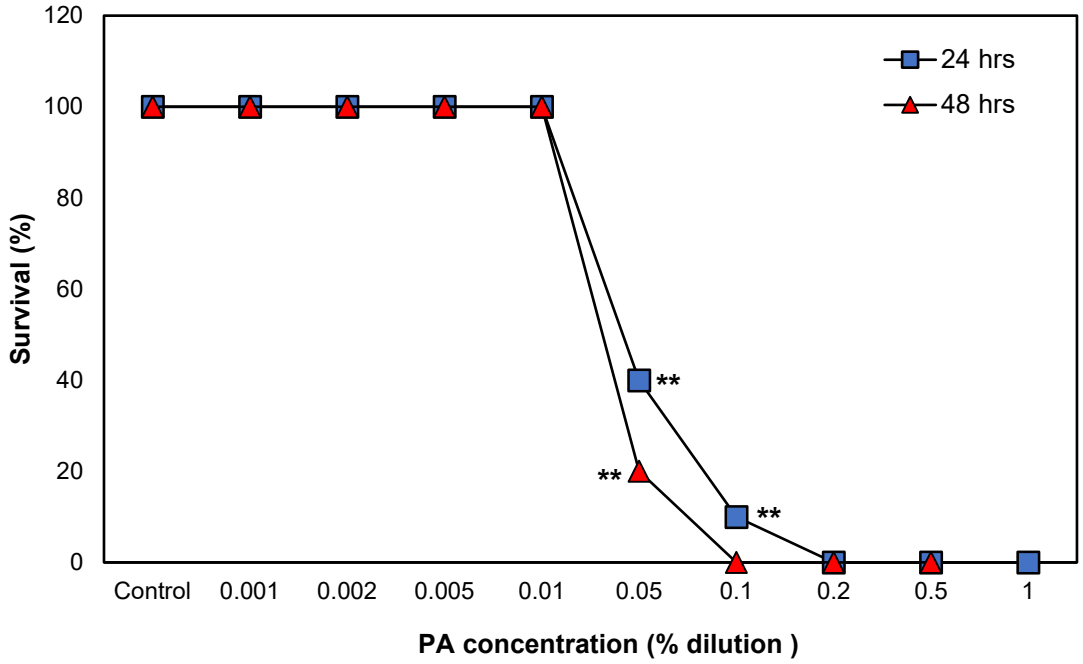
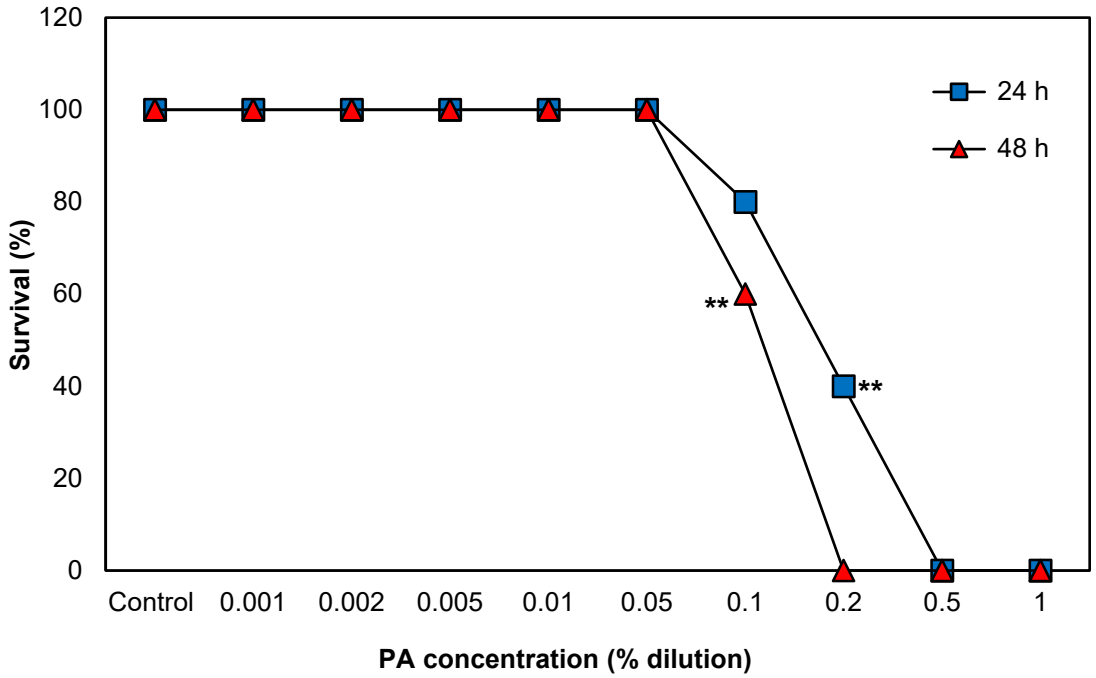
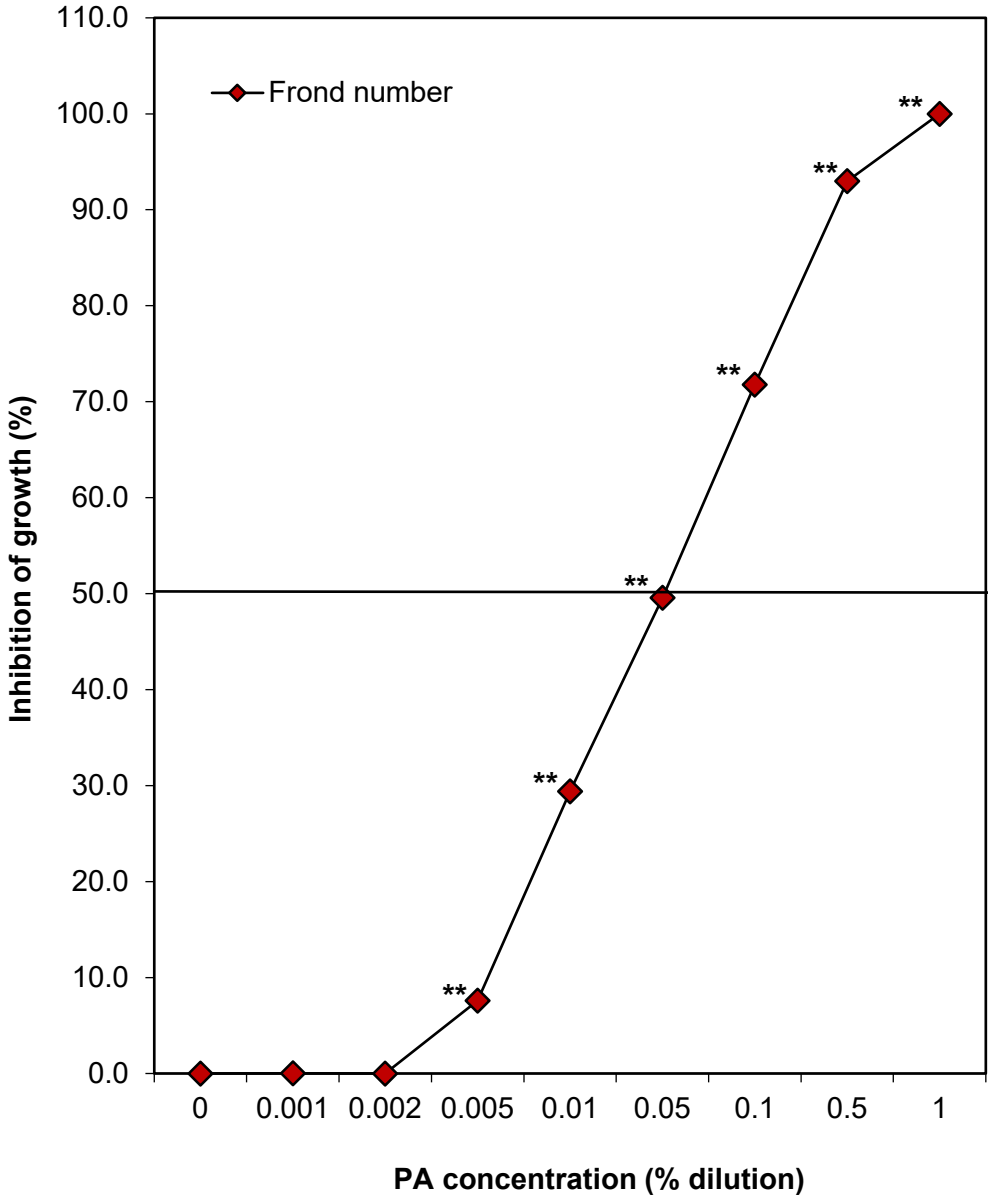



**Fig.3b** Effect of PA on the survival of *D. carinata* (24 and 48 h) in creek water



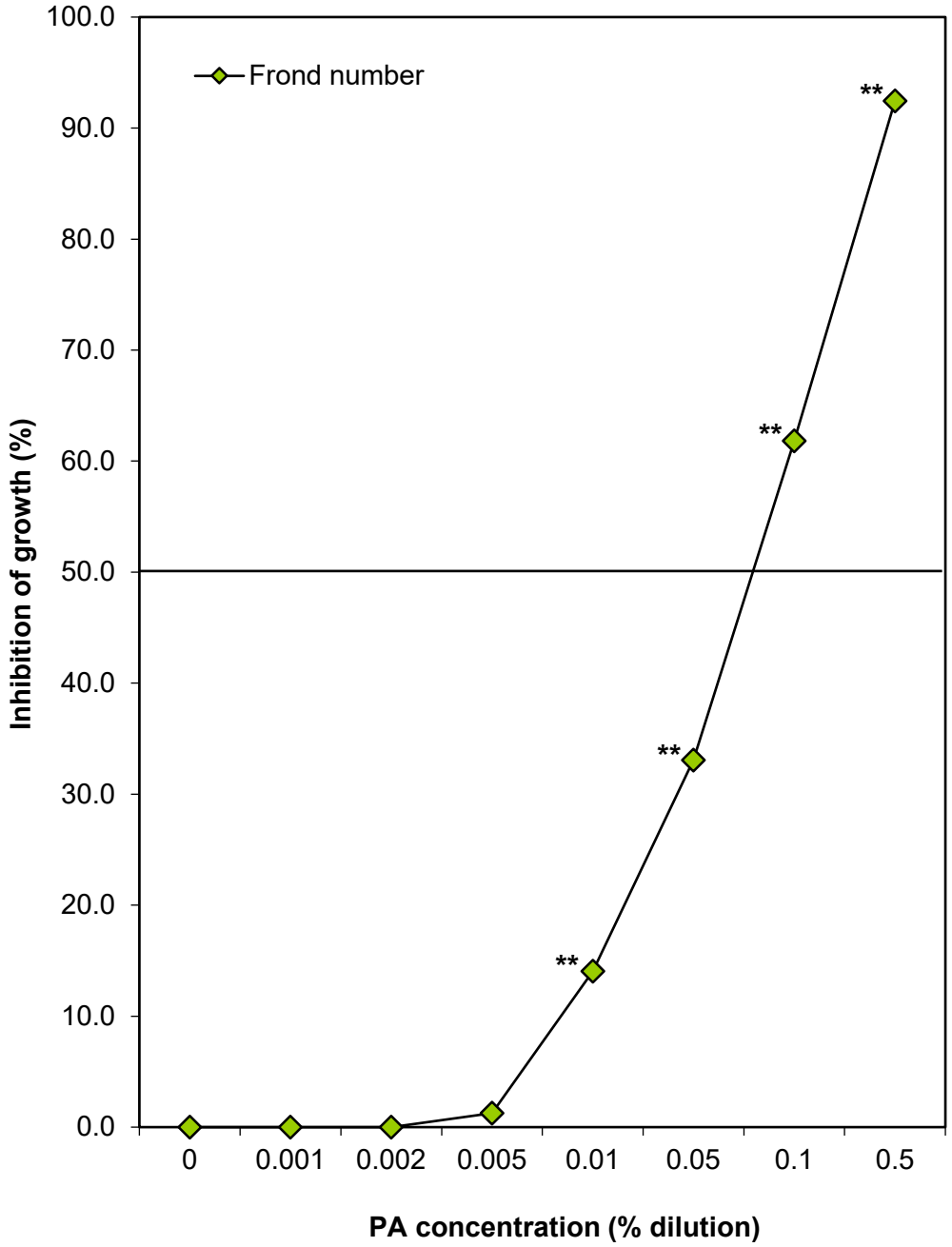

** - Statistically significant based on Student's *t*-test at *P* = 0.01

**Fig. 4a** Growth inhibition in *L. minor* (based on frond number) after exposure to different concentrations of PA (for 7 days) in Steinberg medium.



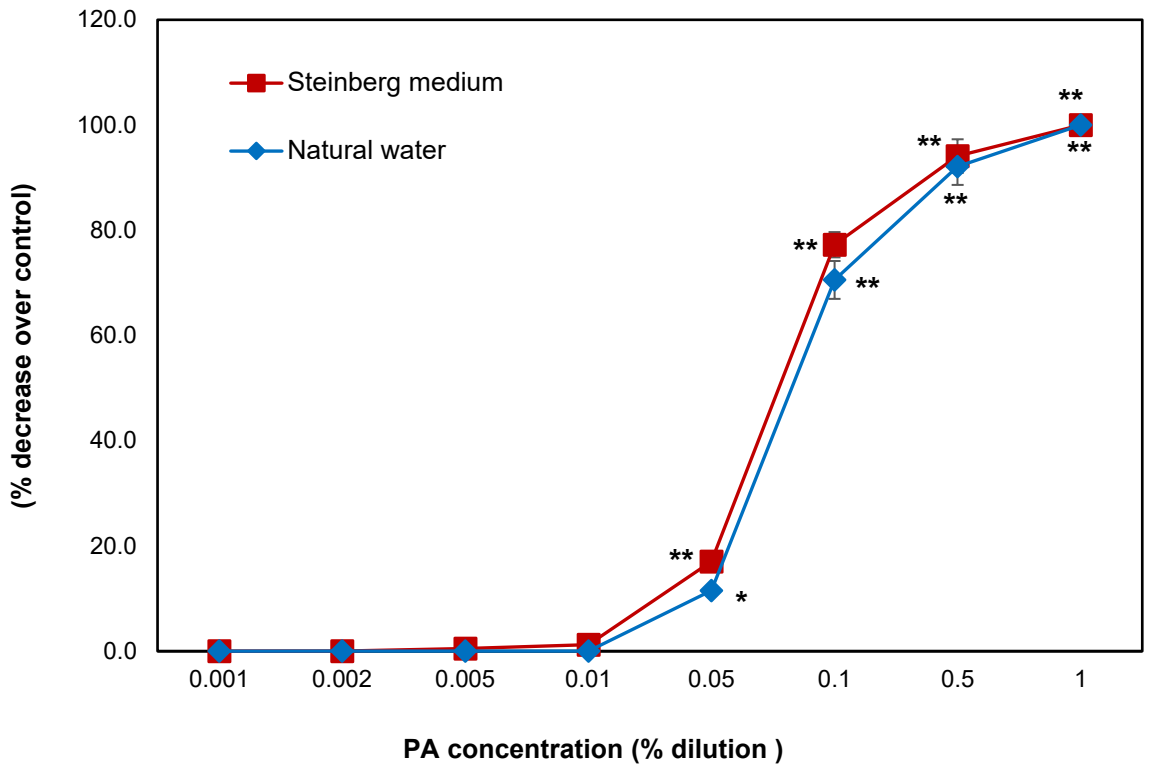

*- Statistically significant based on Student's *t*-test at *P* = 0.05, ** - *P* = 0.01

**Fig. 4b** Growth inhibition in *L. minor* (based on frond number) after exposure to different concentrations of PA (for 7 days) in natural creek water.




*- Statistically significant based on Student's *t* test at *P* = 0.05, ** - *P* = 0.01

**Fig.5** Effect of PA on the total chlorophyll content of *L. minor* in Steinberg medium and natural creek water




*- Statistically significant based on Student's *t* test at *P* = 0.05, ** - *P* = 0.01

**Fig. 6** Effect of PA on *L. minor* root cell viability

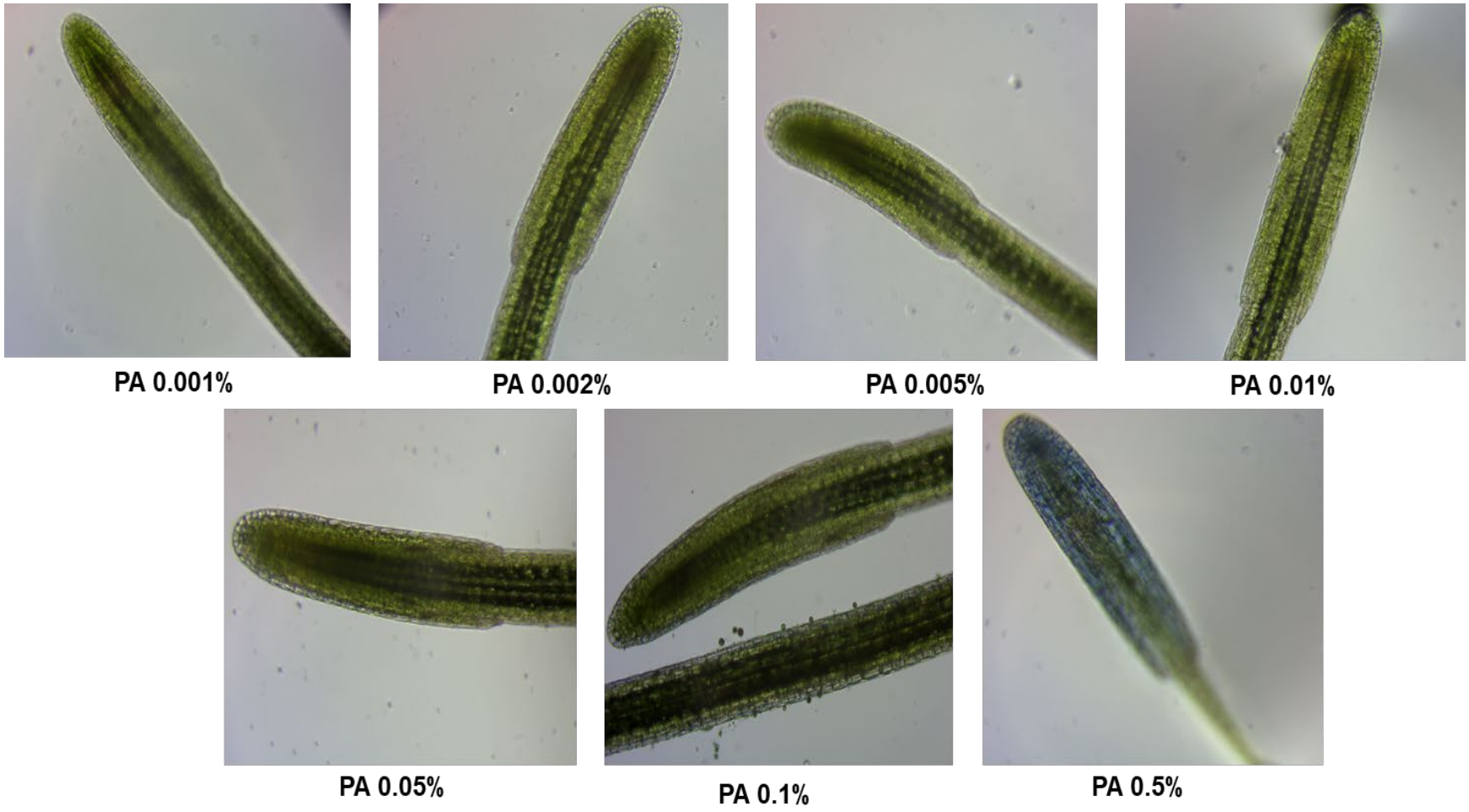
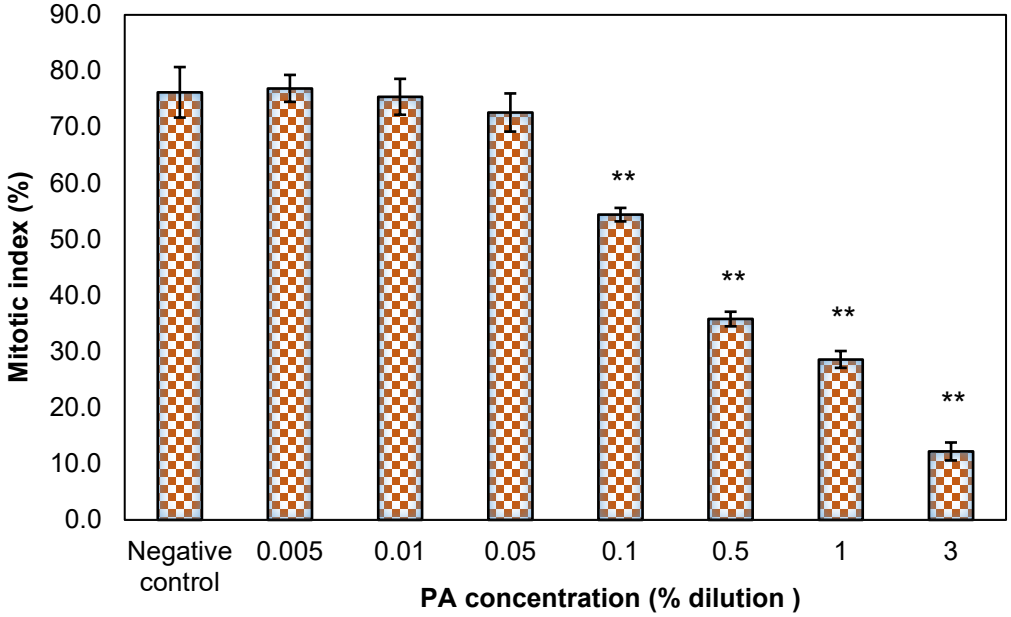


**Fig.7** Effect of PA to *A. cepa* cell division




*- Statistically significant based on Student's *t* test at *P* = 0.05, ** - *P* = 0.01

**Fig.8** Effect of PA to *A. cepa* (chromosomal aberrations)

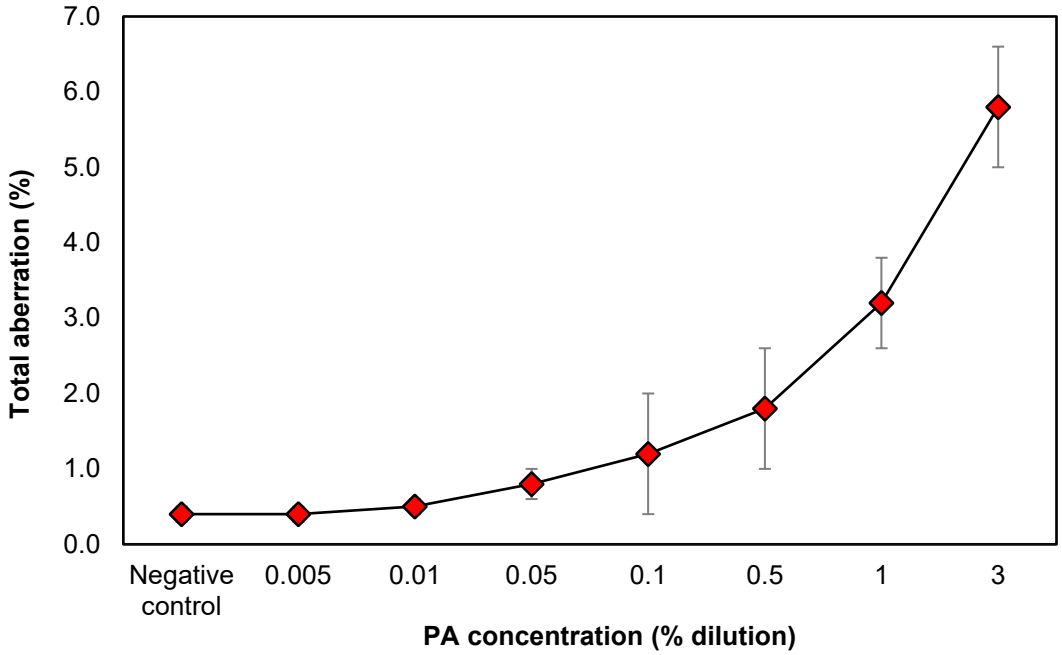


**Fig.9** Effect of PA to *A. cepa*

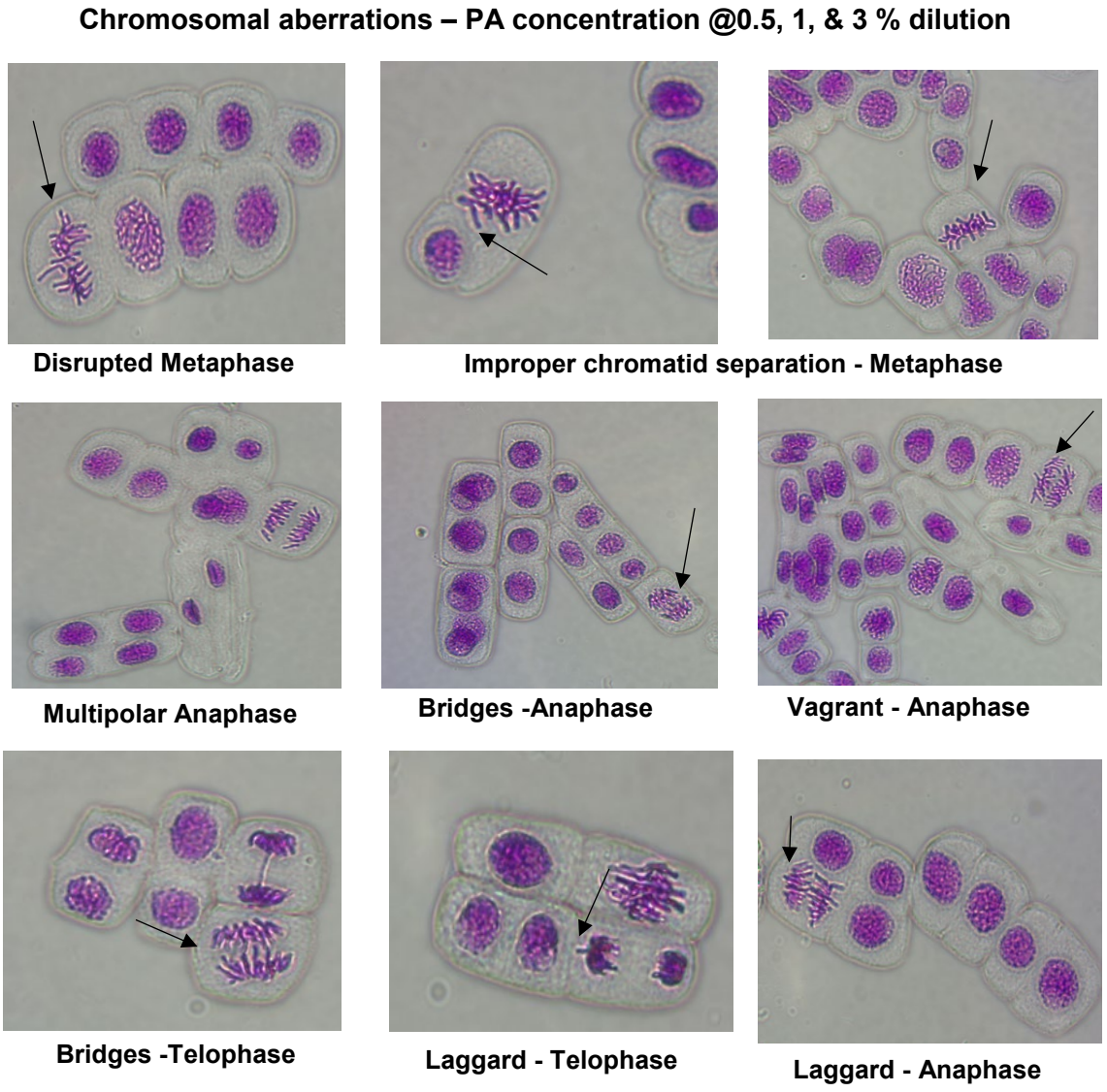


**Fig.10a** Effect of PA to *E. fetida* survival – Soil A

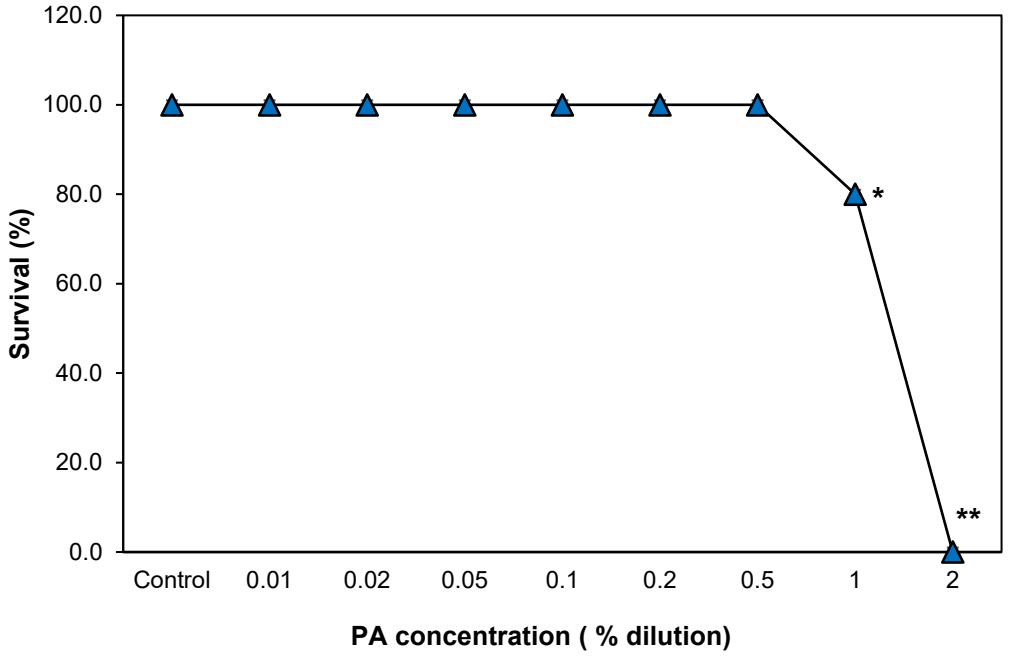
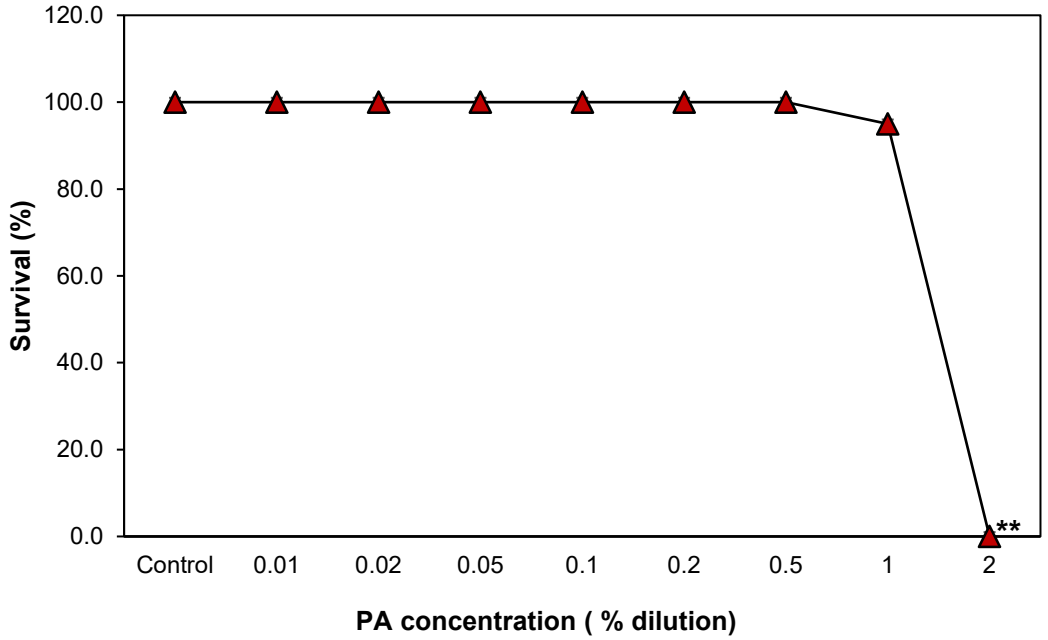



**Fig.10b** Effect of PA to *E. fetida* survival – Soil B




*- Statistically significant based on Student's *t* test at *P* = 0.05, ** - *P* = 0.01

**Fig.11a** Effect of PA to *E. fetida* weight loss – Soil A

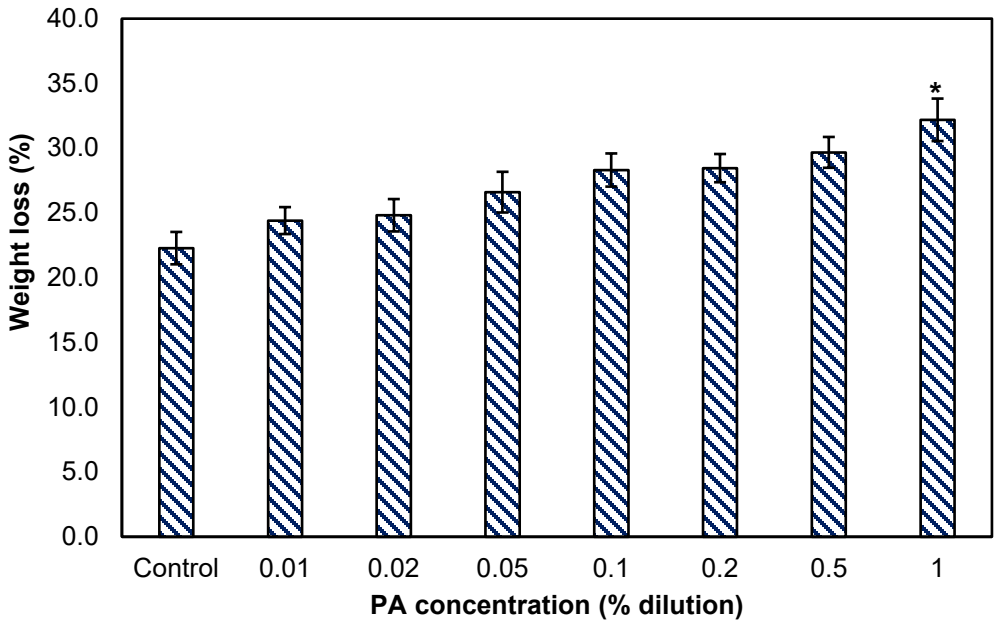
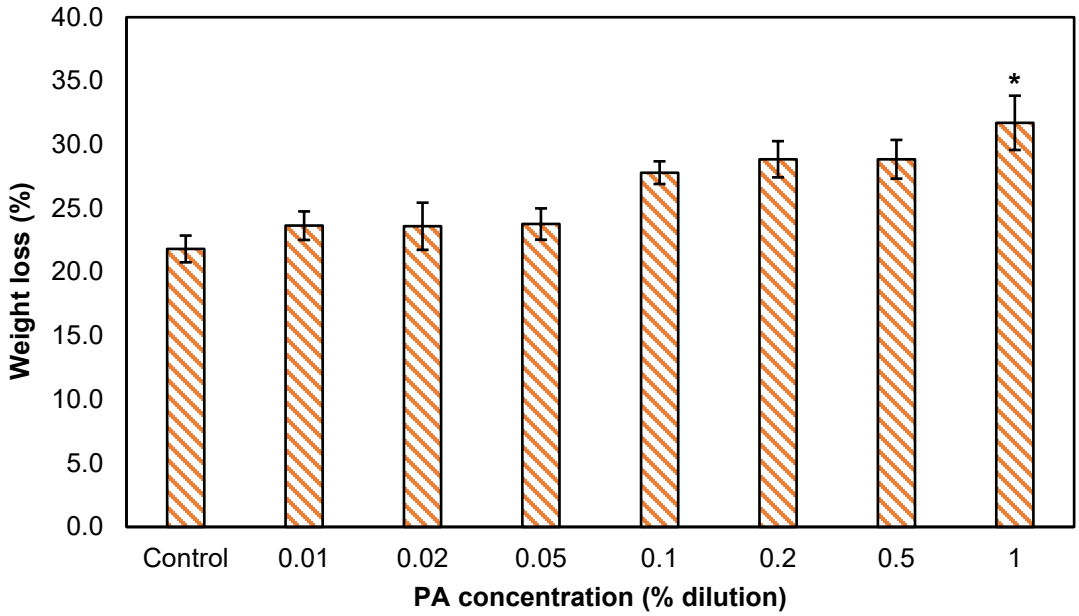



**Fig.11b** Effect of PA to *E. fetida* weight loss – Soil B




*- Statistically significant based on Student's *t*-test at *P* = 0.05

**Fig.12a** Effect of PA to *E. fetida* cocoon and juvenile production - Soil A

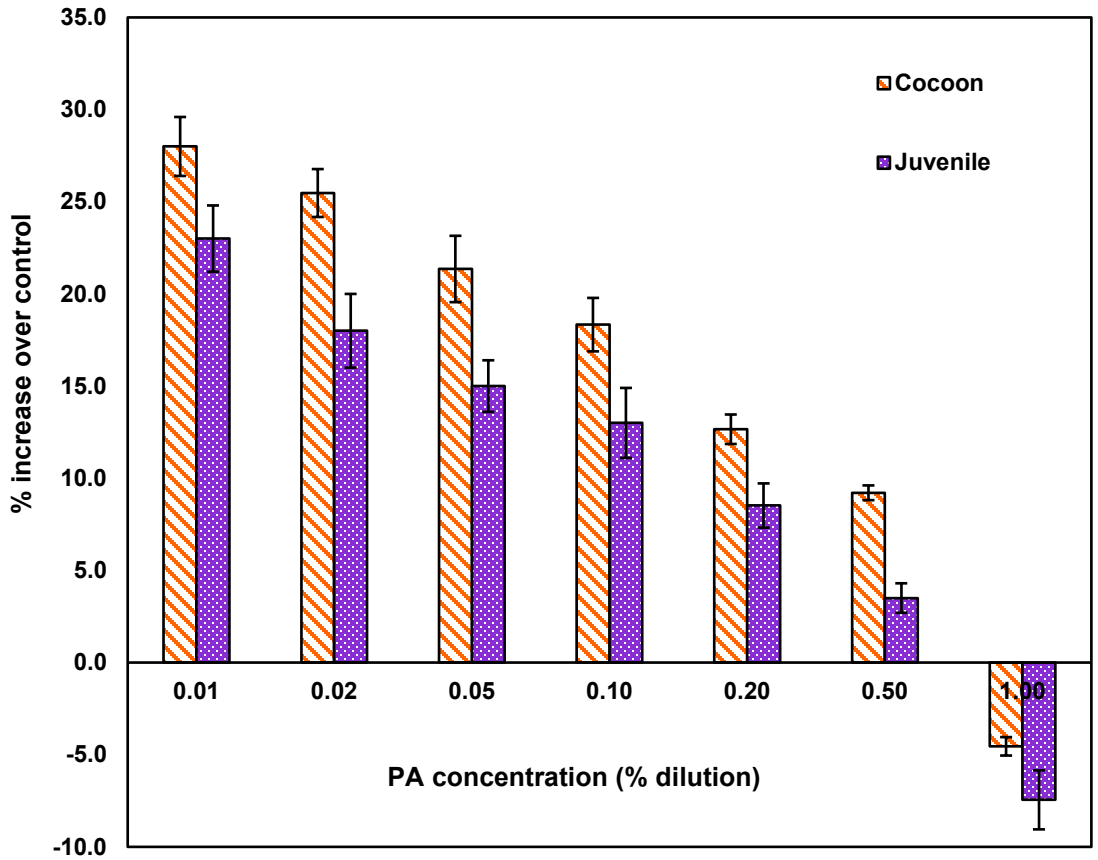


**Fig.12b** Effect of PA to *E. fetida* cocoon and juvenile production - Soil B

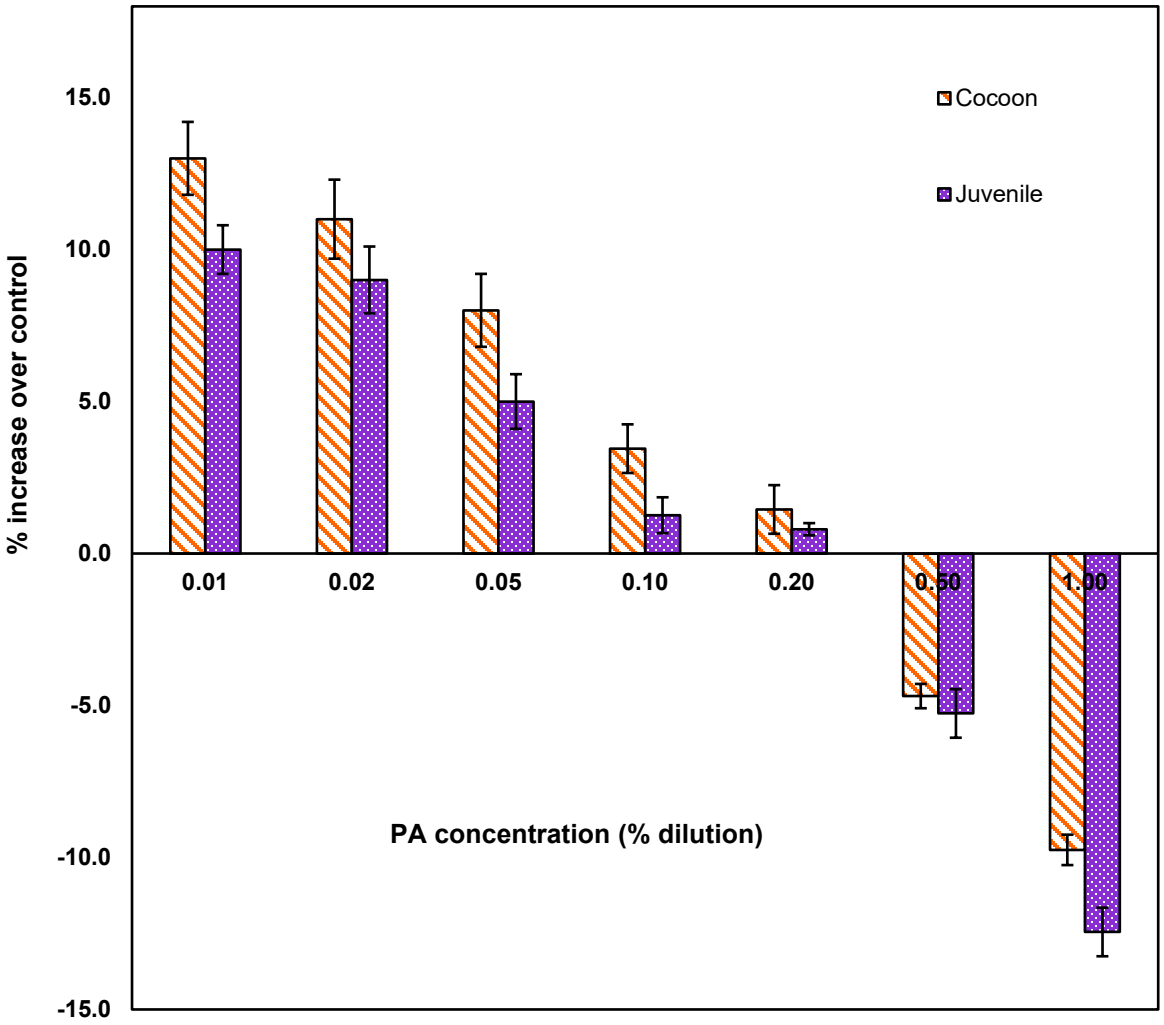
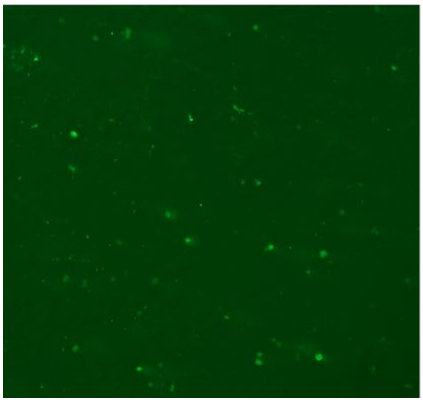
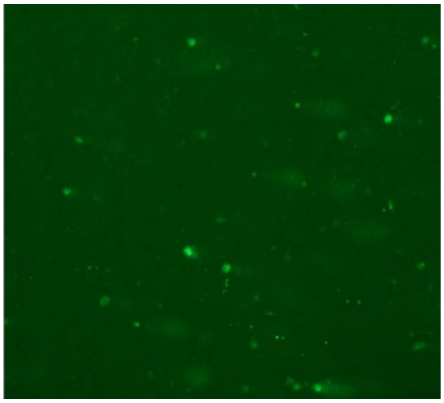


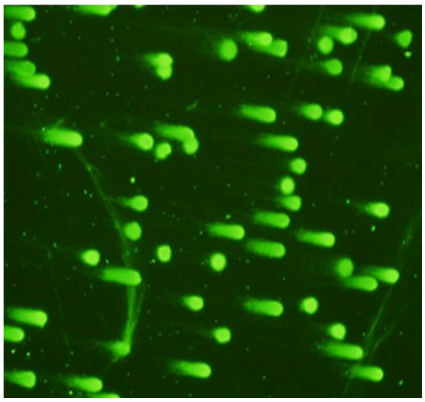
**Fig.13** Cytotoxicity of PA to *E. fetida*



**Negative control**



**PA @ 1% dilution**



**Positive control (BaP @1 mg kg$^{-1}$)**

**Fig.14a** The relative abundance of the most abundant bacterial phyla in Soil A spiked with different concentrations of PA

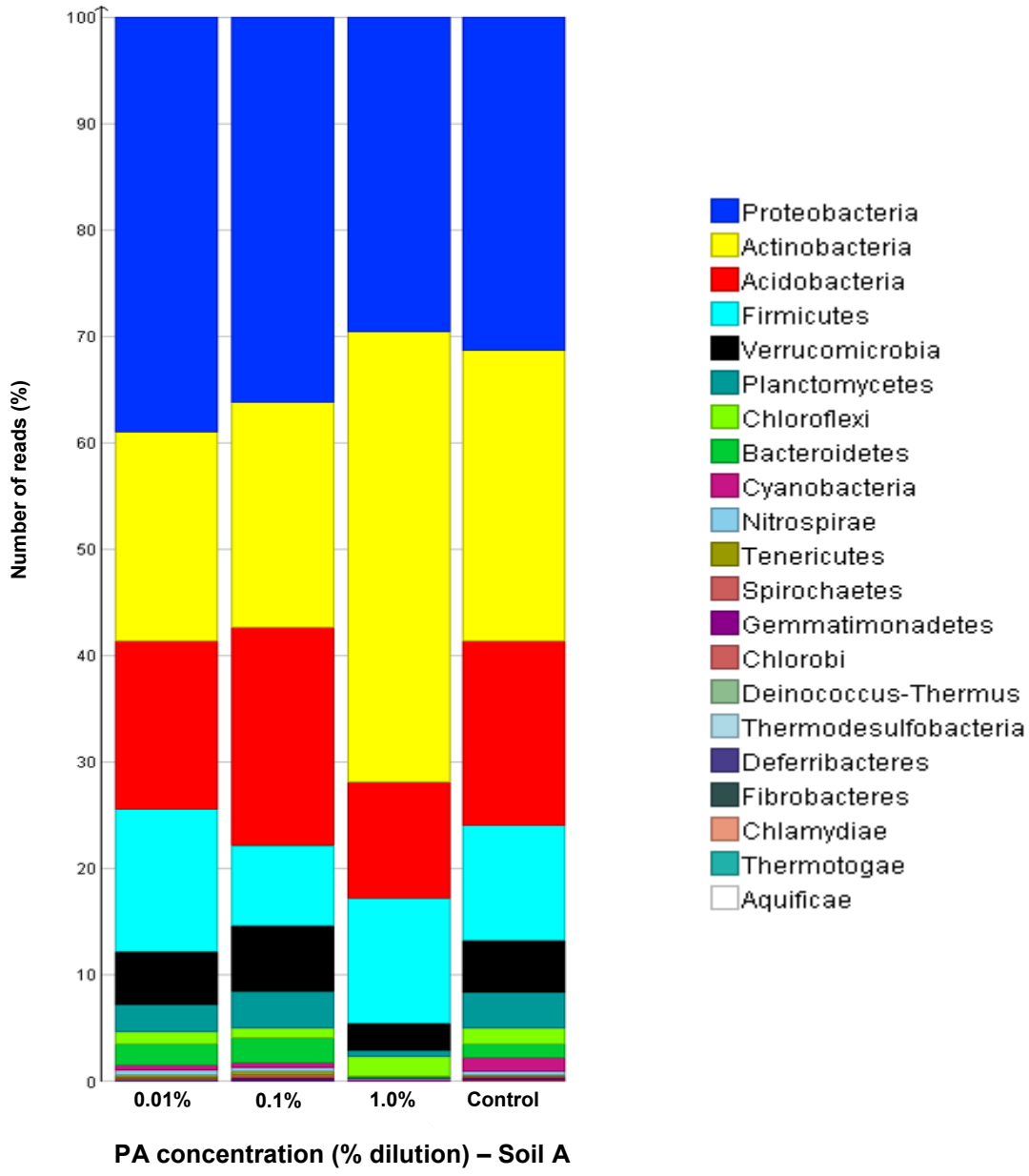


**Fig.14b** The relative abundance of the most abundant bacterial phyla in Soil B spiked with different concentrations of PA

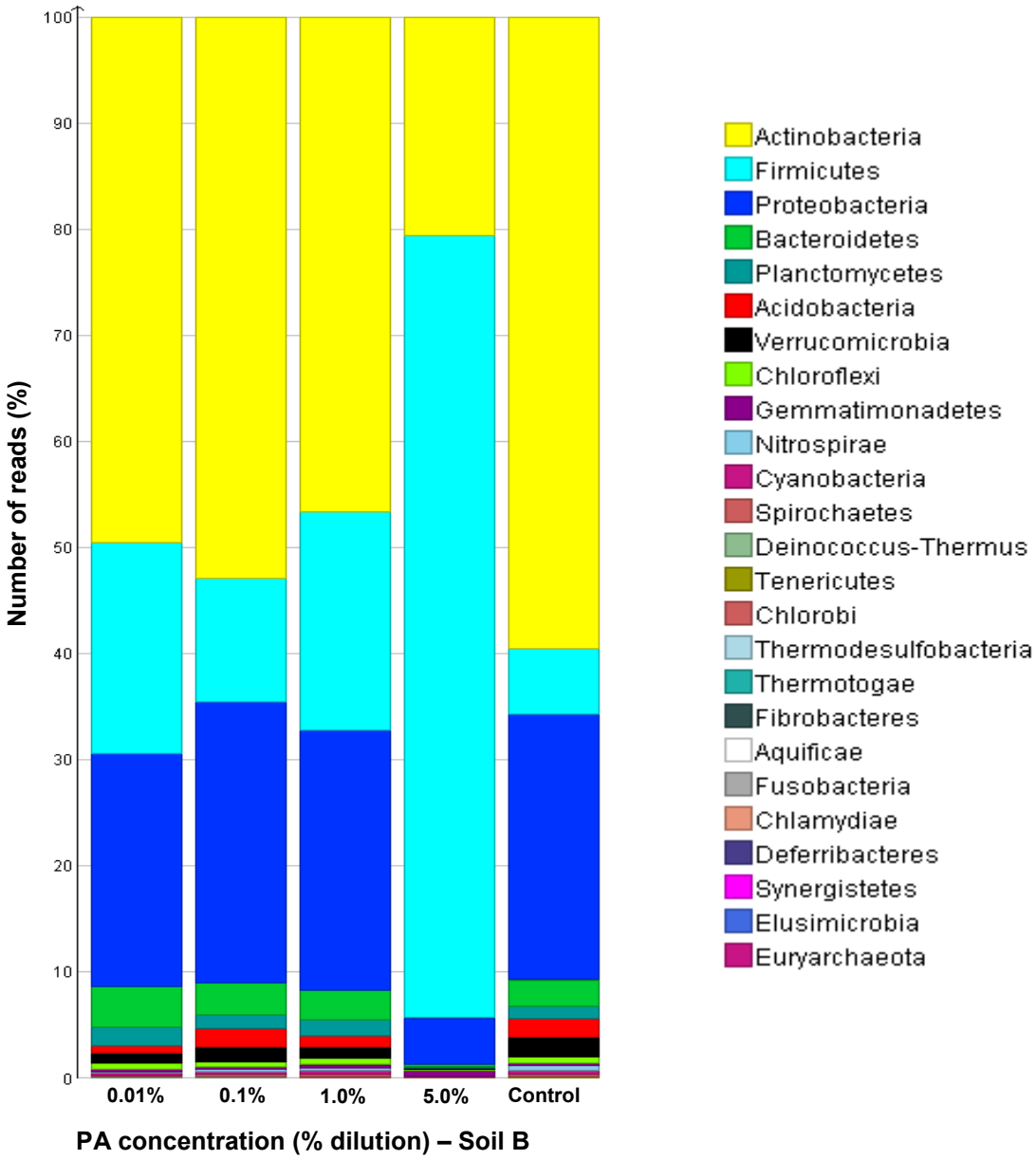


**Fig.15a** The relative abundance of the important plant growth-promoting rhizobacteria in Soil A spiked with different concentrations of PA

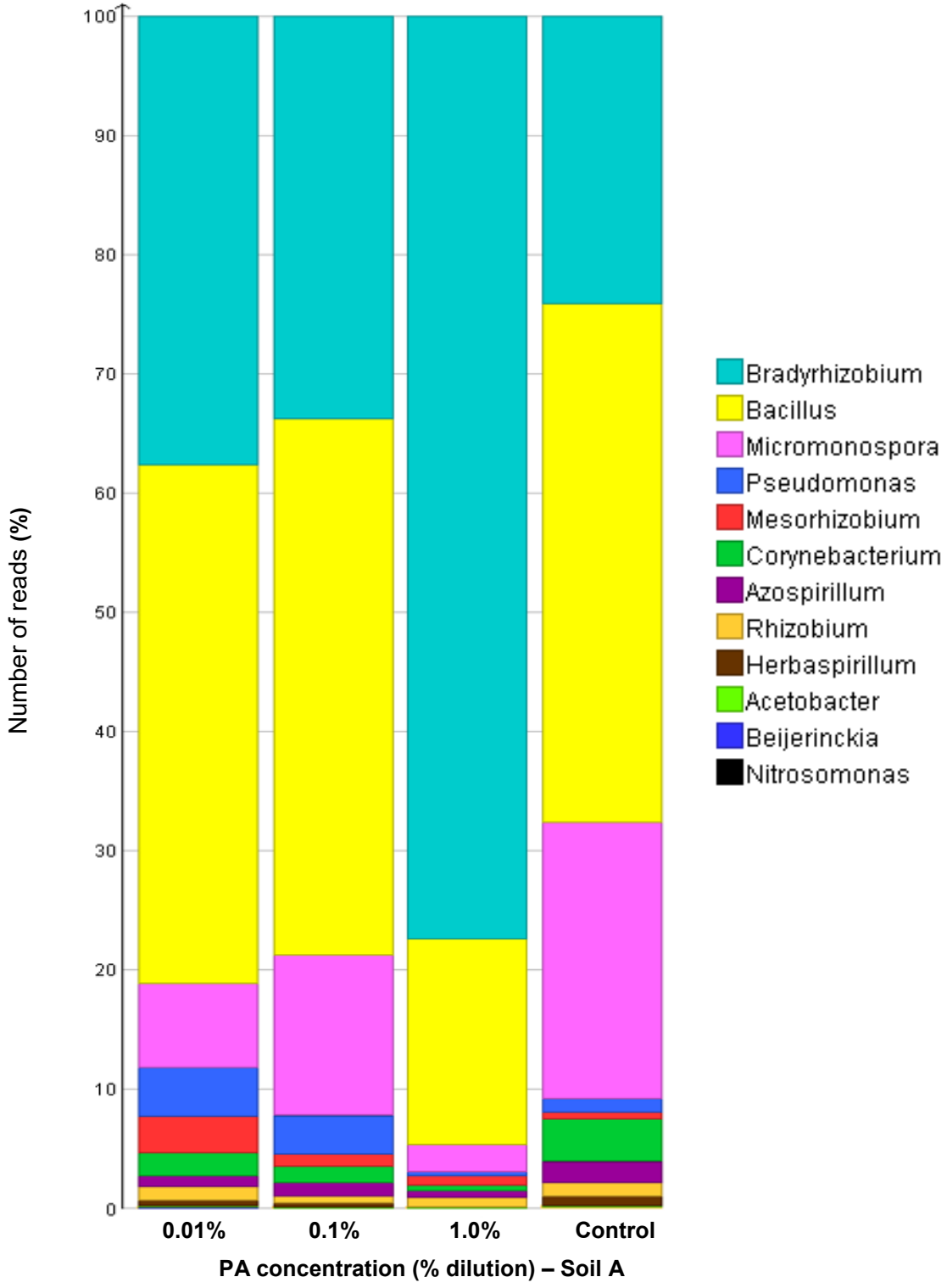
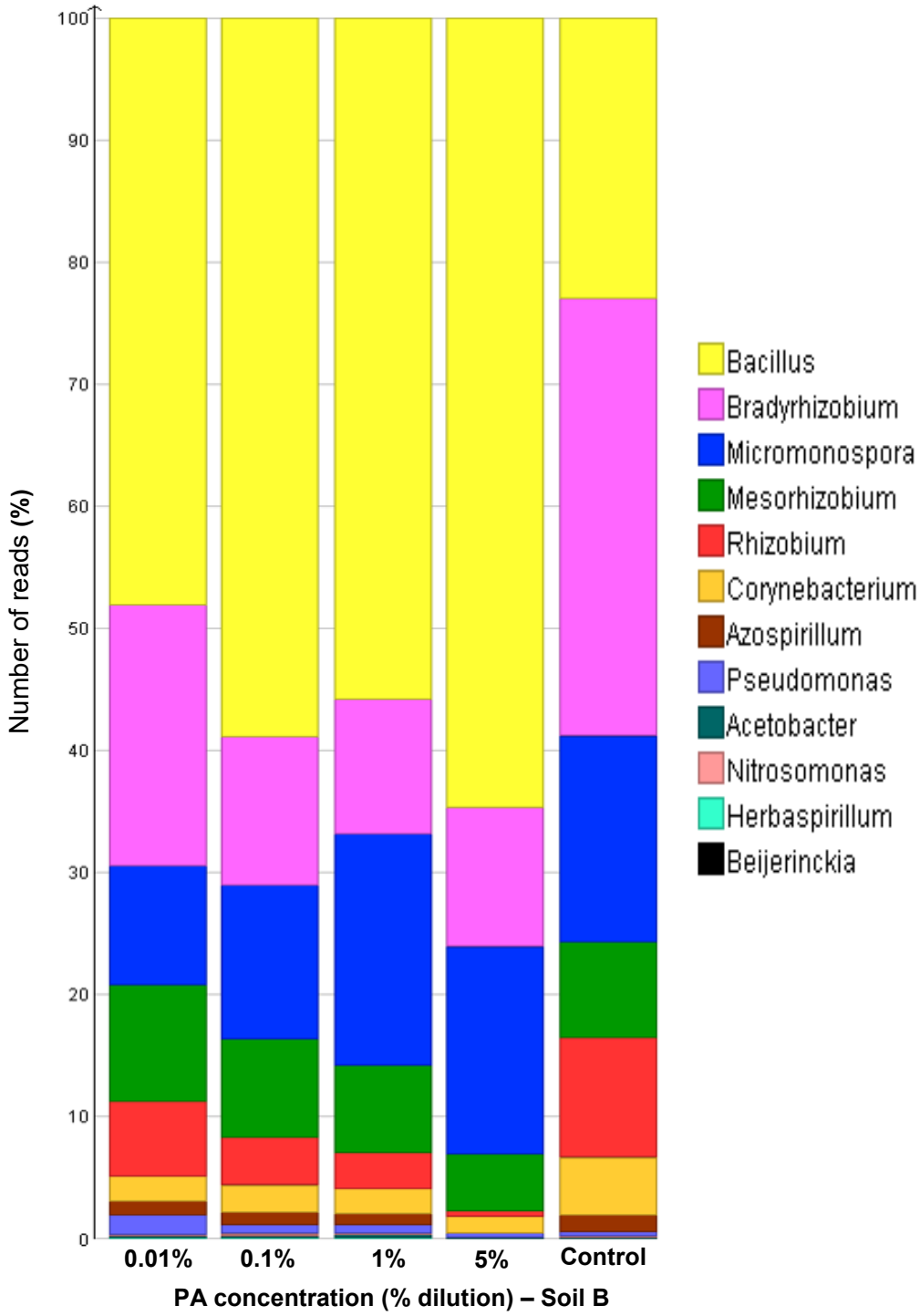


**Fig.15b** The relative abundance of the important plant growth-promoting rhizobacteria in Soil B spiked with different concentrations of PA



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