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Research Article

Ecotoxic response of *Rhizobium* sp. to the commonly used pesticides (paraquat and dichlorvos)

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Abstract
This study investigated the 96-hour ecotoxicity response of *Rhizobium* sp. to paraquat and dichlorvos at application concentrations ranging from 0.01 mg/L to 100 mg/L. The test organism was isolated and enumerated using yeast extract mannitol agar. Paraquat proved to be more toxic than dichlorvos based on the toxicity parameters established using probit regression analysis. *Rhizobium* sp. showed no growth (100% mortality) at the higher concentrations of 10 mg/L and 100 mg/L for paraquat; for dichlorvos, this absence of growth was only seen at the highest application concentration of 100 mg/L. Overall, *Rhizobium* sp. tolerated the insecticide, dichlorvos, better than the herbicide, paraquat. At the lowest concentration of 0.01 mg/L, it displayed moderate tolerance indices of between 0.534 and 0.589 for both pesticides. At 0.1 mg/L and 1.0 mg/L pesticide concentrations, the 96-hour tolerance indices obtained were less than 0.39, indicative of very low tolerance. Neither pesticide was tolerated at 10 mg/L and 100 mg/L. Statistical comparisons of the tolerance indices between the two pesticides and from one pesticide concentration to the other showed significant differences both within and the between groups ($p \leq 0.05$). The median lethal concentrations (LC_{50}) were 0.0158 mg/L and 0.0350 mg/L for paraquat and dichlorvos, respectively. The lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) were 0.0011 and 0.0027, respectively for paraquat and 0.0017 and 0.0047, respectively for dichlorvos. The results indicated that pesticides have adverse acute effects on key microbial ecosystem service providers like *Rhizobium* sp. and should, thus, be used with restraint.

***Keywords*:** Emerging pollutants, median lethal concentration, paraquat, pesticide stress, toxicity

Introduction
Pesticides are employed in modern agricultural practice to counteract the problems of compromised yield due to attack by pests and pathogens. They are chemicals or biological agents whose use is intended to protect crops and produce in a bid to maximise farmers' profit. These xenobiotic compounds are well known to persist in soil and water systems impacting on the physical, chemical, and biological characteristics of these environments. Their fate is any ecosystem is largely contingent on their interaction with the native microbial communities (Maldani et al., 2018; Hamuda, 2020). Studies have shown that about 99.9 % of applied pesticides is lost to the receiving environment where it induces pesticide stress on non-target organisms in that environment due to its accumulation and persistence (Ahemad & Khan, 2011). Microbial biomass and activity are limiting factors for the effectiveness of microorganism-driven ecosystem functions (Lin et al., 2007). Though, certain microbial groups have been known to utilise pesticides as a source of carbon and energy, pesticides and their degradation by-products will often affect the cell densities, enzymatic activities, and physiology of exposed microorganisms within an ecosystem (Moawad et al., 2014; Ahemad & Khan, 2011). Maldani et al. (2018) corroborate the toxicity of pesticides to groups of microorganisms and state that toxicity will often be most severe in the interval immediately following application or exposure.

One such group of microorganisms impacted by pesticides are the Rhizobia. They are aerobic, Gram negative, non-spore forming, often flagellated, rods known for their ability to convert atmospheric nitrogen to more biologically available forms within the ecosystem. These microorganisms play a crucial role in sustainable agriculture. Most members establish symbiotic relationships with certain leguminous plants through the formation of root nodules while others occur as free-living saprophytes in the environment. *Rhizobium* is the most popular member of this important group of nitrogen-fixing microorganisms; other known members include, *Mesorhizobium, Bradyrhizobium, Ensifer, Allorhizobium, Azorhizobium, Neorhizobium* and *Pararhizobium* (Hamuda, 2020; Burul et al., 2022). Pesticide-use is considered one of the leading threats to biological nitrogen fixation and nitrogen fixing bacteria; this is particularly true of herbicides. Pesticides trigger DNA, protein, oxidative or membrane damage in Rhizobia and negatively impact their nitrogen fixation capabilities and their ability to form symbiotic relationships with legumes (Zahran, 1999; Ahemad and Khan, 2011; Burul et al., 2022).

Paraquat (1,1–dimethyl–4,4–bipyridinium dichloride, $C_{12}H_{14}Cl_2N_2$) and dichlorvos (2,2–dichlorovinyl dimethyl phosphate, $C_4H_7Cl_2O_4P$) are the active ingredients in numerous pesticides widely employed in agriculture in many developing countries including Nigeria although they are banned internationally by the European Union (EU) and the US Environmental

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Protection Agency (USEPA) (USEPA, 1997; European Commission, 2011; Bang et al., 2015; Adeniyi, 2022). Paraquat is commonly used in developing countries for the treatment of numerous food crop variants and is rated the second most toxic herbicide after glyphosate. Both dichlorvos and paraquat have considerably high aquifer pollution capabilities and have shown half-lives of over 3 months in the environment (Pizutti et al., 2015; Silva et al., 2015). Their persistence in soil has been attributed to adsorption on soil organic matter and clay (Gondar et al., 2012). In spite of their extensive use, the ecotoxic effects of their presence in the ecosystem have not been adequately quantified. De Lorenzo et al. (2009) and Hamuda (2020) both confirm the scarcity of toxicity data for pesticides against autochthonous microbial populations in the pesticide-receiving ecosystems.

The productivity of soil and water ecosystems is constantly threatened by pesticide use. Growing global populations translate to increased largescale crop production and, thus, greater dependence on pesticides to effectively maintain yield quality. This trend, in turn, drives the need for more comprehensive research into the ecotoxic effects of these chemicals on niche players within the ecosystem. This study fills the gap by investigating the 96-hour ecotoxicity response of *Rhizobium* sp. to the organochlorine herbicide, paraquat and the organophosphate insecticide, dichlorvos at application concentrations ranging from 0.01 mg/L to 100 mg/L. The effect of the different pesticide concentrations on the growth of bacterium and their toxicity parameters were determined.

Materials and Methods

Sample collection

The pesticides used (paraquat and dichlorvos) were obtained from the local market in Port Harcourt, Nigeria. Sterilisation of the pesticides was done via filtration using a 0.22 µm membrane filter (Merck Millipore, Germany). Based on the manufacturers' instructions, the recommended field application rate (RFAR) for both paraquat and dichlorvos was 2 – 5 kg/ha translating to about 20 – 50 mg/L.

The *Rhizobium* sp. employed in the study was isolated from the semi-aquatic environment around the New Calabar River in Choba, Port Harcourt, Nigeria. The water samples were collected in pre-rinsed glass bottles which were immediately sent to the Laboratory of the University of Port Harcourt, Nigeria for analysis.

Isolation and characterisation of *Rhizobium* species

The water samples were subjected to six-fold serial dilution then 0.1 mL aliquots were plated out on sterile yeast extract mannitol agar (YEMA) containing 0.25 % congo red dye (HiMedia, India) in plates (with replicates) using the spread plate technique. The inoculated plates were incubated at 37°C and observed regularly for the whitish to pale pink mucoid colonies indicative of *Rhizobium* sp. Discrete colonies were purified by streaking multiple times unto fresh agar plates. The pure isolates obtained were stored on slants in Bijou bottles.

Biochemical tests and microscopic analysis were used as confirmatory tests to verify the identity of the isolates obtained as *Rhizobium* sp. the tests employed include microscopic examination for Gram's stain reaction, cell shape, presence of a capsule, flagella and spore formation via staining; others are catalase test, citrate test, coagulase test, Methyl Red – Voges Proskauer (MRVP), haemolysis, motility test, gelatin hydrolysis, hydrogen sulphide production, indole, oxidase, nitrate reduction, oxidative-fermentative, triple sugar iron agar (TSIA) test, urease, fermentation of several simple and complex sugars, starch hydrolysis as well as phenylalanine deaminase, acetate utilisation, arginine dehydrolase, lipase and ornithine decarboxylase reactions (Cheesborough, 2006).

96-hour acute toxicity bioassay

An acute toxicity bioassay was employed in the determination of the response of the test isolate to paraquat and dichlorvos. The media dilution method as described by Osadebe and Ubochi (2022) was used. The *Rhizobium* isolate, stored on the slant, was aseptically introduced into fresh yeast extract mannitol broth using a sterile wire loop. This set up was incubated at 37 °C for 48 h with agitation. About 0.1 mL of the 48h broth culture was, again, inoculated into fresh broth and incubated under the same conditions. The process was repeated for three cycles in order to ensure that the isolates used for the toxicity bioassay were within the exponential phase of their growth cycle. The final broth culture had an absorbance of 0.6 at 600 nm.

The replicated set-up for the bioassay, for each pesticide, consisted of 6 flasks per set and 3 sets in all resulting in a total of 18 flasks. Each set of 6 flasks consisted of five flasks of pesticide-incorporated broth using application levels of 100 mg/L, 10 mg/L, 1 mg/L, 0.1 mg/L, and 0.01 mg/L for each pesticide in sterile yeast extract mannitol broth and the Control study which entailed inoculated broth with no pesticide added. Precisely 1 mL of the 24h old actively growing cells of *Rhizobium* sp. was aseptically transferred into each flask containing 99 mL of the broth. The flasks were then plugged with non-absorbent cotton wool and incubated at 37°C for 96 h with agitation (Laboa, China). Testing was carried out at 24-hour intervals for the 96-hour period of the study.

Enumeration of the *Rhizobium* isolate was done by drawing 0.1 mL aliquots from each flask and inoculating on YEMA plates (with replicates) via the spread plate method. Plates that had visible colonies within the range of 30 – 300 at the end of the 48h incubation period were used to determine the bacterial abundance. A 0.1 ml sample was drawn from each flask immediately after inoculation and spread on YEMA plates (with replicates) to obtain the 0 h count. The counts obtained were expressed as colony forming units per millilitre.

Survival and mortality quotients

Equations (1) and (2) were used to calculate the percentage survival and mortality at the various time periods.

$$Survival\ (\%) = \frac{B}{A}\ x\ 100\ \% \qquad \text{(Eq. 1)}$$

Where A = Count on Day 0; B = Count at specific pesticide concentration and time

$$Mortality\ (\%) = 100 - \%\ Survival \qquad \text{(Eq. 2)}$$

Toxicity parameters and 96-hour tolerance indices

Probit regression analysis was used to establish the toxicity parameters – the median lethal concentration (LC_{50}), No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC). The control study was used as a reference as put forward by Finney (1952). The percentage Log survival employed for probit regression analysis was estimated using Equation (3) as per Williamson and Johnson (1981). The slope line of regression from the probit curve of log percentage survival against the logarithmic value of concentration at different concentrations defined the LC_{50}.

$$\%\ Log\ Survival = \frac{\text{Log C}}{\text{Log c}}\ x\ 100\ \% \qquad \text{(Eq. 3)}$$

Where C = Mean counts in each pesticide concentration; c = Mean counts in the control (0 mg/L pesticide concentration)

The 96-hour tolerance indices for the test pesticides were determined by comparing the abundance of the test isolate in pesticide-tainted medium to that in untainted medium as outlined in Equation (4) below.

$$TI_{96} = \frac{\text{Bacterial counts in pesticide–amended medium}}{\text{Bacterial counts in the Control study}} \qquad \text{(Eq. 4)}$$

Where TI_{96} = 96h tolerance index

Statistical analysis

The data was analysed to determine if there were significant differences in the tolerance indices from one pesticide type to the other and from one application concentration to the other. Analysis was conducted using Microsoft Excel® 2016 and SPSS® 23.0.

Results and Discussion

Rhizobium, in the current study, showed a similar response to both paraquat and dichlorvos. The two pesticides both seemed to induce pesticide stress in the bacterium as demonstrated in the results obtained. Abundance was noted to drop with increases in both exposure period and pesticide concentration. The effect of paraquat and dichlorvos on the growth of *Rhizobium* in the current study is depicted in Figures 1 and 2. The test isolate, *Rhizobium* sp., did not show any growth whatsoever at higher concentrations (10 mg/L and 100 mg/L) of paraquat neither did it grow at 100 mg/L of dichlorvos. The isolate clearly thrived better in dichlorvos-tainted medium than in that modified with paraquat.

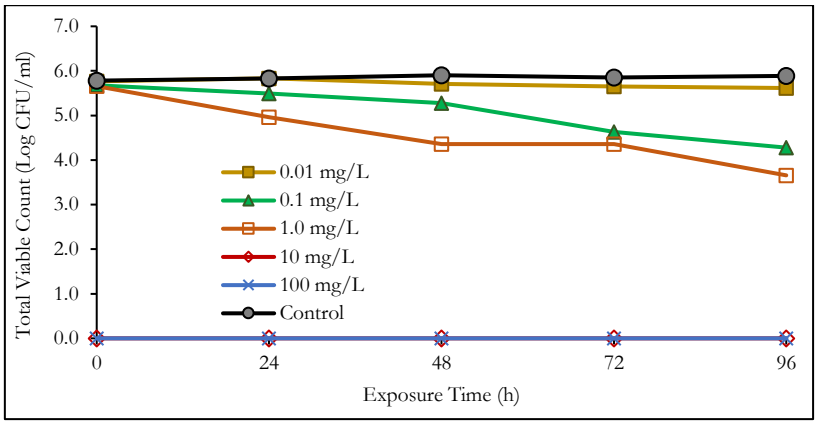

Figure 1 Growth of *Rhizobium* sp. in response to different concentrations of paraquat (herbicide). The test isolate did not grow at higher concentrations (10 mg/L and 100 mg/L) of the herbicide.

A look at the mortality levels obtained during the study (Figures 3 and 4) confirmed the stronger inhibitory effect of paraquat over dichlorvos. For paraquat, 100 % mortality was seen at 96 h at 1.0 mg/L and from 24 h to 96 h at both 10 mg/L and 100 mg/L. This was not the case for dichlorvos where 100 % mortality was only seen at 96 h for 10 mg/L and 24 h – 96 h at the highest application concentration of 100 mg/L.

The acute toxicity response of *Rhizobium* sp. to the test pesticides as ascertained from the probit regression model is shown in Table 1. Paraquat had lower median lethal concentration, LOEC and NOEC values which denote greater toxicity compared to dichlorvos.

Overall, *Rhizobium* sp. tolerated the insecticide, dichlorvos better than the herbicide, paraquat. Based on its 96h-tolerance indices outlined in Table 2, similar

tolerance levels were obtained for the two pesticides studied at the lowest pesticide concentration. It neither tolerated dichlorvos nor paraquat at the higher application concentrations of 10 mg/L and 100 mg/L as revealed by the tolerance indices of 0.00; however, at the lowest concentration of 0.01 mg/L, it displayed a moderate level tolerance (this is in the range of 0.60 – 0.79) for both pesticides. At 0.1 mg/L and 1.0 mg/L, the 96 h tolerance indices obtained were indicative of very low tolerance (0.00 – 0.39) for both pesticides. Statistical comparison of the tolerance indices between the two pesticides and from one pesticide concentration to the other showed significant differences both within and the between groups at 95 % confidence interval. The response of *Rhizobium* to the two pesticides at 0.01 mg/L concentration, based on the tolerance indices obtained, did not differ significantly from one another ($p \leq 0.05$).

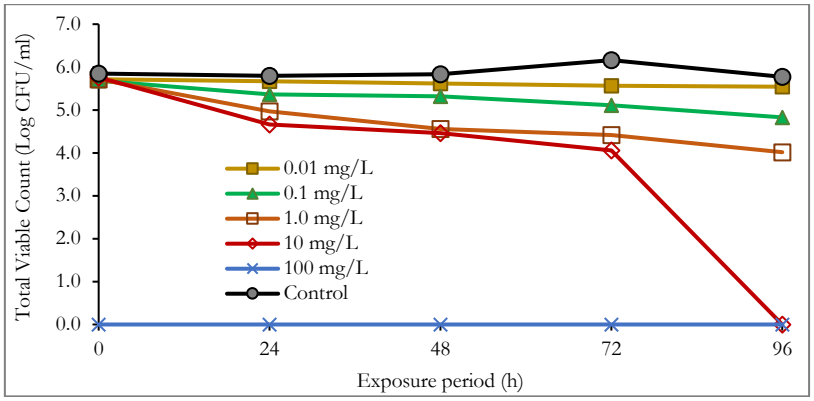

Figure 2 Effect of different concentrations of dichlorvos (insecticide) on the abundance of *Rhizobium* sp. No growth was observed at 100 mg/L

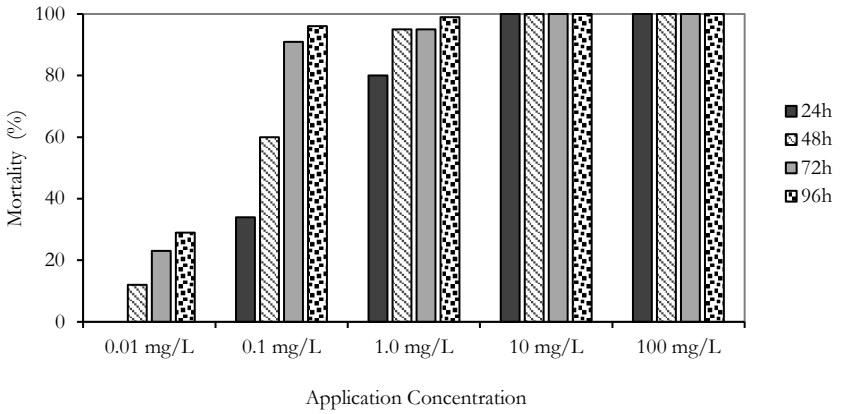

Figure 3 The 96-h mortality of *Rhizobium* sp. in response to exposure to the organochlorine herbicide, paraquat

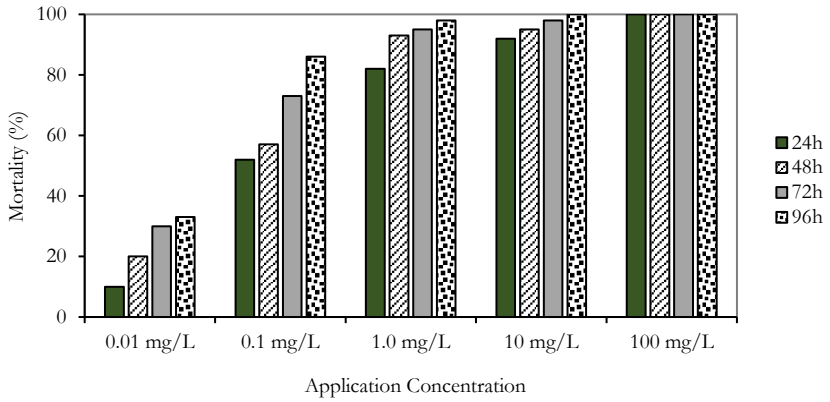

Figure 4 The 96-h mortality of *Rhizobium* sp. in response to exposure to the organophosphorus insecticide, dichlorvos

The findings from the present study are supported by several similar studies which also established that paraquat, dichlorvos and other pesticides negatively influenced the growth of microorganisms in the receiving ecosystems (Nur et al., 2013; Maldani et al., 2018; Mazhari and Ferguson, 2018; Osadebe and George, 2022; Osadebe and Ubochi, 2022). Margino et al. (2000), in their study on paraquat in peat land, reported that paraquat concentrations of 20 mg/kg had a strong inhibitory influence on the community dynamics of soil bacteria and fungi. Zain et al. (2013) equally agreed that the presence of herbicides had a considerable impact on the abundance of soil microorganisms by hampering their growth and that the extent of inhibition correlated directly with pesticide concentration. Maldani et al. (2018) studied the effect of paraquat at concentrations up to 12 g/L on four species of nitrogen fixers including three *Rhizobium* spp. and confirmed an inhibition in the growth of *Rhizobium* spp. with increasing paraquat concentration. They further stated that *Rhizobium tibeticum* showed the greatest tolerance amongst isolates from the genus while *R. radiobacter* displayed the least tolerance for the herbicide. In another study, the plant growth promoting qualities of *Rhizobium* alongside its abundance were impacted by exposure to multiple herbicides, insecticides and fungicides at concentrations over the recommended field application rate (RFAR). A stronger impact was seen at higher pesticide concentrations akin to what was obtained in the current study (Ahemad and Khan, 2011).

Table 1 Acute toxicity response (96 h) of *Rhizobium* sp. to paraquat and dichlorvos

Toxicity Parameter	*LC_{50} (mg/L)	LOEC (mg/L)	NOEC (mg/L)	Degree of Freedom
Paraquat (Herbicide)	0.0158	0.0027	0.0011	3
Dichlorvos (Insecticide)	0.0350	0.0047	0.0017	3

*Determined at 95 % confidence interval ($p \leq 0.05$)
LC50 – Median lethal concentration; LOEC – Lowest Observed Effect Concentration; NOEC – No Observed Effect Concentration

Table 2 96-h-tolerance indices of *Rhizobium* sp. for the test pesticides

Pesticide Concentration (mg/L)	**Paraquat** (Herbicide)	**Dichlorvos** (Insecticide)
0.01	$0.634^{a} \pm 0.0977$	$0.689^{a} \pm 0.0198$
0.1	$0.024^{b} \pm 0.0003$	$0.114^{c} \pm 0.0082$
1.0	$0.006^{d} \pm 0.0001$	$0.018^{b} \pm 0.0026$
10.0	0.000	0.000
100.0	0.000	0.000

Values are means of triplicates ± standard deviation.
Different letters represent figures that are significantly different ($p \leq 0.05$)

Isolates of *Rhizobium* were reported to show a gradual decline in cell density with increasing concentration of the herbicide glyphosate. A survival rate of 19 % – 22 % (78 % – 81 % mortality) was obtained at glyphosate concentrations of 100 µg/L – 150 µg/L. This rate dropped to 16 % – 19 % survival (81 % – 84 % mortality) when the concentration was raised to 200 µg/L (Aynalem and Assefa, 2017). Similarly, Dos Santos et al. (2005) also established that glyphosate negatively impacted the growth and survival of *Bradyrhizobium*. They obtained an 87.5% drop in bacterial abundance at 43.2 µg/L glyphosate. With Drouin et al. (2010), the figure was slightly lower with a 67 % decline in *Bradyrhizobium* counts following exposure to about 450 µg/L glyphosate. Akin to the present study, laboratory-based toxicity tests with the fungicide, mancozeb, revealed that growth of rhizobial isolates declined by 90 % – 96 % at an application concentration of 100 mg/L compared to the set-up with no fungicide included (Aynalem and Assefa, 2017). Cevheri et al. (2011) also reported a 98 % drop in *Rhizobium* sp. abundance when it was subjected to the RFAR of mancozeb in field studies and 97 % inhibition in growth following exposure to a mixture of mancozeb and carbendazim at the RFAR.

The greater acute toxicity shown by paraquat over dichlorvos in this study buttresses reports from other comparable studies. For example, in tandem with the findings of the present study, an ecotoxicity investigation in Brazil involving *Escherichia coli* found that paraquat had the most repressive impact on the growth of *E. coli* compared to other pesticides and control studies without any herbicide (Botelho et al., 2012). Another similar investigation by Adomako and Akyeampong (2016) in Ghana further confirmed that paraquat impeded the growth of soil bacterial populations the most at half its recommended field application rate (RFAR), the RFAR and double the RFAR compared to other herbicides. These reports regarding the superior toxicity of paraquat over dichlorvos, however, do not tally with reports by Kim et al. (2017) and Damalas and Eleftherohorino (2011) who concluded that organochlorine herbicides, like paraquat, exhibit low toxicity when compared with the organophosphate pesticides such as dichlorvos. They also state that organochlorines have low persistence in the environment. The differences may stem from the fact that these studies referenced field-based testing compared with the *in vitro* testing carried out in the current study. Furthermore, the response of Rhizobia to pesticides has been linked to, not only to their in-built chemical resistance systems but to the geographical area from which the isolates are obtained as well (Zablotowicz and Reddy, 2007).

The toxicity levels observed in the current study may be considered somewhat contrary to findings from previous studies that have linked strains of *Rhizobium* to the degradation of pesticides in environmental media. Moawad et al. (2014) found that *Rhizobium* was able to utilise two different types of fungicides as sources of

carbon in laboratory studies. Likewise, Sabourmoghaddam et al. (2015) reported the effective degradation of the insecticide, imidacloprid in soil by *Rhizobium* isolates obtained from vegetable farms in Malaysia while *R. leguminosarum* was implicated in the breakdown of the pesticides, malathion and methomyl (Hassan, 2010). The answer here likely lies with the variances between *in situ* and *in vitro* studies. Studies have revealed that results from laboratory-based analyses with cultivable microorganisms may not necessarily be representative of the findings that would be obtained in field studies. Additionally, a decline in the diversity and abundance of microorganisms within an ecosystem, as occurs following pesticide exposure, would typically be accompanied by a corresponding decrease in particular functions like resistance to invasion by pathogenic species. This phenomenon may also apply to biodegradative capacity whereby the use of a single species in seclusion from its natural habitat may impact on its ability to effectively degrade the hydrocarbon pesticides (Van Elsas et al., 2012; Jacobsen and Hjeslmø, 2014). Furthermore, there is the possibility that some level of pesticide biodegradation may be going on in the current study in spite of the decrease in microbial abundance seen.

Conclusions

Ecosystems serve as habitats to an extensive assortment of microorganisms. These systems are regularly exposed to pesticide pollution mainly directly but also due to run-off from surrounding areas. Rhizobia are key inhabitants of terrestrial and aquatic ecosystems where they drive primary production and nutrient cycling. The findings from the study demonstrated that both paraquat and dichlorvos triggered an acute ecotoxic response from *Rhizobium* sp. even at levels below their recommended field application rate with paraquat eliciting a slightly stronger toxicity response. Precaution must, therefore, be taken in the sustained application of pesticides as these toxic effects observed could have subsequent ripple effects on primary production and higher trophic levels within the ecosystem.

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Author Contributions: Author AUO conceived and designed the study and wrote the first draft of the manuscript. Both authors carried out literature searches as well as laboratory and data analyses.

Conflict of Interests: The authors declare no conflict of interest.

Data Availability Statement: The data that support the finding of this study are available from the corresponding author, upon reasonable request.

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