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Toxicity of Selected Pesticides on the Microbial Pollution Bio-indicator, *Pseudomonas aeruginosa*

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Abstract

Pesticides are well known to impact on non-target organisms such as vulnerable bio-indicator species in aquatic and non-aquatic environments. *Pseudomonas aeruginosa* is a known bioindicator for heavy metal, wastewater and polycyclic aromatic hydrocarbon pollution. This study explored the effects of varying concentrations (0.01 mg/L – 100 mg/L) of the organophosphorus insecticide, dichlorvos and the organochlorine herbicide, paraquat on *P. aeruginosa* isolated from river water to ascertain mortality rates, median lethal concentrations (LC_{50}), lowest observed effect concentrations (LOEC) and the no observed effect concentrations (NOEC) using a 96 h acute toxicity bioassay. *P. aeruginosa* was isolated and enumerated using cetrimide agar while the toxicity parameters were determined via probit regression analysis. There was a general decline in abundance of *P. aeruginosa*, and correspondingly, a rise mortality levels, as exposure period and pesticide concentrations increased. Survival rates of 0% were obtained at 72 h and 48 h and above for paraquat and dichlorvos respectively at the highest pesticide concentration of 100 mg/L. Dichlorvos was found to be more toxic to *P. aeruginosa* than paraquat as the LC_{50}, LOEC and NOEC were determined as 0.032 mg/L, 0.0042 mg/L and 0.0015 mg/L respectively for dichlorvos. The values obtained for paraquat were 0.44 mg/L, 0.0053 mg/L and 0.0017 mg/L for LC_{50}, LOEC and NOEC respectively. The findings of this study raise concerns for the indiscriminate use of pesticides as they may impact negatively on vital ecosystem players.

1. Introduction

The term pesticide covers a range of natural and synthetic substances or biological agents employed in the annihilation, repulsion or management of unwanted flora or fauna associated with cultivated crops, stored produce and animal husbandry. Increasing global food security issues inevitably mean increased pesticide use to boost the quality of agricultural yield. The application of pesticides to soil comes with several undesirable secondary consequences both in the soil ecosystem and in surrounding aquatic systems due to surface run-offs. These compounds are of relatively limited concern when they are used appropriately; their misuse, however, could result in environmental pollution. These emerging pollutants become an environmental concern when they are employed consistently over extended periods or used in excess. The indiscriminate application of pesticides has been linked to ecological impacts that often require remediative interventions since, normally, over 95 – 99 % of applied pesticides are absorbed into the ecosystem where they exert toxic effects on non-target flora and fauna **(Javaid *et al.*, 2016; Doolotkeldieva *et al.*, 2018; Degrendele *et al.*, 2022)**. At the ecosystem level, indiscriminate pesticide use often results in a decline in biodiversity and disruption of ecosystem services. Even readily biodegradable pesticides could pose an environmental threat if their degradation by-products and residues accumulate across trophic levels and in food crops **(Vasquez *et al.*, 2021)**.

De *et al.* (2014) and **Adeniyi (2022)** confirmed that herbicides and insecticides were the most commonly used pesticide groups in the developing world with paraquat and dichlorvos mentioned amongst the principal active ingredients in use. Paraquat ($C_{12}H_{14}Cl_2N_2$) is a halogenated aliphatic compound that is classed amongst the viologen salts. It is well known for its recalcitrance in both aquatic and terrestrial ecosystems and has, thus, been banned by the European Union (EU) while other countries restrict its use **(USEPA, 1997; European Commission 2011)**. Its redox-active properties function to inhibit photosynthesis in green plants. The herbicide is not only relatively immobile in soil but is also particularly resistant to environmental degradative processes like hydrolysis and photodegradation **(Soriwei *et al.*, 2021)**. Dichlorvos ($C_4H_7Cl_2O_4P$), in comparison, is an organophosphate insecticide most commonly used in developing countries for the management of insect pests post-harvest and is typically applied as an aerosol. Though it readily degrades in atmospheric, edaphic and aquatic systems, it exhibits high toxicity within the environment. It is a well-known carcinogen and poison having an acceptable daily intake (ADI) of 0.004 mg/kg body weight (bw) and an acute reference dose of 0.1 mg/kg bw **(Adeniyi, 2022)**. The use of dichlorvos has been prohibited in the EU since

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1998 **(EC, 2000)** but is still used extensively in Nigeria and other developing countries. Both dichlorvos and paraquat are listed on the Pesticide Action Network International list of highly hazardous pesticides (HHP) and have been earmarked to be phased out globally by 2024 **(PAN International, 2019).** They are further noted as class 1B hazardous compounds by the World Health Organisation (2012).

Microorganisms play several niche roles within the ecosystem. Pesticides and their residues modify the microbial community structure in impacted ecosystems. Investigating the impact of pesticides on microorganisms is, therefore, imperative as they are fundamental to a fully functional ecosystem. Microbial bio-indicators serve to proffer clear information as to the qualitative state of an ecosystem at any particular time **(Sudhakar and Karagam, 2011)**. *Pseudomonas aeruginosa* is a rod-shaped, Gram negative, facultatively aerobic bacterium well known for its environmental resilience and xenobiotic degradative capacity. It is also often linked to nosocomial infection **(Januário *et al*., 2020)**. It can be readily isolated from most environments and has been implicated in the degradation of several hydrocarbon compounds. The bacterium has been identified as a bio-indicator for heavy metal pollution, contamination of environmental media by waste water and polycyclic aromatic hydrocarbon pollution **(de Victoria and Galvan, 2001; Niepceron *et al*., 2013; Sumampouw and Risjani, 2014; Teodoro *et al*., 2018; Tovar-Sánchez *et al*., 2019)**.

This ecotoxicity study investigates and compares the effects of varying concentrations of the organophosphorus insecticide, dichlorvos and the organochlorine herbicide, paraquat on *Pseudomonas aeruginosa* to ascertain mortality rates, median lethal concentrations (LC_{50}), lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) using a 96 h acute toxicity assay. The median lethal concentration (LC_{50}) represents concentrations of the pesticides that would eliminate 50% of the test organism while LOEC and NOEC are the lowest tested pesticide concentrations being significantly different from the control that gives rise to adverse effects and no statistically significant effects in the test organism respectively.

2. Material and methods

2.1 Sample Collection

The herbicide (paraquat) and insecticide (dichlorvos) were purchased from a local vendor in Port Harcourt, Rivers State, Nigeria. The recommended field application rates (RFAR) for paraquat and dichlorvos as stated by the product manufacturers is 2 – 5 kg/ha which corresponds to about 20 – 50 mg/L. The pesticides were sterilised by filtration (0.22 mm) prior to use.

The test isolate was obtained from freshwater samples from the New Calabar River in Choba, Port Harcourt, Nigeria. Water samples were collected using clean glass containers. The containers were first rinsed with river water twice before collection. Sampling was done along the water table. Analysis was done within 8 hours of samples collection.

2.2 Isolation of *Pseudomonas aeruginosa*

Isolation was done following a six-fold serial dilution; after which, 0.1 mL aliquots of suitable dilutions were inoculated unto cetrimide agar plates (with replicates) via the spread plate technique. Incubation was at 37 °C for about 48 hours. The presence of blue-green colonies on cetrimide agar were considered indicative of *P. aeruginosa*. Discrete colonies were purified by sub-culturing twice via streaking unto fresh agar plates. The pure isolates obtained were stored on slants in Bijou bottles until required for further analysis.

2.3 Confirmatory Test and Characterisation of *P. aeruginosa* Isolates

The identity of the *P. aeruginosa* isolate was confirmed via microscopic analysis and biochemical tests. Following microscopic examination for Gram's stain reaction, cell shape, presence of a capsule, flagella and spore formation; biochemical tests were carried out as listed: 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. Enzymatic reactions – phenylalanine deaminase, acetate utilisation, arginine dehydrolase, lipase and ornithine decarboxylase – were also tested **(Cheesborough, 2006)**.

2.4 The 96-hour Acute Toxicity Bioassay

The toxicity of the selected pesticides was determined using an acute toxicity bioassay technique over 96 hours. The pesticides were each tested in triplicate sets at concentrations of 100 mg/L, 10 mg/L, 1 mg/L, 0.1 mg/L, and 0.01 mg/L with sampling every 24 hours for 96 hours. The control consisted of the set-up with no pesticide added (0 mg/L) making a total of 6 flasks per set. The isolate was aseptically transferred from the agar slant to sterile nutrient broth (Merck, Germany) in an Erlenmeyer flask. After 48 h incubation at 37 °C, a loopful of the isolate in broth culture was introduced into fresh nutrient broth. The culture was again incubated at 37 °C for 48 h with agitation. Aliquots of about 1 ml from this stock culture was then inoculated into fresh nutrient broth and incubated at 37 °C for 24 h in order to ensure that the cells employed in the toxicity bioassay were within the exponential growth phase with an optical density of about 0.06 at 600 nm.

The set-up for the bioassay consisted of Erlenmeyer flasks containing nutrient broth (Merck, Germany) with the test pesticides applied at the relevant concentrations and plugged with non-absorbent cotton wool. About 1 ml of the 24 h old *P. aeruginosa* culture was introduced into the pesticide-tainted broths with incubation at 37 °C for 96 h with agitation. A 0.1 ml sample was drawn immediately and plated out (with replicates) unto nutrient agar in order to obtain the 0 hour count. Subsequently, samples were drawn for enumeration at 24 h intervals for 96 h.

2.5 Enumeration of *P. aeruginosa* during the Toxicity Bioassay

Enumeration of *P. aeruginosa* from the set-ups was done using cetrimide agar via the spread plate method. Approximately 0.1 mL aliquots of relevant dilutions were plated out on the cetrimide medium in Petri plates. The plates were incubated at 37 °C for 48 h. Plates with visible colonies ranging from 30 – 300 were enumerated using an automated digital colony counter (Balance Instrument Co., China) and expressed as colony forming units per milliliter.

2.6 Determination of Survival and Mortality Quotients

In the determination of the survival and mortality, the control was used as a reference as recommended by **Finney (1952)**. The percentage survival and percentage mortality at different time intervals was determined using Equations (1) and (2) respectively.

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

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

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

2.7 Determination of Toxicity Parameters

The median lethal concentration (LC_{50}), No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined by probit regression analysis. A probit curve of log percentage survival against the logarithmic value of concentration was drawn and the line of regression fit. The regression analysis was done at different concentrations of pesticides. The LC_{50} was determined using the slope of the regression curve.

The percentage Log survival employed for probit regression analysis was estimated using Equation (3) according to **Williamson and Johnson (1981)**.

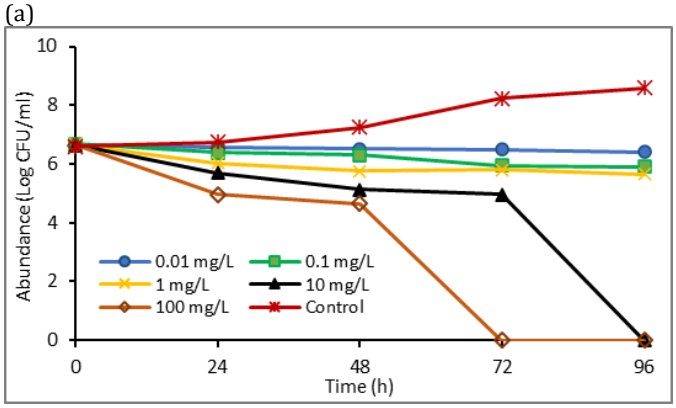
$$\% \text{ Log Survival} = \frac{\text{Log C}}{\text{Log c}} \text{ x } 100\% \quad \text{Eq. (3)}$$

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

3. Results

A general decline in abundance of *P. aeruginosa* was obtained as exposure period and pesticide concentration increased (Figure 1). Correspondingly, mortality quotients obtained for the test isolate rose with progressing pesticide concentrations and exposure periods as depicted in Figures 2 and 3. A total elimination of the test isolate (0 % survival rate) was seen from 72 h and 48 h respectively for paraquat and dichlorvos at the highest pesticide concentration of 100 mg/L. For pesticide concentration of 10 mg/L, a 0 % survival rate was obtained only at 96 h for both paraquat and dichlorvos.

(a)



(b)

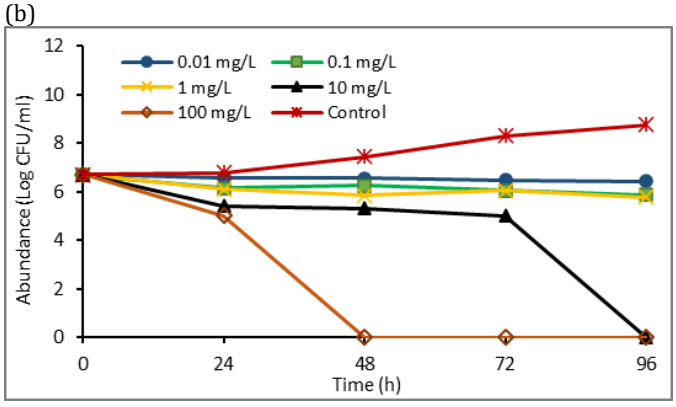


Figure 1 Variation in abundance of *Pseudomonas aeruginosa* in response to (a) paraquat (herbicide) and (b) dichlorvos (insecticide)

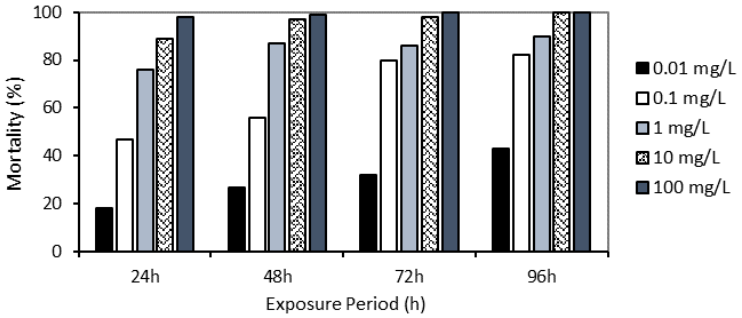


Figure 2 Mortality of *P. aeruginosa* in response to different concentrations of paraquat (herbicide)

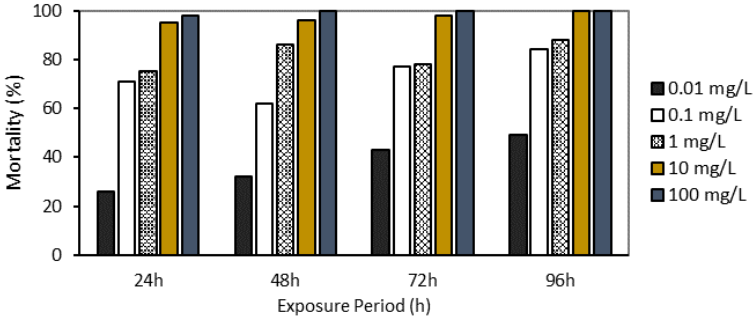


Figure 3 Mortality of *P. aeruginosa* in response to different concentrations of dichlorvos (insecticide)

The toxicity response of the bio-indicator – *P. aeruginosa* – to the test pesticides as determined from the probit regression model is outlined in Table 1. Overall, dichlorvos was shown to be relatively more toxic than paraquat having lower median lethal concentration, LOEC and NOEC values.

Table 1 Toxicity Response of *P. aeruginosa* to the Test Pesticides

Toxicity Parameter	**Paraquat** (Herbicide)	**Dichlorvos** (Insecticide)
*LC_{50} (mg/L)	0.44	0.032
LOEC (mg/L)	0.0053	0.0042
NOEC (mg/L)	0.0017	0.0015
Degree of Freedom	3	3

*Determined at 95 % confidence interval
LC50 – Median lethal concentration; LOEC – Lowest Observed Effect Concentration; NOEC – No Observed Effect Concentration

4. Discussion

The test isolate, *Pseudomonas aeruginosa*, showed diminished proliferation in the presence of both paraquat and dichlorvos, especially at higher concentrations. These results are buttressed by reports that the application of herbicides generally results in a drop in the microbial population in that affected environment. Paraquat was shown to inhibit about 82% of soil bacterial population in a study in Malaysia when applied at twice the recommended field rate (RFR) and over 42 % when applied at the RFR **(Nur *et al.*, 2013)**. Comparable results were also obtained by **Jacobsen and Hjeslmø (2014)** who confirmed that the presence of applied pesticides in soil, resulted in a relative decline in overall species richness. In the research by **de Oliveira *et al.* (2021)**, *Pseudomonas* sp. CMA-7.3 displayed decreased viability and growth rates in response to increased

concentrations of the herbicide, 2, 4-Dichlorophenoxyacetic acid. Another study subjected *P. aeruginosa* to varying concentrations of seven different pesticides and found that the plant growth-promoting characteristics of the bio-indicator diminished steadily when exposed to the recommended field application rates (RFAR) and over. The greatest toxicity was demonstrated at levels three times the RFAR **(Ahemad and Khan, 2011)**. These studies, though comparable with the current study, were conducted in terrestrial ecosystems.

While there are no clearly defined toxicity categories for microorganisms, based on the categories outlined by the **USEPA (2022)** for aquatic organisms, LC_{50} values of less than 0.1 are classed as "very highly toxic" while those in the range of 0.1 – 1.0 belong to the "highly toxic" category. The LC_{50} obtained in the current study, therefore, indicate very high toxicity and high toxicity for *P. aeruginosa* with regards to dichlorvos and paraquat respectively. The toxicity findings of the present study are in contrast to the conclusions of **Kim *et al.* (2017)** and **Damalas and Eleftherohorino (2011)** who both state that organochlorine pesticides typically possess low acute toxicity unlike the organophosphates that are fairly toxic to higher organisms like mammals and have low persistence. Their conclusions are somewhat corroborated by the **APVMA (2008)** report where it was highlighted that even at concentrations of more than 250 mg/L, dichlorvos was relatively non-toxic to isolates from sewage and soil including *Pseudomonas* and *Bacillus* spp.

Unlike in the current study where dichlorvos proved to be more toxic to *P. aeruginosa* than paraquat, a toxicity study in Brazil found paraquat to have the greatest inhibitory effect on aquatic *E. coli* isolates when compared with other similar pesticides and herbicide-free control studies in an investigation. The study concluded that paraquat could be the most toxic herbicide **(Botelho *et al.*, 2012)**. This toxic effect of paraquat is confirmed in another similar study in Ghana where the organochlorine herbicide was identified to exert the greatest impact on soil bacterial populations (compared to other herbicides) following exposure to half the recommended field rate (RFR), the RFR and double the RFR **(Adomako and Akyeampong, 2016)**. A comparable study in Rivers state, Nigeria compared the toxic effect of paraquat to the insecticide γ–cyhalothrin against the phosphate solubiliser, *Pantoa dispersa* and concluded that paraquat was more toxic in freshwater, brackish and marine systems. In this case, 100 % mortality was seen at paraquat concentrations of 50 % – 75 % after 4 h exposure **(Nrior *et al.*, 2022)**. This might elude to enhanced ecotoxic effects in aquatic systems compared to soil and would further explain the higher toxicity in the present study compared to other studies.

The greater toxicity of dichlorvos may be linked to its mechanism of action. The toxicity of pesticides have been rationalised by their mode of action. They cause DNA damage in the cell and cell membrane rupture by varying means or prevent cell division. Certain pesticides function as metal chelators diminishing the amounts of crucial cell micronutrients that are required as co-factors for enzyme function and biomolecular constituents **(Singh *et al.*, 2020)**. Herbicides are typically more toxic to organisms and cells that have photosynthetic capacity as they function mainly by interrupting the process or by stopping the synthesis of carotenoid, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. Paraquat, specifically, intercepts electrons from the photosystem I, a system that is not necessarily present in *P. aeruginosa*. Herbicides are also known to inhibit the microtubule-forming unit in the dimeric protein tubulin at cellular level which hampers spindle microtubule formation. In the absence of spindle microtubules, nuclear division and the separation of chromosomes are impacted. Insecticides, on the other hand, inhibit enzymatic and cellular pathways, that are present in all organisms. The organophosphorus pesticides function principally by phosphorylation of the acetylcholinesterase enzyme. They are also known to interrupt normal mitotic divisional processes **(De Lorenzo *et al.*, 2009; Casida, 2009; Binukumar and Gill, 2010; Combarnous, 2017)**.

The levels of toxicity obtained in the present study are noteworthy as *Pseudomonas* sp. has been implicated in the biodegradation of dichlorvos **(Gaonkar *et al.*, 2019)** and several other pesticides like, methyl parathion **(Archana and Thatheyus, 2020)** endosulfan **(Nwokem *et al.*, 2017)**, diazinon **(Najavand *et al.*, 2012)**, glyphosate **(Singh *et al.*, 2020)** and deltamethrin **(Gür Özdal and Algur, 2020)** amongst others. **Archana and Thatheyus, (2020)** noted that *P. aeruginosa* was able to actively utilise and degrade organophosphorus insecticide, methyl parathion at 50 – 200 ppm. The microorganism (*P. aeruginosa*) was also recommended for the bioremediation of pesticides as it exhibited a strong capacity for the degradation and elimination of the organophosphorus insecticide chlorpyrifos occurring at around 120 µg/L. A 99 % removal efficiency was reported after 6 days **(Hashim *et al.*, 2022)**. **Kharabsheh *et al.* (2017)** confirmed the ability of *P. aeruginosa* to utilise the organophosphate insecticide, Chlorpyrifos as well.

The disparity between laboratory- and field-based studies may be the key here. Studies have shown that findings from *in vitro* investigations with cultivable bacteria may not be representative of the results that would be obtained in the field **(Jacobsen and Hjeslmø, 2014)**. Furthermore**, Van Elsas *et al.* (2012)** noted that when there is a drop in microbial community diversity and abundance, certain functions like resistance to invasion by pathogenic species are impacted; this phenomenon may also apply to biodegradative capacity. **Nielsen *et al.* (2011)**, however, dispute this; they opine that changes in species richness gives rise to only a limited impact on mineralisation of organic compounds. Another possibility lies in the use of axenic *P. aeruginosa* cultures for testing. When a microorganism occurs in a functioning ecosystem, the different species work in synergy with one another to both effect biodegradation and to resist toxicity such as ensues when an organism occurs within a biofilm. Furthermore, the lack of prior exposure to pesticides by the P. aeruginosa isolate may also increase its sensitivity to the test pesticides resulting in an increased susceptibility to pesticide toxicity as opposed to isolates from pesticide-polluted environments.

5. Conclusion

Pseudomonas aeruginosa is an important ecosystem player that is regularly employed as a bio-indicator of heavy metal, wastewater and polycyclic aromatic hydrocarbon pollution. Unfortunately, its abundance in the environment may be impacted by the presence of pesticides as highlighted in the current study. Although both the organochlorine herbicide, paraquat and the organophosphorus insecticide, dichlorvos, investigated were toxic to the pollution bio-indicator, dichlorvos proved to be more toxic. This study underscored the need for proper regulation of pesticide use to minimise the impact on both aquatic and non-aquatic ecosystems.

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Declaration of interest

The authors report no conflicts of interest.

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