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Pesticides and bacterial density - analysis of a dam situated in agricultural area

Pesticidas e densidade bacteriana – análise de uma represa situada em área agrícola

Plaguicidas y densidad bacteriana - análisis en una represa ubicada en una zona agrícola

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Abstract
The present study aimed to detect the types and concentration of pesticides in the surface water of a dam located in an agricultural region, and relate them to aquatic bacterial density. 1L of water were collected six times, three in the dry season (D1, D2 and D3) and three in the rainy season (R1, R2 and R3). The pesticide analysis was performed using CG/MS. The bacterial density was analyzed by means of CFU counting. The most abundant colonies that were cultivated in all studied samples were isolated and identified by molecular methods. The bacterial density had a significant difference between the rainy and dry seasons. In the dry season, the average bacterial density ($5.69 \times 10^5 \pm 2.86 \times 10^5$ UFC mL^{-1}) was higher than in the rainy season ($2.31 \times 10^5 \pm 1.73 \times 10^5$ UFC mL^{-1}). In the dry season, lambda cyhalothrin (0.3 µg L^{-1}) and endrin (0.23 µg L^{-1}) were found in D1 collection and endosulfan sulfate in D1 and D2 (0.05 - 0.27 µg L^{-1}). In the rainy season, DEA (0.02 - 0.09 µg L^{-1}) and atrazine (0.02 - 0.06 µg L^{-1}) were detected in all collections, metalachlor (0.2 µg L^{-1}), chlorpyrifos (0.2 µg L^{-1}) in R1 and R2 and endosulfan sulfate (0.1 µg L^{-1}) in R1. In the dry season, the bacterial density in R1 was significantly lower than R2 and R3. High correlation among those pesticides and the bacterial density was observed. Endrin, lambda cyhalothrin and endosulfan sulphate are toxic and persistent pesticides, and may therefore have influenced the decrease in aquatic bacterial density. Molecular analyzes identified the bacterium *Bacillus weihenstephanensis*, known to be present in environments contaminated by pesticides and for its biodegradable potential.
Keywords: Endrin; Lambda cyhalothrin; Endosulfan sulphate.

Resumo
O presente estudo teve como objetivo detectar os tipos e concentrações de agrotóxicos nas águas superficiais de uma represa localizada em uma região agrícola, e relacioná-los com a densidade bacteriana aquática. Foram coletadas seis amostras de 1L de água, três na estação seca (D1, D2 e D3) e três na estação chuvosa (R1, R2 e R3). A análise de pesticidas foi realizada usando CG/MS. A densidade bacteriana foi analisada por meio de contagem de UFC. As colônias mais abundantes cultivadas em todas as amostras estudadas foram isoladas e identificadas por métodos moleculares. A densidade bacteriana apresentou diferença significativa entre as estações chuvosa e seca. Na estação seca, a densidade bacteriana média (5,69x105±2,86 x105 UFC mL-1) foi maior do que na estação chuvosa (2,31x105±1,73 x105 UFC mL-1). Na estação seca, lambda cialotrina (0,3 µg L -1) e endrin (0,23 µg L -1) foram encontrados na coleta D1 e sulfato de endosulfan em D1 e D2 (0,05 - 0,27 µg L -1). Na estação chuvosa, DEA (0,02 - 0,09 µg L-1) e atrazina (0,02 - 0,06 µg L -1) foram detectados em todas as coletas, metalacloro (0,2 µg L -1), clorpirifós (0,2 µg L -1) em R1 e R2 e sulfato de endossulfam (0,1 µg L -1) em R1. Na estação seca, a densidade bacteriana em R1 foi significativamente menor que R2 e R3. Observou-se alta correlação entre esses pesticidas e a densidade bacteriana. Endrin, lambda cialotrina e sulfato de endosulfan são pesticidas tóxicos e persistentes e podem, portanto, ter influenciado a diminuição da densidade bacteriana aquática. As análises moleculares identificaram a bactéria Bacillus weihenstephanensis, conhecida por estar presente em ambientes contaminados por agrotóxicos e por seu potencial biodegradável.
Palavras-chave: Endrin; Lambda cialotrina; Sulfato de endossulfan.

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Resumen
El presente estudio tuvo como objetivo analizar los tipos y concentraciones de pesticidas en el agua superficial de una represa ubicada en una región agrícola, y relacionarlos con la densidad bacteriana acuática. Se recolectaron seis muestras de agua de 1L, tres en época poco lluviosa (D1, D2 y D3) y tres en época lluviosa (R1, R2 y R3). El análisis de pesticidas se realizó usando GC/MS. La densidad bacteriana se analizó utilizando recuentos de UFC. Las colonias más abundantes cultivadas en todas las muestras estudiadas fueron aisladas e identificadas por métodos moleculares. La densidad bacteriana mostró una diferencia significativa entre las estaciones lluviosa y seca. En época poco lluviosa, la densidad bacteriana promedio (5,69x105±2,86 x105 UFC mL-1) fue mayor que en época de lluvias (2,31x105±1,73 x105 UFC mL-1). En época seca se encontró lambda cihalotrina (0.3 µg L-1) y endrina (0.23 µg L-1) en la colecta D1 y endosulfán sulfato en D1 y D2 (0.05 - 0.27 µg L-1). En época de lluvias se detectó DEA (0.02 - 0.09 µg L^{-1}) (0.02 - 0.09 µg L-1) y atrazina (0.02 - 0.06 µg L -1) en todas las colectas, metalacloro (0.2 µg L-1), clorpirifos (0.2 µg L -1) en R1 y R2 y sulfato de endosulfán (0,1 µg L -1) en R1. En la estación seca, la densidad bacteriana en R1 fue significativamente menor que en R2 y R3. Hubo una alta correlación entre estos plaguicidas y la disminución de la densidad bacteriana. La endrina, la lambda cihalotrina y el sulfato de endosulfán son pesticidas tóxicos y persistentes y, por lo tanto, pueden haber influido en la disminución de la densidad bacteriana acuática. Los análisis moleculares identificaron la bacteria Bacillus weihenstephanensis, conocida por estar presente en ambientes contaminados por pesticidas y por su potencial biodegradable.
Palabras clave: Endrín; Lambda cihalotrina; Sulfato de endosulfán.

1. Introduction

The modern agriculture is dependent not only of mineral fertilization, but also requires the control of insects, pathogenic fungi and pests. For the chemical control of these organisms, more than a hundred pesticides are available on the market and millions of tons have been used worldwide (Stolp, 1988). Thereby, the use of pesticides is increasing worldwide. Brazil has an extensive planting area which makes the country the largest consumer of pesticides in the world since 2008 (Rebelo et al., 2010; Rigoto et al., 2014; Pignati et al., 2017; Guida et al., 2018), being responsible for approximately 20% of total global use (Albuquerque et al., 2016). In addition, for the agricultural production growth, Brazil has adopted permissive laws to expand its agribusiness (Guida et al. 2018). Organochlorines (endrin, endosufan), Organophosphates (chloropyrifos), Carbamates (carbaryl), synthetic pyrethroids (permethrin, lambda cyhalothrin), and neonicotinoids (imidacloprid) pertains to the prime range of insecticides. Phenoxy herbicides (2,4- D), and triazines (atrazine) and chloroacetanilide (metalachlor) are the principal series of herbicides (Yadav et al., 2020).

Many pesticides are stable over time in the environment and, therefore, can be transported to many areas far from their source (Lofrano et al., 2020). These Pesticides, which are composed of insecticides, herbicides and fungicides, are a group of potentially toxic substances capable of disrupting microbial structure and function in aquatic habitats (uturi et al., 2017). With the increase in the use of pesticides, aquatic environments receive inputs from these compounds which negatively affect all biological communities dependent on this environment (Staley et al., 2015). Microorganisms are important inhabitants of aquatic ecosystems, where they fulfill critical functions in primary productivity, nutrient cycle and decomposition (DeLorenzo et al., 2001). Biological changes in this group arising from these chemicals can cause drastic changes in all biological functions present in this environment.

The effects of pesticides in each organism are difficult to predict since the chemical acts differently depending on the studied species. For some microorganisms, pesticides can be a carbon source, for others, they can be toxic (Pham et al., 2004). In addition, aquatic ecosystems are usually exposed to a mixture of pesticides, which lead to different effects on the microbial community9. Several studies have pointed out that exposure to pesticides modifies - temporarily and chronically - bacterial biomass, abundance of pesticide degraders and the entire dynamics of the aquatic bacterial community (Hoagland et al., 2009, Muturi et al., 2014, Mauffret et al., 2017). Recent study observed that some pesticides severely inhibit bacterial growth (Rosic et al., 2020), influenced on decreasing of both microbial abundance and diversity (Onwana-Kwakye et al., 2020).

Monitoring the concentration of these compounds in aquatic environments and study their effects on the bacterial community becomes of great importance for understanding the toxic effects of pesticides on this biological group, thus being able to promote economically viable and environmentally safe strategies for a more sustainable agricultural development.

2. Methodology

2.1 Study area

The waters sampling were carried out in a dam located on a farm in the Campo Verde – Mato Grosso, Brazil (latitude S15º37'18.8 "and longitude W55º10" 24.8). Campo Verde is an important agricultural center in Brazil, with a population of approximately 31 thousand inhabitants. The region has a rainy season from november to april and a dry season from may to october. Agriculture in these areas is dominated by the intensive cultivation of soy, cotton and corn (Nogueira et al., 2012).

2.2 Sample collection

In total, six samples were collected for the present study, three in the dry season and three in the rainy season. The water samples were collected in triplicate, using dark glass bottles, properly sterilized, with capacity for 1 liter. The samples were stored under refrigeration until they arrived at the laboratory which were immediately processed.

2.3 Pesticide Analysis

Assuming that there are large varieties of pesticides used in agriculture, the most used in the study region were tested: Alpha HCH; Trifluralin; Alachlor; Aldrin; Endosulfan alpha; Dieldrin; DDE; Endrin; Endosulfan beta; Endosulfan sulfate; DDT1; DDT; Methoxychlor 1; Methoxychlor; Lambda cyhalothrin; Permethrin 1; Permethrin 2; Cirpermethrin 1; Cypermethrin 2; Deltamethrin; Terbuthylazine; DEA; Simazine; Atrazine; Metribuzin; Ametrine; Metolachlor; Cyanazine; Methyl paration; Malation; Chlorpyrifos, and quantified by the internal standard Ditalimfós 1.00 ug.

The multi-residue extraction method was based on a procedure described elsewhere.10 Briefly, SPE cartridges were made by packing glass tubes (8 mL) with 1000 mg of Bakerbond C18 (J.T. Baker, Grossgerau, Germany). The cartridges were conditioned with 10 mL of methanol followed by 10 mL of water. An aliquot of 500 mL of dissolved water phase (0.47 μm glass fiber filter), pH previously adjusted to 6.5-7.5, was transferred to the cartridge (ca. 5 mL min-1). Analytes were eluted with 10 mL portions of ethyl acetate and hexane:ethyl acetate (7:3) and a 5 mL portion of hexane. Different mixtures of solvent were required since the analytes presents not similar properties. Eluate was concentrated and the residue was redissolved in toluene (1 mL) with phenanthrene-d10 employed as internal standard. An HP 6890 series gas chromatograph coupled to an HP 5973 mass spectrometer (Hewlett-Packard GmbH, Germany), equipped with an HP 7683 autosampler, a split/ splitless injector and an HP-5MS (5% phenylmethylsiloxane) column (30 m × 250 μm id × 0.25 μm phase thickness) was used for pesticide identification and quantification (Nogueira et al., 2012).

2.4 Bacterial cultivation and count

The samples were homogenized and subsequently diluted in series (Neder, 1992). After serial dilution, the samples were inoculated, using the pour-plate method, on standard agar and cultured at 35ºC for 48 hours. Subsequently, colonies were counted (Gomes et al., 2020) and isolated on nutrient agar.

2.5 Molecular identification

Bacterial identification was carried out in four of the isolated colonies, by amplifying total DNA using universal 16S primers (5'AACGCGAAGAACCTTAC3' F and 5'CGGTGTGTACAAGGCCCGGGAACG3' R). The PCR (polymerase chain reaction) reactions were performed using the DYEnamic ™ - GE Kit (Fabiano et al., 2017). To analyze the identified sequences,

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the program developed by Embrapa Electropherogram quality analysis was used, which uses the Phred index (Aburjaile et al., 2015). The National Center for Biotechnology Information bank was employed to identify the species.

2.6 Statistical analysis

To determine the existence of a significant difference between bacterial densities in the different collections, the one-way ANOVA and Kruskal-Wallis variance test were performed for normal and non-normal data, respectively. To identify which of the limnological variables would be associated with bacterial density, the data were correlated by Pearson's linear correlation coefficient (r) when the data were normal, and Sperman when they did not meet the assumption of normality. The assumption of normality was verified by the Shapiro-Wilk test ($p > 0.05$). For all tests, a significance level of $p < 0.05$ was used.

3. Results

According to Table 1, in all water sampling seven pesticides were detected. There was a clear difference between the pesticides detected in each season. In the dry season, only insecticides were detected, such as endosulfan sulfate (D1 and D2), lambda cyhalothrin (D1) and endrin (D1). In the D3 sample collection, none of the studied pesticides was detected. On the other hand, in the rainy season, herbicides and insecticides were detected. The herbicides DEA and atrazine were detected in all samples collections. As observed in Table 1, the herbicide metalachlor was detected in collections R1 and R2, as well as the insecticide chlorpyrifos. The endosulfan sulfate insecticide was detected only in R1. The highest concentrations of pesticides were detected in sample D1.

Table 1 - Concentration of pesticides (µg L^{-1}) detected in water samples collected in the dry season (D1, D2 and D3) and in the rainy season (R1, R2, and R3). Caption: ND – Not Detection.

PESTICIDE	D1	D2	D3	R1	R2	R3
DEA	ND	ND	ND	0.09	0.04	0.02
ATRAZINE	ND	ND	ND	0.08	0.06	0.02
METALACHLOR	ND	ND	ND	0.06	0.02	ND
CHLORPYRIFOS	ND	ND	ND	0.05	0.02	ND
ENDOSUFAN SULPHATE	0.27	0.05	ND	0.1	ND	ND
LAMBDA CYHALOTHRYN	0.3	ND	ND	ND	ND	ND
ENDRIN	0.23	ND	ND	ND	ND	ND

Source: Authors.

Bacterial densities for all studied periods are demonstrated in Figure 1. These densities significantly differed between the studied samples (ANOVA, $F = 10.46$; $p = 0.005$). In the dry season, the sample D1 presented the highest concentration of pesticides and consequently, the lowest bacterial density (2.5×10^5 CFU $L^{-1} \pm 1.25 \times 10^5$). Contrary, the sample D3 had the highest bacterial density and no detected pesticides. There was a significant difference in bacterial density in D1 comparing to D2 and D3 (ANOVA; $F = 14.46$; $p = 0.02$). In the rainy season, the lowest bacterial density was observed in R3 (1.1×10^5 CFU $L^{-1} \pm 4.62 \times 10^4$), followed by R1 (1.5×10^5 CFU $L^{-1} \pm 2.65 \times 10^4$) and the highest value was found in R2 (4.3×10^5 CFU $L^{-1} \pm 1.51 \times 10^5$).

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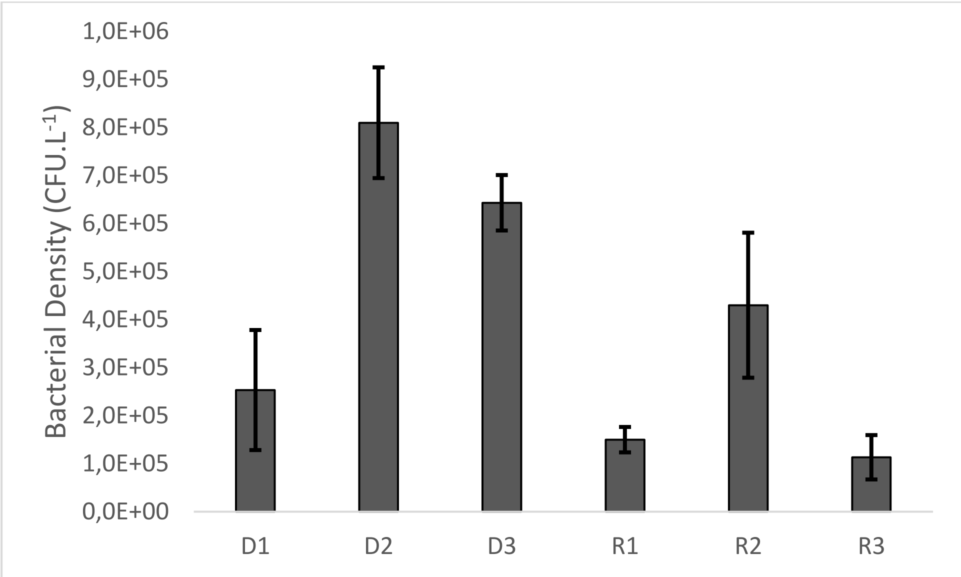
Figure 1 – Bacterial density in the 3 collections of the dry season (D1, D2 and D3) and in the three collections of the rainy season (R1, R2 and R3).



Source: Authors.

To assess the correlation between the pesticides concentration and bacterial density (BD), Pearson's correlation analysis was performed. A significant negative relationship between BD and the concentration of endosulfan sulphate ($r = -0.89$; $p<0.05$), endrin ($r = -0.95$; $p<0.05$) and lambda cyhalothrin ($r = -0.95$; $p<0.05$) was observed for samples from the dry season. On the other hand, in the rainy season, no significant correlation was identified between BD and the detected pesticides.

The bacterial strains identified by molecular analyzes were *Endophytic bacterium* (97%), *Bacillus weihenstephanensis* (97%), Uncultured gamma proteobacterium (87%) and *Citrobacter* sp. (94%).

4. Discussion

From the 31 pesticides and their metabolites observed in this study, seven compounds were found in at least one sample. These pesticides are commonly used in agriculture. In the first sample collection, three chemicals were detected and with the highest concentrations: endosulfan sulfate, lambda-cyhalothrin and endrin. Endosulfan sulfate is a metabolite of the insecticide endosulfan, belonging to organochlorines group, used to control pests in soybean, cotton, coffee and sugarcane crops, by spraying on leaves or incorporating into the soil (Scorza-Júnior, Franco & Moraes, 2013). This compound is commonly found in the environment, as it is persistent, with half-lives ranging from approximately 9 months to 6 years (Kataoka & Takagi, 2013). In this study, the concentration of endosulfan sulfate was 0.01- 0.27 μg L^{-1}, lower values than that observed in other studies which verified 0.036μg L^{-1} to 62.3 μg L^{-1} of the compound (Mohammed et al., 2019). The relatively high aqueous concentrations and the persistence of endosulfan sulfate in the water compared to the endosulfan isomers indicate that this metabolite has higher toxicity for a non-target aquatic biota in natural water bodies (Leonard et al., 2001).

Lambda-cyhalothrin was found with the highest concentration in the present study (0.3 μg L^{-1}). This compound is used as an ingredient in insecticidal products to control cotton pests (Wu & Smith, 2015). Other studies have detected 0.02 mg L^{-1} and 0.087 lambda-cyhalothrin in water bodies (Senoro et al., 2016; Riaz et al., 2018). Another pesticide also detected in the studied region was endrin (0.23 μg L^{-1}). It is an organochlorine pesticide used against insects, rodents and birds, mainly in maize, cotton, rice and sugarcane crops (Xiao & Kondo, 2019). Due to its toxicity, it has been banned in many countries since 1970[31]. However, it is still found in environmental samples due to its great persistence in the environment (Matsumoto et al., 2009).

In the rainy season, the chlorpyrifos pesticide was detected in R1 and R2. This substance is mainly applied to corn, soy and cotton crops, to combat various insect infestations, such as the cotton borer and aphid, cartridge caterpillar and soy caterpillar (Oliveira et al., 2020). Some studies have observed the presence of chlorpyrifos (38.94 µg L^{-1}; 1.01 µg L^{-1} and 2.86 ng L^{-1}) in river waters near crops (Oliveira et al., 2020). In Brazil, chlorpyriphos was already found in São Paulo, Mato Grosso, Rio Grande do Sul and Sergipe states (Albuquerque et al., 2016).

Among the herbicides analyzed, three substances were found in the present study, all in the rainy season: atrazine, its metabolite DEA and metalachlor. Atrazine is used to control weeds in sugarcane and corn crops (Umar et al., 2012; Velisek et al., 2012). In this study, the highest concentrations of atrazine and DEA were 0.08 and 0.09 µg L^{-1}, respectively. Surface water studies are also accustomed to high concentrations of those compounds in rainy season, ranging from 0.6-2.7 µg L^{-1} (Armas et al., 2007). On the other hand, metalachlor is a substance belonging to the chemical group of chloroacetamides. In Brazil, this chemical is mostly used in the cultures of beans, corn, soybeans and sugarcane (Procópio et al., 2001).

Bacteria and Pesticides

There was a significant difference in bacterial density between the studied periods (dry and rain). In a study developed in water rivers, the authors also observed that the density of bacterial species has been significantly altered (Pearson's: $p <0.05$) as the seasonal change which affected the physical-chemical variables (Adewoyin % Okoh, 2020).

The lowest bacterial density was observed in the dry season (D1), being up to three times lower than the other samples collections for the same period. In D1, the bacterial density decreases as the main pesticide (high concentration) increases. This decline was observed when comparing endosulfan sulfate, lambda cyhalothrin and endrin with bacterial density during the dry season, suggesting a toxic effect of these substances on the bacterial community.

Several studies describe the toxic effect of endosulfan and its metabolites on non-target aquatic organisms. Toxic effect on fish has been observed in some studies (Capkin et al., 2006, Nordin et al., 2018, Muazzam et al., 2019) including in low concentrations (0.08 µg L^{-1}) (Carriger et al., 2010). Other studies show the toxic effect on gastropods (Alonso-Trujillo et al., 2010), and tadpoles (Jones, Hammond & Relyea, 2009). A positive correlation between the concentration of endosulfan sulfate and the abundance of algae was observed (Braga et al., 2014). The application of endosulfan also reduced the biomass of microorganisms in soil (Pereira et al., 2008). Endrin also has a serious toxic effect for crustaceans (Nebeker et al., 1989, You, Schuler & Lydy, 2004) and fish (Hansen et al., 1977).

In the present study, the colonies selected as the three most common among all analyzed samples were identified as *Bacillus weihenstephanensis* (97%), uncultivated proteobacterium range (87%) and *Citrobacter* sp. *Bacillus weihenstephanensis* stands out due to the presence in environments contaminated with pesticides, and for its degradation capacity. This specie had a positive result for degradation of the fungicide azoxystrobin, even at high concentrations (22.50 mg kg^{-1}) (Bacgama et al., 2016). It was also detected in soils contaminated with pesticides, and is known to be efficient in degrading polycyclic aromatic hydrocarbons (Zhao et al., 2018). Another study isolated this bacterium from a soil contaminated with azoxystrobin (22.50 mg kg^{-1}) (Bacgama et al., 2015). *Bacillus weihenstephanensis* was isolated from an environment contaminated with pesticides and the authors observed a good resistance to them due to its capacity of specific enzymes production (Shetti & Kaliwal, 2016). This strategy justify the presence of this bacteria specie in this study, even in periods with high pesticides concentrations.

5. Conclusion

The aquatic environment is increasingly occupying a smaller portion in relation to the agricultural area, allowing a greater “run-off”, directly and indirectly receiving pesticides to which, the microorganisms are exposed. While pesticides can cause a variety of chronic and acute toxic effects, microorganisms also have the ability to accumulate or metabolize some pesticides. Harmful effects on microbial species may also have a subsequent impact on higher trophic levels. It should be noted that microbial species occupy functional niches that, may determine, if altered, losses of ecological processes in that place, although the bacterial richness is high.

Ecosystems considered "out of equilibrium", periodically disturbed, tend to have a higher diversity than "equilibrium" ones, where competitive dominance and exclusion are more intense. Thus, the increase in diversity over the analyzed period may suggest the influence of pesticides residues on microbial communities, since this has been a common practice that is repeated at each planting period of the crop.

In this way, the monitoring of microbial communities in agricultural areas has a strong appeal, consisting of an attempt to guarantee immediate detections of local biodivesity.

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