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Original

A Toxicological Evaluation of Activated Bioproduct IHPLUS® in *Artemia sp.* Larvae

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ABSTRACT

Background: Rational water use and the application of residual water treatment systems is an alternative to mitigate the pollution of aquatic systems. Activated IHPLUS® is an alternative for the bioremediation of polluted water sources. **Aim**. To determine toxicity after a single exposure of Artemia sp. larvae to activated IHPLUS®. **Methods:** The study evaluated toxicity in cysts of *Artemia sp* larvae in artificial seawater medium after exposure to a single treatment of activated IHPLUS®. The experimental groups of Artemia sp. were exposed for 24 hours at five concentrations of the product, and were compared with the unexposed control group. The bioproduct's LC_{50} was calculated, and the influence of possible factors of toxicity/mortality in the presence or absence of microorganisms and the pH was determined. **Results:** The product's CL_{50} was 0.042% in artificial seawater, thus classifying it as nontoxic for crustaceans. The activated IHPLUS® was toxic at concentrations higher than 0.032% in Artemia sp larvae in the assay conditions. The pH was responsible for toxicity in Artemia sp. **Conclusion:** The activated IHPLUS® was not dangerous to crustaceans in marine environments.

Keywords: aquatic environment, crustaceans, toxic levels, pH, toxicity (Source: *MESH*)

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INTRODUCTION

The poor state of ecosystems has a direct effect on many animals and plants living in water, as well as other species and humans in need of clean water (EEA, 2021). Different methods are used to mitigate this problem, including bioremediation, an interesting and promising alternative to conventional techniques to treat pollutants (Montenegro, 2019). IHPLUS® was registered in 2011 by OCPI (the Cuban Office of Industrial Property), starting a Cuban technology based on efficient microorganisms (EM). These productions have been established in different production plants with multiple purposes. In animal and plant health, as a probiotic for nutrition, the removal of undesirable smells in livestock facilities, and bioremediation (Suárez, 2015).

In the area of liquid residues, it plays a relevant role in removing micro alga that used to clog irrigation systems, and its application in the bioremediation process of Josone Lake, in Varadero, Matanzas, improved the concentrations of water quality (Palma and Cruz, 2021). Research and development of this product entail possible risks due to exposure to humans and representative species in ecosystems where its application has been estimated.

Concerning evaluations of microbial pesticides, the Environmental Protection Agency in the USA has issued a set of guides for harmonized assays (EPA, OCSPP) for studying and developing microbial pesticides. Among these methods are the guides for ecotoxicological assays, including the evaluation of toxicity in aquatic invertebrates (EPA, 2015).

Today, *Artemia sp,* an invertebrate living in saline aquatic reservoirs, is used to study the toxicity of chemical, physical, or biological substances, and the effect of residues or complex mixtures. It is also part of a screening assay in a toxicity evaluation program for the study of models of toxic actions of substances and studies of trophic transference of pollutants (Persoone, 1987 and Artoxkit, 2003). It is one of the most commonly used models to characterize the impact of pollution on aquatic environments. Its cryptobiosis and euryhaline capacity make it a particularly advantageous organism with several applications. The utilization of this species in basic and applied research in ecotoxicology offers a cost-effective model with biological stability, and a capacity for use in different laboratories (Persoone, 1987).

Accordingly, this study aimed to determine the toxicity caused by a single exposure to activated IHPLUS® in Artemia sp larva in saline aquatic environments, to collect important data about the prospects for the safe utilization of this organism in interaction with aquatic ecosystems.

MATERIALS AND METHODS

Experimental location

The study took place at the toxicology and ecotoxicology laboratories of the Department of Biological Research of the Center for Chemical Bioactives, the Martha Abreu Central University of Las Villas, Santa Clara, Villa Clara, Cuba. The experiments were performed between March 12th and April 9th, 2019.

Item studied

An activated IHPLUS® sample supplied by the Indio Hatuey Experimental Station of Pastures and Forages was evaluated. It resulted from the anaerobic liquid fermentation of native microorganisms from solid fermentation containing natural, non-genetically engineered aerobic and anaerobic bacteria, yeasts, fungi, and lactobacilli used as inoculants for liquid fermentation.

Besides, the activated biopreparation obtained by sterilization for 1 hour in the autoclave (Hirayama, 121ºC and 1.216 x 10^6 Pa were evaluated. All the samples were used immediately after preparation, and the assay was performed at room temperature in the lab (22-25ºC).

Culture medium

An artificial seawater (ASW) medium was used to keep the Artemia sp larva, which was prepared using a mix of salts, according to the formula stated by Dietrich and Kalle (1957).

Acute toxicity study

An acute toxicity study was performed in Artemia sp. using the method by Artoxkit (2003), the ICT Guide (2002), and the protocol suggested by Banti and Hadjikakou (2021). The Artemia sp larvae were obtained from dried cysts, at Argent Chemical Laboratories (Washington, USA), which conferred optimum hatching conditions and homogeneous populations of nauplii. The assay was based on the determination of toxic effects produced in *Artemia sp.* larvae following exposure to activated IHPLUS® biopreparation.

During the initial experimental tests, the Artemia sp nauplii were exposed to concentrations of the product obtained in serial dilutions at 1:2 and 1:10. Then the assay was performed using five dilutions with a diluting factor that enabled 3 dilutions between the minimum dilution (100% mortality) and the maximum dilution (0 mortality). That procedure allowed for the estimation of the Mean Lethal Concentration (LC_{50}). Each assay included an unexposed control group containing the same quantity of larvae in ASW.

The larvae were observed using a magnifying glass or stereoscope microscope, and group mortality was recorded. The individuals unable to move for ten seconds were considered dead. The mortality percentage was calculated using the total number of individuals exposed. Mortality was evaluated 24 hours following exposure.

In another study, the groups were made with the same number of larvae and repetitions to evaluate possible factors or parameters that could determine or influence mortality in the individuals. Accordingly, a specific IHPLUS concentration (6%) was evaluated under three different conditions (see Table 1).

Table 1. Toxicity assays of activated IHPlus under different conditions

Group	Concentration	Altered condition	Effect on toxicity
IHPLUS®	6%	-	Real toxicity
Inactivated IHPLUS®		Inactivation	Absence of viable microorganisms
Adjusted IHPLUS®		pH set to 7	pH
Control	-	-	-

The pH of the third variant was adjusted to 7 by adding sodium carbonate (Merck) to the ASW.

Each group in the assays was supplied with 40 larvae at random, in four repetitions consisting of 10 individuals each. Each repetition remained in the corresponding well on the 24-well sterile polypropylene wells. The plates were labeled with a permanent marker, through a code that enabled blind readouts by trained staff.

The study was considered valid if the control group showed no larval mortality equal to or greater than 10%.

Calculations and analysis

The lethal percentage was calculated in each group. The data gathered at different concentrations were used to lay out the dose-response curve, and the Mean Lethal Concentration (LC_{50}) was calculated through non-linear adjustment to a sigmoidal curve with Statística 10.

Based on the results, the bioproduct was classified using the method suggested by the Globally Harmonized System for Classification and Labeling of Chemical Products (GHS), for short-term hazards (acute) (UN, 2015), as follows:

Table 2. Classification chart of aquatic environment hazardous substances (crustaceans)

Categories for crustacean classification	
Short-term hazard (acute)	
Acute category 1	$C(E)L_{50} \leq 1.00$ mg/L
Acute category 2	1.00 mg/L $< C(E)L_{50} \leq 10.0$ mg/L
Acute category 3	10.0 mg/L $< C(E)L_{50} \leq 100$ mg/L

RESULTS AND DISCUSSION

The tests were valid since no mortality was observed in the control groups at 24 hours following exposure (see Table 3).

Table 3. Mortality during the tests at concentrations of activated IHPLUS® in *Artemia sp.* larvae

Group	**Dilution**	**Concentration (percentage)**	**Mortality (percentage)**
First test (1:2 dilution)			
1	1:2	50	100
2	1:4	25	100
3	1:8	12.5	100
4	1:16	6.25	100
5	1:32	3.125	100
1-I	1:2	50	100
2-I	1:4	25	100
3-I	1:8	12.5	100
4-I	1:16	6.25	100
5-I	1:32	3.125	100
Control	-	-	0

Second test (1:10 dilution)			
6	1:10	0.1	100
7	1:100	0.01	100
8	1:1000	0.001	0
9	1:10000	0.0001	0
10	1: 100000	0.00001	0
6-I	1:10	0.1	100
7-I	1:100	0.01	100
8-I	1:1000	0.001	0
9-I	1:10000	0.0001	0
10-I	1: 100000	0.00001	0
Control	-	-	0

I: Inactivated bioproduct

In the initial studies of activated and inactivated IHPLUS®, it induced 100% mortality in Artemia sp nauplii, up to a 1:100 dilution (0.1%), in ASW medium. At 0.01% concentrations and higher, no larval death was observed, a similar behavior was found when the inactivated product was evaluated.

The results of the main assay, which included 0.1% and 0.01% concentrations, are shown in Table 4. The mortality results with the use of activated IHPLUS® showed that the LC_5 was estimated at 0.042%, equal to 420 mg/L, in a saline medium like ASW.

Table 4. ***Results of the acute toxicity study of*** **activated IHPLUS® in Artemia sp**

Group	Dilution factor	Concentration (percentage)	Mortality (percentage)	CL_{50}
1	1.78	0.01	100	0.042% (420 mg/L)
2		0.056	100	
3		0.032	0	
4		0.018	0	
5		0.001	0	
Control	-	-	0	

Model is: M=100*C**n/(Cl50**N+C**N) (Spreadsheet1)

Dep. Var. : MLevel of confidence: 95.0% (alpha=0.050)

	Estimate	Standard	t-value	p-level	Lo. Conf	Up. Conf
n	30.94473	3093.743	0.010002	0.992647	-9814.73	9876.616
Cl50	**0.04231**	1.338	0.031622	0.976760	-4.22	4.300

Concerning the product's toxicity with variations in the mix for elucidating the responsible factor, the results are shown in Table 5.

Table 5. Toxicity assay results of activated IHPLUS® in Artemia sp. under different conditions

Group	Concentration	Altered condition	Mortality (percentage)
IHPLUS®	6%	-	100
Inactivated IHPLUS®		Inactivation	100
Adjusted IHPLUS®		pH set to 7	0

Control	-	-	0

The previous results demonstrated that the main factor responsible for the product's toxicity is the pH. The pH of activated IHPLUS® is very low, affecting larval viability in the medium. PH determinations at different levels showed that the unaltered product had 3.2, whereas the 6% product had 3.9. When the biotic fraction of the product was inactivated, mortality continued. Still, when the pH was set to neutral, the closest to ASW conditions, no mortality was observed in the larvae.

Considering the results of the experiment, and according to the classification system for short-term hazards (acute) in aquatic environments (UN, 2015), IHPLUS® does not qualify within the acute toxicity categories since the LC_{50} value was much higher than the set levels for this category, with substances of up to 100 mg/L. However, our product was determined at 420 mg/L. These results prove that the product is not hazardous under acute exposure to aquatic organisms. However, other species should be studied to set up a hazard level in the aquatic environment.

The literature shows no reports about the toxicity of similar products in Artemia sp. However, the utilization of EM to treat several sources of wastewater in the environment where other species close to *Artemia sp.* live, has been widely studied. As shown in Table 6, several authors recommend using this product in 1:1000 and 1:10000 dilutions. In this study, these concentrations were evaluated (Table 2) and produced no mortality in Artemia sp.

Table 6. Reports of EM use in water treatment

Concentrations used	Uses	Contributors
1L EM/1000 L water (1:1000)	Residual water treatment by bioremediation	Mendietta L. (2015)
Activation dose: 1L EM/m^3 water (1:1000) Maintenance dose: 1L EM/10 000 L water (1:10 000)	Treatment in ponds	OISCA (2009)
Activation dose: 1L EM/m^3 water (1:1000) Maintenance dose: 1L EM/10 000 L water (1:10 000)	Distillery dumping	Karthick. S (2014)
1 mL EM / 1000 mL residual water (1:1000)	Residual Water from Swine Farms	Toc R.M. (2012)

Note. EM: Efficient Microorganisms

CONCLUSIONS

The activated IHPLUS® was toxic to concentrations higher than 320 mg/L in Artemia sp larvae in the assay conditions.

This microbial product is harmless since the LC_{50} was 420 mg/L in Artemia sp.

The pH was the main factor responsible for activated IHPLUS® toxicity in Artemia sp.

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: MSW, OMC, EAJ, ZACH, MDS, LLP; data analysis and interpretation: MSW, OMC, EAJ, ZACH, MDS, LLP; redaction od the manuscript: MSW, OMC, EAJ, ZACH, MDS, LLP.

CONFLICT OF INTEREST STATEMENT

The authors state there are no conflicts of interest whatsoever.