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Evaluating the Combined Toxicity of Chlorhexidine and Dibutyl Phthalate on Daphnia magna: Implications for Aquatic Ecosystem Safety and Environmental Risk Management

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Abstract **Introduction:** Pollutant accumulation in aquatic ecosystems has become a growing environmental concern, posing serious risks to aquatic organisms. While the toxic effects of individual chemicals are well-studied, limited research has explored their combined effects. This study investigates the synergistic and antagonistic toxicity of chlorhexidine (CHX) and dibutyl phthalate (DBP) on *Daphnia magna*, a commonly used ecotoxicological model. The objective was to assess toxicity levels of CHX and DBP at varying concentrations and evaluate their combined effects using the Bliss Independence Model through an immobilization assay. **Methodology:** Different concentrations of CHX and DBP were tested both individually and in combination (ratios of 2:1 and 3:1) over a 48-hour period. Immobilization rates were recorded at 24 and 48 hours. A total of 50 organisms per concentration were assessed. The Bliss Independence Model was applied to evaluate synergistic, additive, or antagonistic interactions. **Results:** The individual toxicity thresholds for CHX were IC30 = 22.85 mg/L, IC50 = 47.93 mg/L, and IC70 = 1.90 mg/L. For DBP, the respective values were IC30 = 8.31 mg/L, IC50 = 14.86 mg/L, and IC70 = 2.52 mg/L. At a 2:1 ratio, the combination demonstrated synergistic effects with combination indices (CI) of 0.718 (IC30), 0.734 (IC50), and 0.758 (IC70). The 3:1 ratio exhibited antagonism at IC30 (CI = 1.42) and IC50 (CI = 1.48), but showed synergism at IC70 (CI = 0.85). **Conclusion:** The findings demonstrate that chemical interactions vary with concentration and combination ratios. The observed synergistic effects at the 2:1 ratio highlight potential ecological risks, while the 3:1 ratio shows complex interaction patterns. These results underscore the need for comprehensive assessments of chemical mixtures to guide environmental risk management and improved water treatment strategies.

Key Words Environmental toxicity, Combined chemical effects, Bliss Independence Model, Aquatic ecosystem safety, Chemical interaction studies

INTRODUCTION

Water is a vital resource that sustains life on Earth, making its safety and environmental protection crucial for maintaining ecological balance. Achieving the Sustainable Development Goals (SDGs), particularly those targeting environmental conservation, emphasizes the need to preserve water quality to ensure ecosystem sustainability and human well-being. However, the escalating levels of pollutants in aquatic environments pose a growing threat to these objectives. Both natural and synthetic chemicals have infiltrated water systems, posing significant risks to aquatic organisms and ecosystems [1].

Globally, approximately 40% of pollutants are discharged into aquatic environments through industrial waste, pharmaceutical disposal, and household runoff (UN Environment Program). Among these pollutants, chemicals such as antiseptics, solvents, and plasticizers-which are extensively used for their functional properties-may unexpectedly contribute to environmental toxicity when released into water bodies [2].

Chlorhexidine (CHX), a widely used antimicrobial in dentistry, is one such emerging contaminant. CHX is extensively used in oral health products such as mouthwashes due to its ability to disrupt cellular membranes and inhibit bacterial growth. Its effective antibacterial properties make it a common choice for dental treatments [3]. Despite its benefits, the rising presence of CHX residues in water sources has raised environmental concerns. CHX has been detected in concentrations between 0.3 to 16 µg g^{-1}, highlighting its persistence in aquatic environments and underscoring the need for acute and chronic toxicity evaluations [4,5].

Dibutyl phthalate (DBP), a plasticizer commonly used in consumer products such as sealants, paints, adhesives, cosmetics, and dental materials, is another concerning contaminant. DBP is widely incorporated in dental resins and polymers for its flexibility and durability [6]. While DBP enhances product performance, its potential to interfere with the endocrine system raises concerns about its environmental impact. DBP has been classified as an endocrine disruptor, capable of causing severe aquatic toxicity when accumulated over time [7]. A concentration as low as 3.4 mg/L has been shown to cause harmful effects in aquatic organisms, particularly *Daphnia magna* [8,9].

The combined presence of CHX and DBP in aquatic ecosystems introduces further complexity. Although the individual toxic effects of these compounds are well-documented, studies exploring their synergistic or antagonistic interactions remain limited. Synergistic toxicity occurs when the combined impact of two or more chemicals exceeds the sum of their individual effects, potentially amplifying environmental harm [10]. Conversely, antagonistic interactions may mitigate the toxic effects of individual compounds. Understanding such interactions is crucial since real-world environmental conditions often expose aquatic organisms to chemical mixtures rather than isolated pollutants.

Many toxicology studies have explored the environmental impacts of pollutants, particularly in fish species [11]. However, there is limited research focusing on aquatic invertebrates like *Daphnia magna*, a critical species in ecotoxicological studies. *Daphnia magna* is a widely recognized model organism due to its high sensitivity to environmental changes, rapid reproduction rate, and ease of laboratory maintenance [12]. Its role as a keystone species in aquatic food chains makes it highly suitable for evaluating pollutant toxicity and understanding potential threats to ecosystem stability [13-15].

Given the increasing contamination of aquatic environments, this study aims to assess the synergistic and antagonistic toxicity of CHX and DBP both individually and in combination on *Daphnia magna* using the immobilization test. By employing the Bliss Independence Model, this research seeks to provide critical insights into chemical interactions that could inform environmental safety protocols and pollution management strategies.

MATERIALS AND METHODS

Test Organism Selection and Maintenance

Daphnia magna, a widely recognized model organism in ecotoxicology, was selected for this study due to its established sensitivity to environmental pollutants and its ecological significance in aquatic food chains. Healthy adult *Daphnia magna* (≤24 hours old) were cultured in a controlled laboratory environment maintained at a temperature of 20±1°C with a 16:8-hour light-dark cycle. The culture medium consisted of aerated, dechlorinated tap water. To maintain optimal health, the daphnids were fed *Chlorella vulgaris* daily. The culture was maintained at a density of ≤10 individuals per litre to ensure sufficient space and resources. Prior to initiating toxicity testing, daphnids were acclimated for 48 hours in identical environmental conditions to minimize stress and ensure consistent physiological states.

Chemical Preparation and Test Concentration Selection

Chlorhexidine (CHX) and Dibutyl phthalate (DBP) were procured from Sigma-Aldrich, India. Both chemicals were diluted in distilled water to prepare stock solutions. To establish appropriate test concentrations, an initial range-finding test was conducted to determine inhibitory concentration (IC) values-IC30, IC50, and IC70-representing 30%, 50%, and 70% immobilization of the *Daphnia magna* population after 24 hours of exposure. The identified concentrations for CHX and DBP were subsequently serially diluted to create the required test concentrations. To ensure consistency, freshly prepared solutions were used in each trial, and their stability was confirmed throughout the experiment to mitigate concentration fluctuations.

Acute Immobilization Assay

The OECD Guideline 202 for acute immobilization assays in daphnids was strictly followed to ensure methodological rigor. Young daphnids (less than 24 hours old) were exposed to CHX and DBP across a series of at least five concentrations for 48 hours. Immobilization was assessed at 24-hour and 48-hour intervals and compared to control groups maintained in dechlorinated tap water without toxicants.

Test groups consisted of:

- Daphnids exposed to CHX and DBP individually at IC30, IC50, and IC70
- Combination treatments where CHX and DBP were mixed in ratios of 2:1 and 3:1

Each test concentration contained five replicates (n = 10 per replicate), with a total of 50 individuals per concentration to enhance statistical reliability. The exposures were conducted in 50-mL glass beakers under static conditions, without feeding during the test period. After 24 hours, immobilized daphnia-defined as those unable to swim for 15 seconds following gentle agitation-were recorded to ensure standardized assessment criteria.

Bliss Independence Model for Interaction Analysis

To evaluate the nature of the interactions between CHX and DBP, the Bliss Independence Model was employed. This widely used model in toxicology predicts the combined effects of independent substances on living organisms.

The expected immobilization effect (E_AB) under independent action was calculated using the equation:

$$EAB = EA+EB-(EA \times EB)$$

Where:
EA = Fractional immobilization caused by CHX alone
EB = Fractional immobilization caused by DBP alone
EAB = Expected immobilization under independent action

The observed immobilization (O_AB) from the combination treatments was compared to the expected values. The type of interaction was classified as follows:

- Synergistic Effect: O_AB>E_AB (greater-than-expected immobilization)
- Additive Effect: O_AB≈E_AB (immobilization aligns with expectation)
- Antagonistic Effect: O_AB E_AB (less-than-expected immobilization)

This model provided a clear framework to identify potential synergistic or antagonistic interactions across varying concentration ratios.

Statistical Analysis

Data analysis was conducted using GraphPad Prism (version 8.0). Dose-response curves were plotted using non-linear regression models to determine IC values. Statistical differences between groups were assessed using a two-way ANOVA with a significance threshold of $p<0.05$. To enhance result accuracy, post-hoc tests such as Tukey's multiple comparisons test were employed to identify specific differences between treatment groups. Bliss interaction scores were calculated to validate the nature of observed chemical interactions.

Quality Control and Replicability Measures

To ensure data reliability and consistency, each experiment was conducted in triplicate. Water quality parameters, including pH, temperature, and dissolved oxygen, were monitored throughout the study to maintain consistent conditions and avoid environmental fluctuations. Additionally, control groups were maintained alongside treatment groups to account for baseline immobilization rates and minimize environmental variability.

RESULTS AND DISCUSSION

Both CHX and DBP demonstrated concentration-dependent immobilization of *Daphnia* (Figure 1) in independent tests. For DBP, the IC_{50} and IC_{70} values were 14.86 mg/L and 2.52 mg/L, respectively, whilst the concentration needed to immobilize 30% of the population (IC_{30}) was 8.31 mg/L. Because lower concentrations were needed at IC_{70} than IC_{50}, these data suggest that DBP has a nonlinear relationship with immobilization thresholds. This could be because of its long-term or cumulative effects on the physiology of the organism.

Similarly, at greater dosages, CHX showed increased immobilization potency (Figure 2). The required dosage was 22.85 mg/L at IC_{30}, 47.93 mg/L at IC_{50} and a precipitous decline to 1.90 mg/L at IC_{70}. This trend implies that CHX might cause acute effects at higher thresholds, possibly through processes like oxidative stress or interference with *Daphnia's* cellular respiration. These outcomes are consistent with research by Tarring *et al.* [16], which found that CHX

Figure 1: *Daphnia magna* underlight microscopy (10x)

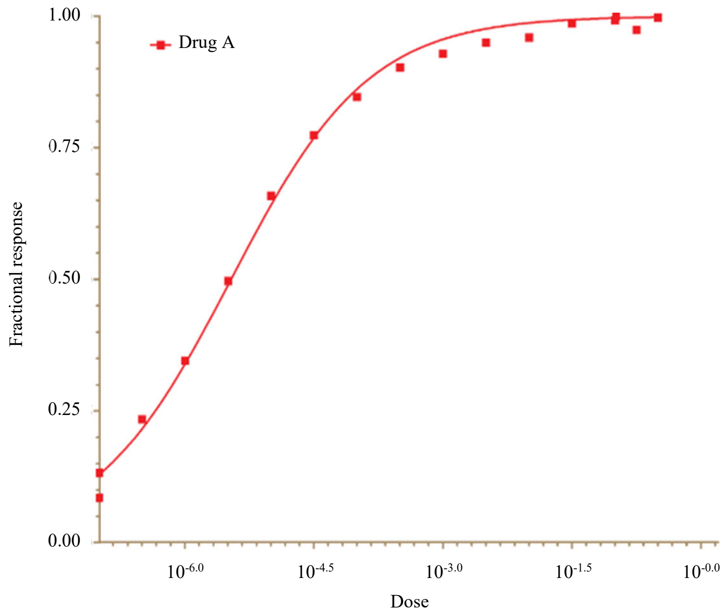

Figure 2: Graph representing the dose response relationship of Chlorhexidine (CHX)

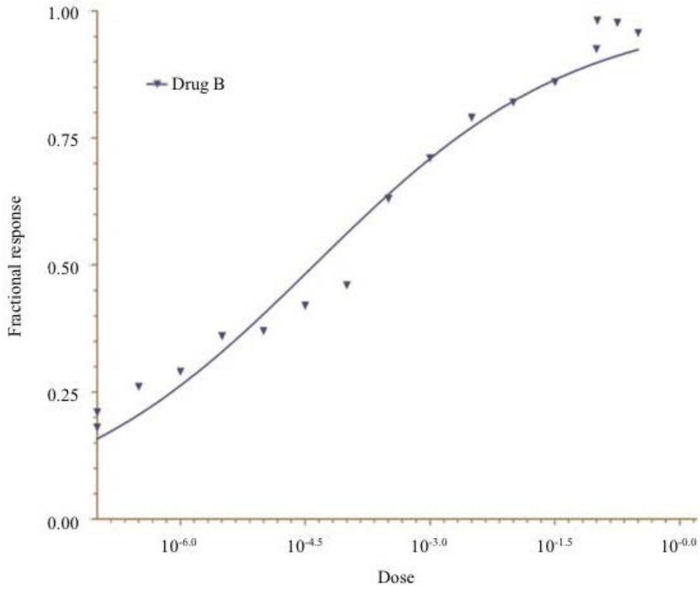

Figure 3: Graph representing the dose response relationship ofDibutyl phthalate (DBP)

and other volatile organic chemicals cause aquatic creatures to become immobile by disrupting their membranes and causing an imbalance in their ions.

Across all inhibitory doses, the 2:1 ratio of CHX to DPB demonstrated steady synergistic effects. With a CI of 0.718, the combination needed 10 mg/L CHX and 1.458 mg/L DBP at IC_{30}. This indicates that the combination was more successful in producing the same immobilizing effect than each chemical alone. Likewise, sustained synergy was shown by the CI values of 0.734 and 0.758 at IC_{50} and IC_{70}, respectively. Recent research by Chen *et al.* [17], which showed that combining two toxicants with complimentary modes of action frequently increases efficacy, supports these findings [17]. Here, the membrane-disrupting qualities of CHX may enhance the inhibitory effects of DBP on cellular metabolism, giving *Daphnia* a more severe toxic effect (Figure 3).

A more intricate interaction profile was shown by the 3:1 ratio. The combination demonstrated antagonism at IC_{30}, requiring 1.73 mg/L CHX and 10 mg/L DBP, with a CI of

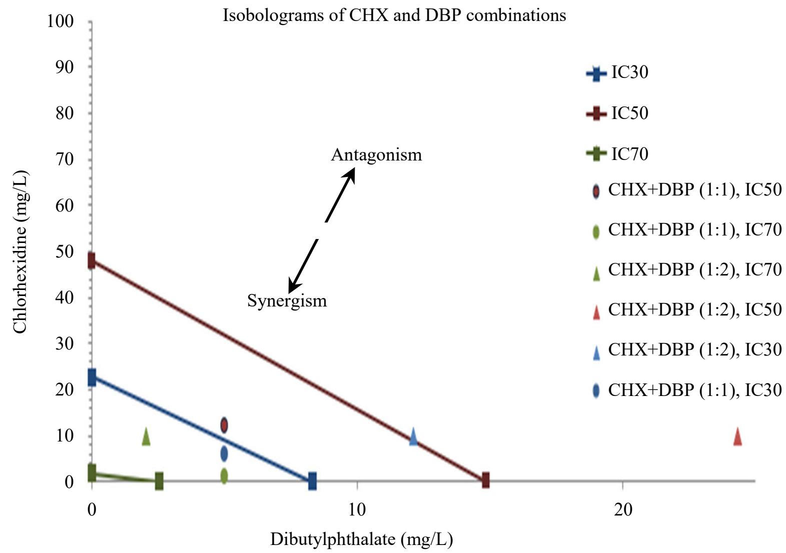

Figure 4: Isobologram of Chlorhexidine (CHX) and Dibutyl phthalate (DBP)

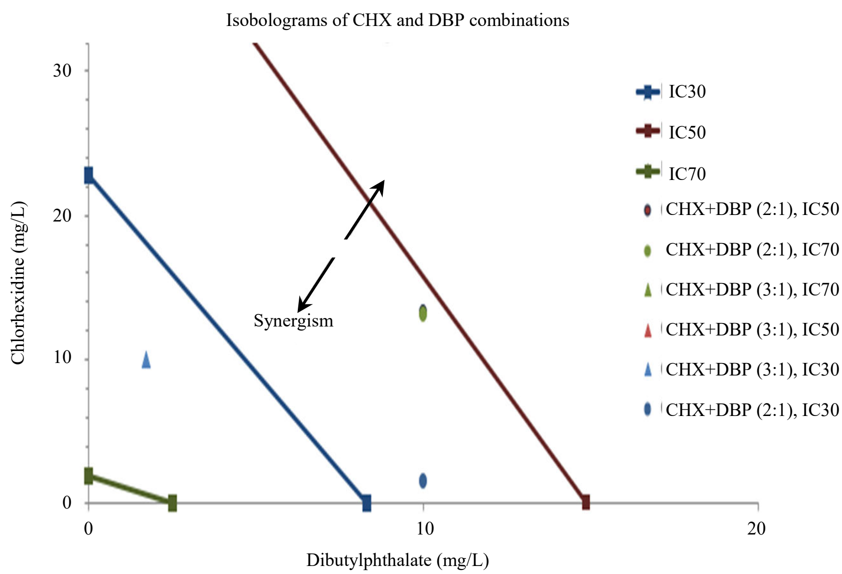

Figure 5:Isobologram of Chlorhexidine (CHX) and Dibutyl phthalate (DBP)

1.42. At IC_{50}, where 10 mg/L DBP and 27.46 mg/L CHX resulted in a CI of 1.48, this antagonistic effect remained. However, with a CI of 0.85, the combination became synergistic at IC_{70}. This change implies that the antagonistic interaction is overcome by larger inhibitory concentrations, possibly as a result of competing routes becoming saturated. In their investigation on pesticide combinations, Pavan *et al.* [18] noted similar trends, with antagonism at lower dosages giving way to synergy at higher thresholds (Figure 4).

Competition between the two drugs for the same toxicological target may be the cause of the antagonistic effects at IC_{30} and IC_{50}. For instance, whereas both CHX and DBP may impair mitochondrial function, their combined presence at below-optimal concentrations may lessen the effectiveness of each of them separately. This emphasizes how crucial dose optimization is for combo toxicity research (Figure 5).

Isobologram visually represented the interactions between the CHX and DBP by plotting their IC values of individual and in combinations (Figure 6). For CHX showed IC_{30}, IC_{50} and IC_{70} were 22.85, 47.93 and 1.9 mg/L respectively. Further IC_{30}, IC_{50} and IC_{70} of DBP were 38.31, 14.86 and 2.52 mg/L, respectively. When combined at an optimized ratio (1:3) CI of combinations were 1.02, 0.86, 1.29, respectively. This value shows the synergistic effect of

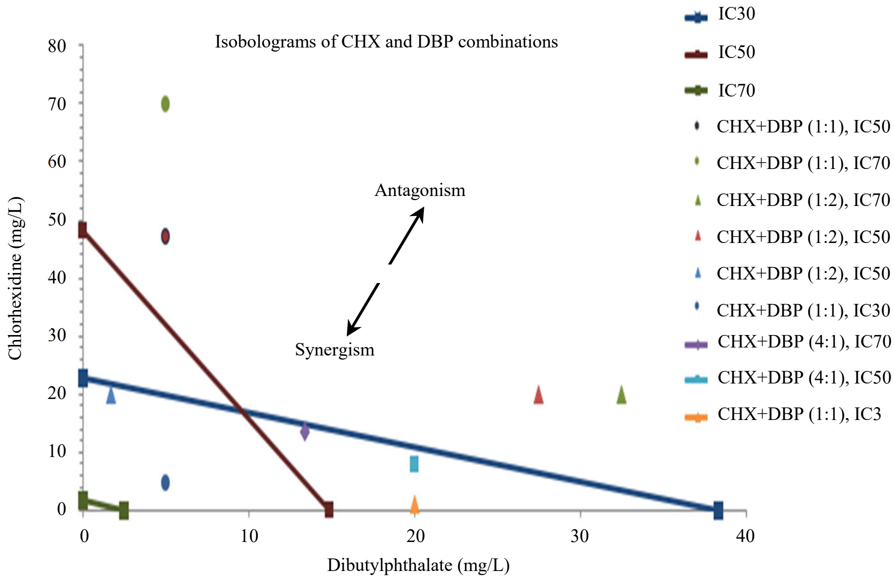

Figure 6: Isobologram of Chlorhexidine (CHX) and Dibutyl phthalate (DBP)combinations

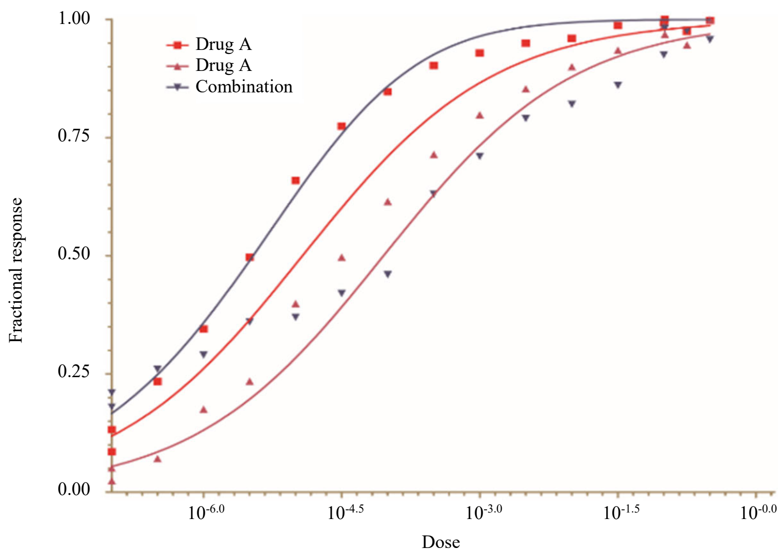

Figure 7: Dose fractional response curve of both Chlorhexidine (CHX) and Dibutyl phthalate (DBP)

IC_{30} and IC_{50}. Meanwhile IC_{70} showed antagonistic effect and combination of CHX and DBP at 1:3 ratio and 1:4 ratio IC_{30} and IC_{70} were antagonistic but IC_{70} was synergistic effect. In the ratio of 4:1, the CHX and DBP showed the combination effect inversely with respect to 1:4 ratio (i.e., IC_{30} and IC_{50} were synergistic but IC_{70} was antagonistic. This result showed CHX and DBP combination play different roles according to their individual concentrations. Hence the persistency in the environment could greatly influence the biological systems, by influencing the toxicity and might result in the species reduction also. Further their combination effects were confirmed by the dose fractional curve. The dose fractional curve is a critical component in analysis used to evaluate the interaction between CHX and DBP (Figure 7).

DISCUSSION

The rapid increase in chemical production globally has heightened concerns about their toxic impacts, particularly in aquatic environments. Many chemicals, commonly used for their functional benefits in daily life, pose significant risks to aquatic ecosystems when discharged into water bodies. Chlorhexidine (CHX), widely used as an oral rinse since 1970 for its effective antibacterial properties, has become a common pollutant in aquatic environments due to its extensive use in oral health products [19,20]. Similarly, dibutyl phthalate (DBP), a plasticizer used in various industries including dentistry, is frequently discharged into aquatic systems via wastewater, runoff, and landfill leachates. Since DBP is not chemically bound to plastic materials, its release into the environment is inevitable.

In this study, the synergistic and antagonistic toxicological effects of CHX and DBP on *Daphnia magna* were evaluated. *Daphnia magna*, commonly referred to as the water flea, is a key species in aquatic ecosystems and is widely used as a model organism in ecotoxicological studies. Its high sensitivity to environmental stressors makes it an ideal candidate for assessing chemical toxicity. Although reproductive studies on *Daphnia magna* are common, immobilization tests have received less attention despite their significance in identifying acute toxic effects. Immobilization bioassays are highly effective for assessing immediate toxic impacts, especially for evaluating the harmful effects of toxicants on an organism's mobility [21].

This study observed a wide concentration range for CHX (1.90-47.93 mg/L), which aligns with the findings of Golpe *et al.* [22], who reported CHX concentrations between 0.3 and 16 µg g^{-1} in environmental samples. Such a drastic increase in CHX levels reflects its rising presence in aquatic ecosystems, potentially posing serious risks to aquatic life. Persistent low-dose exposure to CHX in aquatic environments may cause long-term harm, particularly to micro-level organisms like *Daphnia magna* [23].

Similarly, the presence of DBP in aquatic systems has increased substantially in recent years, with concentrations reaching up to 14.86 mg/L in this study. This observation is consistent with previous reports highlighting DBP as a priority pollutant due to its environmental persistence and endocrine-disrupting properties [24]. The lethal concentration values (LC50) for DBP in aquatic species have been reported at 4.92 and 4.31 mg/L, suggesting that DBP contamination is a growing environmental concern [25].

While the toxic effects of CHX and DBP as individual contaminants have been extensively studied, their combined effects remain underexplored. The results of this study demonstrate that chemical combinations can exhibit significantly different toxicological outcomes compared to individual effects. The observed synergistic interactions in the 2:1 ratio and antagonistic effects in the 3:1 ratio highlight the complexity of chemical interactions in aquatic systems [26].

The synergistic effect observed at the 2:1 ratio suggests that CHX and DBP, when combined at appropriate concentrations, can enhance each other's toxicity. CHX damages cellular membranes, causing ion leakage and osmotic stress, while DBP interferes with critical metabolic processes. Together, these mechanisms create an intensified toxic environment that severely impairs the organism's physiological functions [27].

Conversely, the antagonistic effect observed at the 3:1 ratio may be attributed to overlapping toxicological pathways. When CHX and DBP target similar biological processes, their combined presence may saturate the pathway, reducing the efficiency of individual toxic actions. This aligns with previous research on herbicide mixtures, where high-dose combinations targeting the same enzyme pathways resulted in reduced overall toxicity [28].

Recommendations for Future Research

While this study offers valuable insights into the combined effects of CHX and DBP, further research is required to understand the underlying mechanisms driving these interactions. Mechanistic studies, such as transcriptomic or proteomic analyses, could provide deeper insights into how CHX and DBP influence gene expression and cellular pathways in *Daphnia magna* [29,30].

Additionally, future studies should investigate the effects of chronic exposure to CHX and DBP on aquatic populations. Unlike acute exposure studies, chronic exposure may reveal different patterns of synergistic or antagonistic effects over prolonged periods. Expanding the analysis to include different ratios, concentrations, and environmental conditions (e.g., pH, temperature) will help predict chemical behavior in real-world ecosystems. Advanced modeling techniques, such as response surface methodology, could improve the accuracy of toxicity predictions and enhance risk assessment strategies [31,32].

CONCLUSION

This study underscores the importance of evaluating combined chemical toxicity in environmental risk assessments. The Bliss Independence Model effectively demonstrated the complex interaction patterns of CHX and DBP. The 2:1 combination consistently exhibited synergistic effects, while the 3:1 combination demonstrated antagonism at lower concentrations but shifted to synergy at higher thresholds. These findings highlight the potential for unexpected toxic effects in real-world scenarios where multiple pollutants coexist.

Understanding such interactions is crucial for developing improved environmental monitoring frameworks, pollution control strategies, and regulatory guidelines. Future studies should focus on long-term ecological consequences and explore mitigation strategies, including bioremediation techniques, to minimize environmental risks associated with chemical combinations.

Ethical Considerations

This study was conducted following ethical standards for environmental and ecotoxicological research. All experimental protocols involving *Daphnia magna* adhered to the OECD Guidelines for the Testing of Chemicals (Test No. 202) to ensure humane and scientifically sound procedures. Appropriate measures were taken to minimize stress and ensure the well-being of the test organisms throughout the study.

Conflict of Interest

The authors declare no conflict of interest in relation to this study. The research was conducted independently, with no financial or personal relationships influencing the outcomes or interpretations presented.

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