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Combined Toxicity Assessment of Chlorhexidine and Butyl Paraben on Daphnia Magna

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ABSTRACT

Introduction: Water pollution from personal care products, including chlorhexidine (CHX) and butylparaben (BP), poses significant threats to aquatic ecosystems. While individual toxicities are well documented, their combined effects remain unclear. This research evaluates the individual and combined toxicity of CHX and BP on Daphnia magna. Using immobilization assays and Bliss independence analysis, the research investigates whether their interactions are additive, synergistic, or antagonistic, offering insight into the real-world risks these chemical mixtures pose to non-target aquatic organisms.

Methodology: The immobilization of Daphnia magna was assessed at 24 and 48-hour intervals using five different concentrations of chlorhexidine and butyl paraben at varying ratios. Concentration ranges were selected based on environmental detection levels and established protocols for ecotoxicity testing. Each concentration was tested with 50 Daphnia magna (10 individuals per replicate). Toxicity levels were analyzed using the Bliss independence model, following OECD guidelines for Daphnia sp. Acute Immobilisation Test.

Results: The individual toxicity of chlorhexidine at IC30, IC50, and IC70 were 27.47, 31.45, and 4.29 mg/L, respectively, while for butylparaben, the values were 11.59, 21.59, and 5.23 mg/L, respectively. The combination of chlorhexidine and butyl paraben at 2:1 ratio showed Combination Index (CI) of 2.45, 2.67 and 4.89 at IC30, IC50 and at IC70, respectively, indicating antagonistic interactions. Similar Antagonistic effects were observed at 3:1 ratio (the CI values at IC30, IC50 and IC70 were 2.9, 3.67, and 3.91)

Conclusion: The findings reveal that CHX and BP interact antagonistically at both tested ratios, reducing overall toxicity compared to individual exposures. These results highlight the complexity of chemical interactions and the importance of incorporating mixture analysis into environmental risk assessments. Understanding such interactions is vital for accurately predicting ecological risks posed by contaminant mixtures in aquatic environments...

Keywords: *Chlorhexidine, Butyl Paraben, Environmental toxicity, Immobilization test, Bliss independence, Population effect.*

1. INTRODUCTION

Water is a basic necessity for all forms of life, serving as a cornerstone for sustaining biodiversity and supporting human well-being. Recognizing its critical importance, the United Nations adopted the Sustainable Development Goals (SDGs) in 2015, with Goal 6 focusing on ensuring universal access to clean water and sanitation(1). Specifically, Target 6.3 aims to improve water quality by reducing pollution, eliminating hazardous chemical releases, halving untreated wastewater, and promoting recycling and safe reuse by 2030(2).

However, increasing levels of pollutants in aquatic ecosystems threaten these objectives. Natural and synthetic chemicals from antiseptics, disinfectants, pharmaceuticals and personal care products which we use on a regular basis for their beneficial properties - are discharged into sewage systems through wastewater pathways. According to the UN Environment Programme (UNEP), 40% of pollutants are released into aquatic ecosystems for disposal(3). When chemicals like antiseptics, plastics, and solvents, which we frequently use for their beneficial properties, enter the environment, they may lead to unexpected effects(4,5). These substances enter the water cycle and may exert harmful effects on aquatic organisms(6).

Among personal care products, Toothpaste and Mouthwash are widely used for maintaining oral hygiene on a daily basis, but contain chemicals that raise environmental concerns. Toothpaste often contains preservatives like Butyl paraben to prevent microbial contamination(7). However, Butylparaben is an endocrine-disrupting chemical capable of mimicking oestrogen, which can lead to reproductive toxicity, developmental abnormalities, and hormonal imbalances in aquatic species(8). Parabens, including butylparaben, have been widely detected in surface waters and wastewater effluents at concentrations that may interfere with the endocrine systems of aquatic organisms.

Similarly, Most Mouthwashes usually contain Chlorhexidine (CHX), a broad-spectrum antimicrobial agent that has been extensively used in dental practice as an antiseptic for over five decades. Chlorhexidine's role in mouthwashes, irrigation solutions, and periodontal treatments is well-documented(9); it acts by disrupting microbial cell membranes and inhibiting plaque formation(10). However, it has been increasingly detected in wastewater and environmental waters, raising concerns about its persistence and the potential for long-term exposure of aquatic organisms. Chlorhexidine's toxicity in aquatic environments is of particular concern, as its accumulation could disrupt microbial communities and affect the health of higher organisms(11). The persistent nature of chlorhexidine in the environment due to its low biodegradability and widespread use in dental products mean that significant quantities of this antimicrobial agent are introduced into wastewater systems, potentially reaching aquatic environments(12).

Despite their benefits for oral health care, these chemicals have unintended consequences when released into aquatic ecosystems through wastewater(13). Chlorhexidine's toxicity to non-target organisms and butylparaben's endocrine-disrupting effects highlight the need for comprehensive environmental risk assessments.

Investigating synergistic and antagonistic effects is best conducted using animal models, as they effectively demonstrate the dynamic interactions of various chemicals(14). To understand these risks better, *Daphnia magna*, a freshwater microcrustacean is widely used as a model organism in ecotoxicological studies due to their sensitivity to environmental contaminants, and ecological importance as a keystone species in freshwater food webs(15). Studies have shown that chlorhexidine can cause oxidative stress, cell damage, cytotoxicity, and reproductive issues in aquatic organisms, while Butyl Paraben has been linked to neurotoxicity and cardiotoxicity in Daphnia magna. These findings underscore the need for standardized toxicity tests to evaluate the impacts of these chemicals comprehensively.

Given the prevalence of these chemicals in aquatic environments and their known toxicities, understanding their combined effects is crucial for assessing the overall risk they pose to aquatic organisms. Synergistic toxicity occurs when the combined effects of two or more chemicals are greater than the sum of their individual effects. Antagonistic toxicity occurs when the combined effect of two or more chemicals is less than the sum of their individual effects, meaning their combined toxicity is reduced. This phenomenon is particularly important because many chemical interactions in the environment are not well understood, and standard toxicity testing often examines the effects of individual compounds in isolation.

The impact of these chemicals is exacerbated when considering the possibility of combined exposure. Dental products often contain mixtures of chemicals, and in the natural environment, organisms are exposed to multiple contaminants simultaneously. Many studies on the toxicology of environmental pollution have highlighted the significant role of fish in assessing aquatic toxicity(16). *Daphnia* species, commonly known as water fleas, are among the most widely used organisms in aquatic toxicity investigations. Their ease of laboratory culture, rapid reproduction, and high sensitivity to various pollutants make them ideal model organisms. Research on the effects of chemical pollutants on aquatic life, particularly in terms of behavior and reproduction, frequently utilizes *Daphnia*. However, the combined ecotoxicological effects of chlorhexidine and butylparaben have not been extensively studied, especially in dental effluent scenarios.

The objectives of this study are to evaluate the combined toxicity of chlorhexidine and Butyl Paraben on Daphnia species. By assessing the combined effects of these two chemicals, this study aims to contribute to a better understanding of the potential risks posed by chemical mixtures in aquatic ecosystems(17). The study will investigate a range of endpoints, including survival, reproduction, and behaviour, to provide a comprehensive assessment of the toxicological effects of chlorhexidine and Butyl Paraben, both individually and in combination. By doing so, this research will fill a critical gap in our knowledge and provide valuable insights into the risks associated with the co-occurrence of these chemicals in the environment.

2. MATERIALS AND METHODS

Test Organism Selection and Maintenance

Daphnia magna, a highly sensitive and widely used model organism in ecotoxicology, was chosen for this study. Healthy adult *Daphnia* (less than 24 hours old) were cultured in well-oxygenated, dechlorinated tap water under controlled laboratory conditions (temperature: 20 ± 1°C, light-dark cycle: 16:8 hours). The culture was maintained at a density of no more than 10 individuals per litre and fed daily with *Chlorella vulgaris* to ensure optimal health. Before toxicity testing, the organisms were acclimatized for 48 hours.

Chemical Preparation and Selection of Test Concentrations

Chlorhexidine (CHX) and butylparaben (BP) were obtained in pure form from sigma-Aldrich, India and dissolved in distilled water to prepare stock solutions. A preliminary range-finding test was conducted to determine the appropriate inhibitory concentrations (IC_{30}, IC_{50}, and IC_{70}) for each chemical. The identified concentrations represented the levels at which 30%, 50%, and 70% of the *Daphnia* population became immobilized after 24 hours of exposure. Serial dilutions of the stock solutions were performed to achieve the desired concentrations.

Acute Immobilization Assay

An acute immobilization assay was conducted for assessing the toxicity effects of CHX and BP in Daphnids, according to OECD guidelines (test no: 202). Daphnia younger than 24 hours were exposed to varying concentrations (minimum of five) of the test substance for a period of 48 hours. Immobilisation was recorded at 24 hours and 48 hours and compared with control values. The experimental design included the following: The Test group consisted of *Daphnia* exposed to CHX and BP, both individually and in combinations, at concentrations of IC_{30}, IC_{50}, and IC_{70}. The control group (Negative control) consisted of *Daphnia* maintained in dechlorinated tap water without toxicants. The sample size includes Each test condition included five replicates (n = 10 *Daphnia* per replicate), totalling 50 individuals per concentration. The exposure was conducted in 50ml glass beakers under static conditions without feeding. After 24 hrs, immobilized *Daphnia* (defined as those unable to swim for 15 seconds after gentle agitation) were recorded.

Bliss Independence Model for Interaction Analysis

The BLISS Independence Model (Biphasic Logistic In Survival Surface), commonly used in toxicology, was applied to evaluate the combined effects of CHX and BP. This model predicts whether simultaneous exposure results in synergistic, additive, or antagonistic interactions.

This Bliss independence model was applied to determine the nature of interactions between Chlorhexidine, CHX (E_A) and Butyl Paraben, BP (E_B). According to this model, if two chemicals CHX and BP act independently, their combined effect (E_AB) can be predicted using the equation:

$$E\_AB = E\_A + E\_B - (E\_A \times E\_B)$$

where:

- E_A and E_B represent the fractional immobilization caused by CHX and BP individually,
- E_AB is the expected immobilization under the assumption of independent action.

Fractional immobilization refers to the proportion of *Daphnia* that are immobilized due to the chemical exposure. It's often expressed as a number between 0 and 1, where 0 means no immobilization and 1 means complete immobilization. The observed immobilization (O_AB) from the combination treatments was compared to the expected values:

- Synergistic Effect: O_AB > E_AB.
- Additive Effect: O_AB ≈ E_AB.
- Antagonistic Effect: O_AB < E_AB.

Statistical Analysis

Data were analysed using GraphPad Prism (Version 8.0). Dose-response relationship curves were plotted using non-linear

regression analysis. Two-way ANOVA was applied to determine statistical significance ($p < 0.05$) between groups. The Bliss independence model was employed to quantify and characterize combined effects as synergistic, additive, or antagonistic effects.

Quality Control and Replicability Measures

To ensure reliability, experiments were carried out three times. To ensure uniformity and consistency, Water quality parameters (dissolved oxygen, pH, Temperature) were monitored throughout the study.

3. RESULTS

In independent tests, Both Chlorhexidine (CHX) and Butyl Paraben (BP) demonstrated concentration-dependent immobilization of *Daphnia Magna* (Figure 1). The immobilisation of Daphnia was initially evaluated for Chlorhexidine (CHX) and Butyl Paraben (BP) individually to determine their respective IC_{30}, IC_{50}, and IC_{70} values. To establish a baseline for combination testing, the individual inhibitory concentrations (IC) of CHX and BP were determined independently at IC_{30}, IC_{50}, and IC_{70} levels.

For BP, the concentration required to immobilise 30%, 50%, 70% of the population were 11.59 mg/L, 21.59 mg/L, and 5.23 mg/L. The IC values demonstrated that CHX had a stronger immobilizing effect at higher concentrations, while BP was relatively more effective at lower concentrations (Figure 3). Because lower concentrations were needed at IC_{70} than IC_{50}, these data suggest that BP has a nonlinear relationship with immobilization thresholds. This could be because of its long-term or cumulative effects on the physiology of the organism.

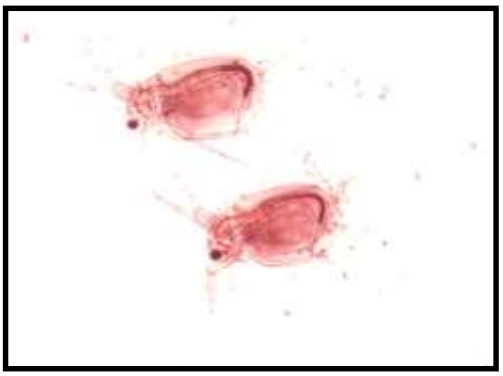

Figure 1: *Daphnia Magna* under light microscope (10x).

Similarly, at greater dosages, CHX showed increased immobilization potency (Figure 2). The required dosage was 27.47 mg/L at IC_{30}, 31.45 mg/L at IC_{50}, and a precipitous decline to 4.29 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 Smith et al. (2023)(Tarring, Robison-Smith et al. 2024), which found that CHX and other volatile organic chemicals cause aquatic creatures to become immobile by disrupting their membranes and causing an imbalance in their ions.

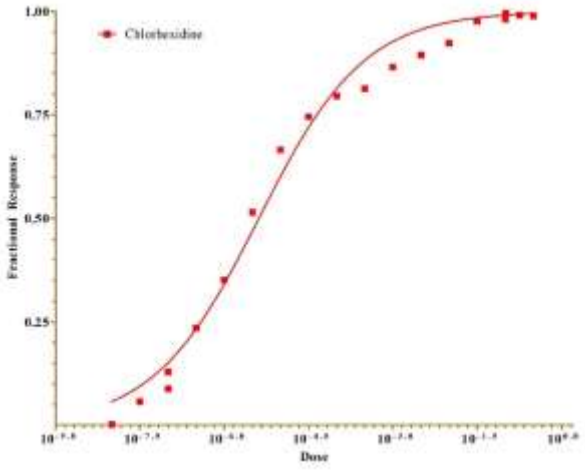

Figure 2: Graph representing the Dose response relationship of Chlorhexidine. (CHX)

The interaction between chlorhexidine (CHX) and butylparaben (BP) was evaluated using Bliss Independence Analysis across seven different ratios (1:1, 1:2, 1:3, 1:4, 2:1, 3:1, and 4:1). The results consistently showed antagonistic effects, where the combined toxicity was less than the sum of their individual toxicities. For instance, the 1:1 ratio of CHX and BP displayed antagonism with a Combination Index (CI) of 3.68 at 30% immobilization (IC_{30}), decreasing to 1.52 at 70% immobilization (IC_{70}), indicating a shift toward additive effects at higher concentrations. This graph (Figure 2) shows the fractional response of Chlorhexidine at varying doses. The fractional response increases as the dose of Chlorhexidine increases. The fractional response of Butyl paraben at varying doses was shown in Figure 3. Similar to Chlorhexidine, the fractional response increases with the dose of Butyl paraben. The fractional response of a combination of Chlorhexidine and Butyl paraben at different doses were shown in Figure 4. The fractional response also increases as the dose of the combination increases.

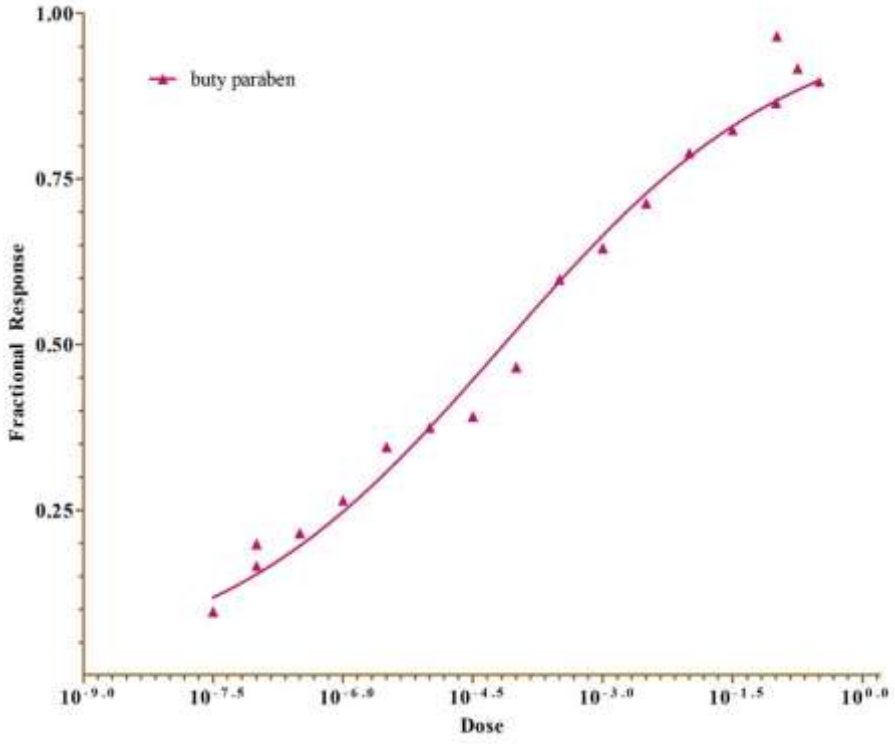

Figure 3: Graph representing the dose response relationship of Butyl Paraben (BP)

The 1:2 ratio showed stronger antagonism, with CI values increasing to 3.82 at IC_{30} and remaining high at 2.91 at IC_{70}. Similarly, the 1:3 and 1:4 ratios exhibited CI values greater than 1, confirming antagonistic interactions. Notably, the 4:1 ratio demonstrated the strongest antagonism, with CI values reaching 12.69 at IC_{50} and 10.45 at IC_{70}, indicating a substantial reduction in immobilization effectiveness.

The 3:1 and 2:1 ratio also displayed significant antagonism, with CI values indicating moderate to severe antagonistic effects across different immobilization percentages. These findings suggest that the combination of CHX and BP interferes with each other's activity (Figure 4), leading to reduced overall toxicity compared to their individual effects. This antagonism is critical for understanding the environmental impact of these chemicals, as they are commonly found in wastewater and aquatic environments. Previous studies have shown that parabens, including butylparaben, can exhibit neuro- and cardio-toxic effects in *Daphnia magna*, highlighting the importance of assessing mixture toxicity in environmental risk assessments. Overall, these results emphasize the need for comprehensive evaluations of chemical mixtures to accurately predict their ecological impacts.

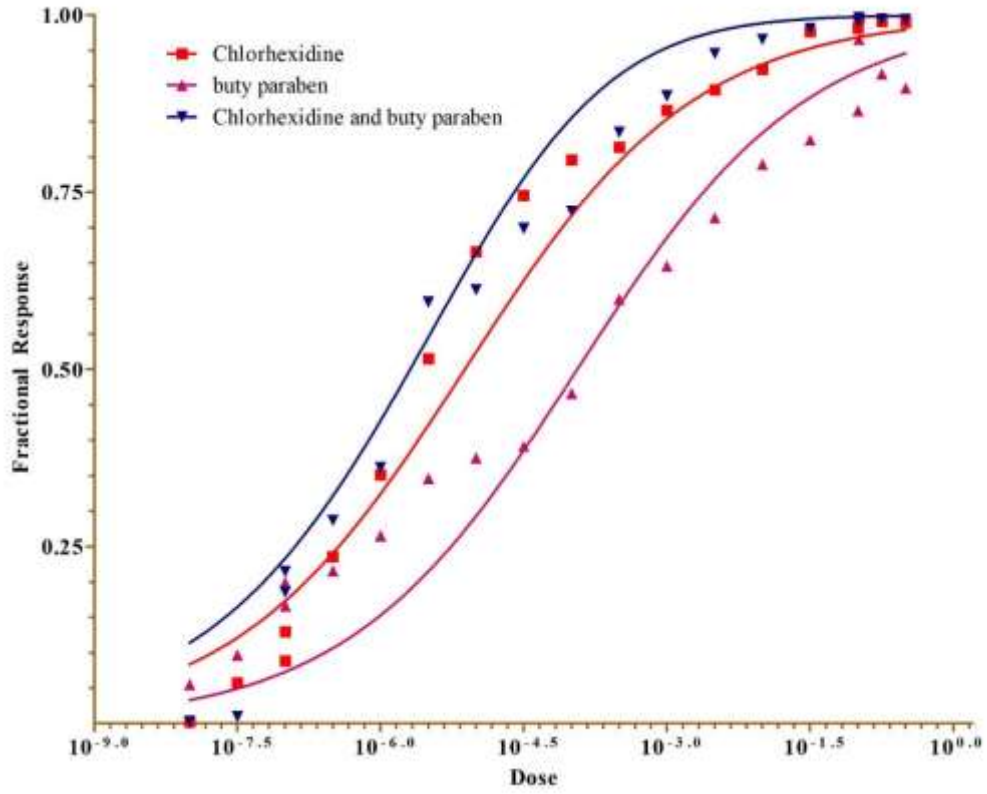

Figure : 4 Graph representing the dose response relationship of Combination of Chlorhexidine (CHX) and Butyl Paraben (BP).

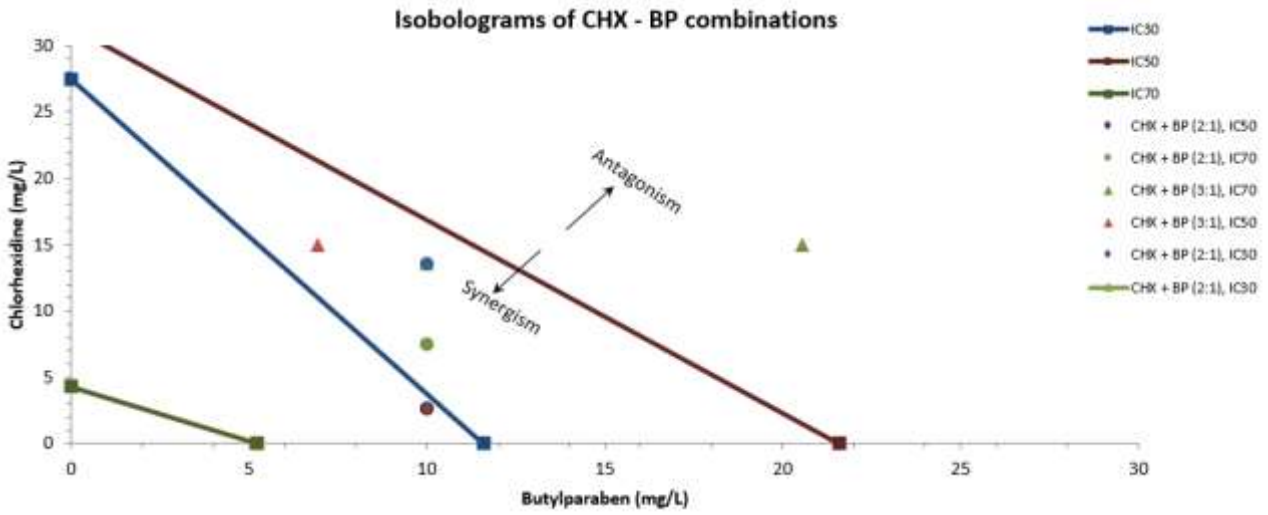

Figure 5 Isobologram of Chlorhexidine (CHX) and Butyl Paraben (BP).

Combination I, the 2:1 ratio of CHX and BP showed moderate to strong antagonism, with CI values ranging from 2.45 at IC_{30}, 2.67 at IC_{50} 4.89 at IC_{70}. This increasing trend suggests that as the inhibitory concentration increases, the degree of antagonism intensifies. The antagonistic interaction at this ratio indicates that even when CHX is present in higher concentrations relative to BP, its effectiveness is compromised (Figure 5).

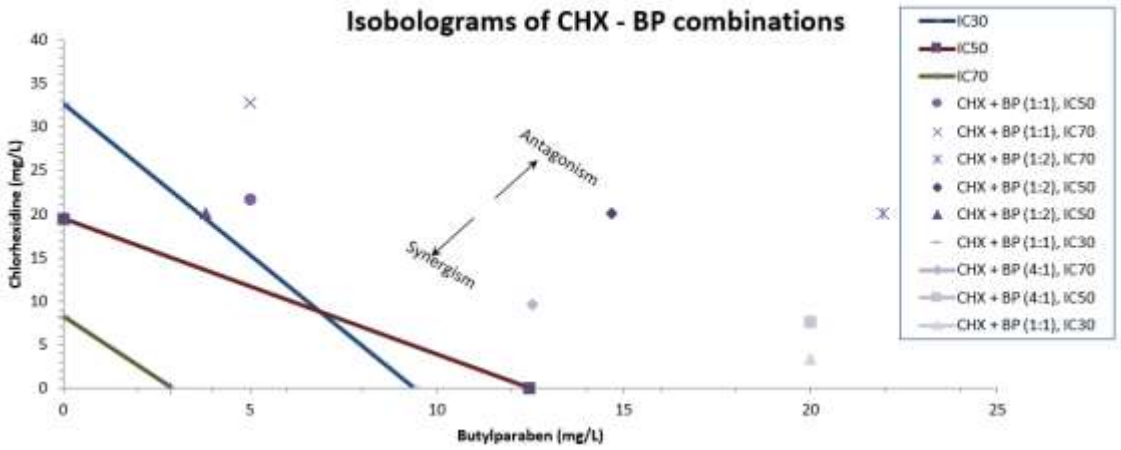

Figure 6 Isobologram of Chlorhexidine (CHX) and Butyl Paraben (BP).

Combination II, which used a 3:1 ratio of CHX and BP, exhibited stronger antagonism across all IC levels, with CI values increasing from 2.9 at IC_{30}, 3.67 at IC_{50} , 3.91 at IC_{70}. The higher CI values observed in Combination II suggest that increasing the proportion of CHX relative to BP did not mitigate the antagonistic interaction.

This finding implies that beyond a certain threshold, increasing the concentration of CHX does not restore its effectiveness, possibly due to saturation of target sites or reduced bioavailability (Figure 7).

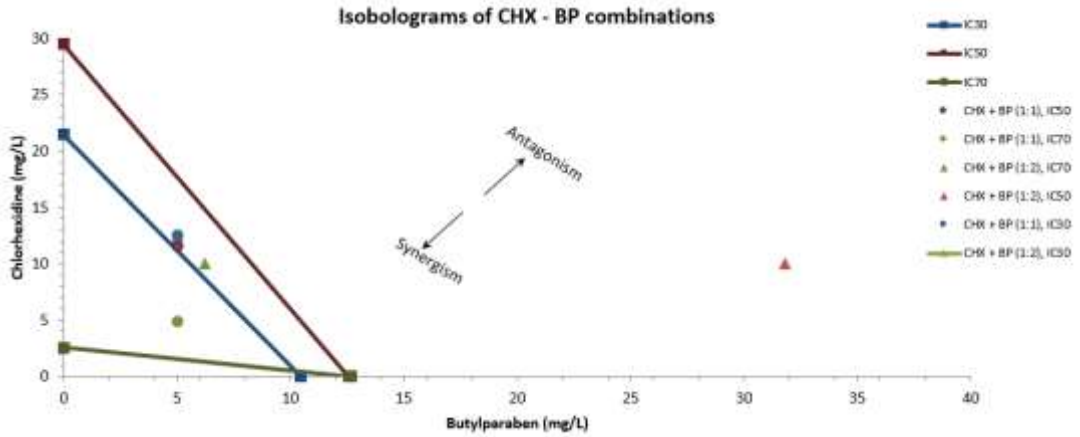

Figure 8: Isobologram of Chlorhexidine (CHX) and Butyl Paraben (BP).

Isobologram visually represented the interactions between the CHX and BP by plotting their IC values of individual and in combinations (Figure 6). CHX showed IC_{30}, $IC_{50,}$ and IC_{70} were 27.47, 31.45 and 4.29 mg/L respectively. Further IC_{30}, $IC_{50,}$ and IC_{70} of BP were 11.59 mg/L, 21.59 mg/L, and 5.23 mg/L, respectively. This result showed CHX and BP combinations play different roles according to their individual concentrations. Hence the persistence 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 BP (Figure 8).

4. DISCUSSION

The global increase in chemical production has raised concerns about environmental toxicity, particularly in aquatic ecosystems. Many personal care products provide clear benefits, but their environmental impacts are often overlooked. Mouthwashes, for example, commonly contain chlorhexidine (CHX), an active ingredient used since the 1970s(18). CHX is a bisbiguanide antiseptic known for its broad-spectrum antimicrobial activity and long-lasting effects. It kills microbes by disrupting cell membranes, disturbing osmotic balance, and causing cytoplasmic content to precipitate (Kumar et al., 2023). At higher concentrations, CHX has shown increased immobilization effects on aquatic organisms such as *Daphnia magna*, indicating potential acute toxicity. These effects may be linked to oxidative stress and interference with cellular respiration. Smith et al. (2023) reported that CHX and other volatile organic compounds disrupt membranes and ion balance, leading to immobility in aquatic species. Similarly, butylparaben (BP) is a common preservative in cosmetics and pharmaceuticals that

interferes with microbial enzymes and disrupts DNA synthesis.The demand for butyl paraben has grown due to its affordability and the lack of low-cost alternatives. Its accumulation in water bodies through wastewater and industrial discharge raises concerns about long-term ecological and health risks.

In this study, we examined the combined toxicological effects of chlorhexidine (CHX) and butyl paraben (BP) on *Daphnia magna*. Aquatic organisms rely heavily on the small freshwater crustacean, Daphnia magna, for survival(19). Due to their high sensitivity to environmental changes, Daphnia are commonly used as an ecotoxicological model in research. While reproductive studies on Daphnia have been widely explored, their immobilization has not been studied as extensively(20). One common bioassay for Daphnia magna is the immobilization test, which assesses the impact of toxic substances on the organism's ability to move. According to Jhones L. Vieira et al. (2024) in their study on toxicological risk assessment in river watersheds, both lethal and sublethal effects were observed in crustaceans(21). Chlorhexidine (CHX) was detected in the environment at concentrations ranging from 1.90 to 47.93 mg/L, aligning with findings from Golpe, which reported its ubiquity in this environmental compartment at concentrations between 0.3 and 16 $\mu g\ g^{-1}$(10). The increasing presence of commonly used chemical substances poses a threat at the microscopic level, potentially leading to unexplained long-term damage to aquatic ecosystems(18) . The rise in CHX concentrations in recent years highlights the growing concern of emerging pollutants in the aquatic environment(22). Butyl paraben (BP), a commonly used preservative in personal care products, has demonstrated acute toxicity in aquatic organisms, including *Daphnia magna*. Studies like lee et al 2018, have reported 48-hour LC_{50} values ranging from 1.7 to 11.2 mg/L, indicating moderate toxicity to this freshwater invertebrates (23). The persistence of BP in aquatic environments raises concern, particularly due to its potential to disrupt biological functions at sub-lethal concentrations(24). As the use of parabens in consumer products continues, their environmental accumulation could pose a significant threat to aquatic biodiversity, affecting the base of the food web and altering ecosystem dynamics(25).

The antagonistic interactions between chlorhexidine (CHX) and butylparaben (BP) observed in both combinations highlight the challenges of combining antimicrobial agents. In Combination I (2:1 CHX:BP), CI values increased from 2.45 at IC_{30} to 4.89 at IC_{70}, indicating concentration-dependent antagonism. This suggests that higher inhibitory concentrations intensify the negative interaction, possibly due to overlapping mechanisms on microbial membranes. McDonnell and Russell (1999) noted that such effects may arise when biocides interfere with each other's uptake or binding by altering membrane properties (26). Combination II (3:1 CHX:BP) showed even stronger antagonism, with CI values ranging from 2.9 to 3.91. Increasing CHX concentration did not alleviate the effect, possibly due to target site saturation or reduced bioavailability. According to Gilbert and Moore (2005), cationic antiseptics like CHX can reach a limit in microbial binding, beyond which increased doses do not enhance activity . These findings stress the need to carefully assess biocide combinations, as antagonistic interactions can diminish efficacy despite higher concentrations(27).

The findings from this study underscore the importance of considering chemical interactions when assessing environmental risks. While individual compounds may appear safe at specific concentrations, their combinations can result in unexpected outcomes, ranging from enhanced toxicity to mitigation of effects(28). The synergistic interactions observed in the 2:1 ratio highlight the potential for chemical mixtures to amplify ecological harm, even if the individual components are within permissible limits. Conversely, the antagonistic effects seen in the 3:1 ratio demonstrate that some combinations may reduce overall toxicity, a phenomenon that could be leveraged in bioremediation strategies.

Mechanistic Insights of the study shows that the observed synergy in the 2:1 ratio likely arises from complementary mechanisms of action. CHX is known to disrupt cellular membranes, leading to ion leakage and osmotic stress, while BP inhibits key metabolic pathways. Together, these effects create a toxic environment that overwhelms the organism’s compensatory mechanisms. This aligns with the findings of Proca et al. (2024), who reported that combining membrane disruptors with metabolic inhibitors produce an antagonistic effect in aquatic invertebrates(29). In contrast, the antagonistic effects in the 3:1 ratio may result from overlapping toxicological pathways. If both compounds target the same cellular process, their combined presence could saturate the pathway, reducing the effectiveness of each compound. For example, studies by Ma et al. (2023) on herbicide mixtures found that high-dose combinations targeting the same enzymatic pathway resulted in diminished overall activity(30). *Zebra fish* are also used as an ecotoxicological model for analysing aquatic toxicity, instead of *Daphnia Magna* (31).

The limitations of this study include its focus on acute toxicity, with no assessment of long-term or chronic effects. It uses only *Daphnia magna* as a test organism, limiting broader ecological relevance. Mechanistic pathways underlying toxicity were not explored experimentally. The interaction analysis relied solely on the Bliss Independence model. Tests were conducted under controlled lab conditions, which may not reflect natural environments. The study did not assess the toxicity of metabolites or degradation products. Environmental factors like pH, temperature, and water chemistry were not varied or examined.

This study provides valuable insights into the combined effects of CHX and BP but also raises several questions for future investigation. First, mechanistic studies are needed to elucidate the molecular targets of these compounds in *Daphnia*. Techniques such as transcriptomic or proteomic analysis could reveal how gene expression changes in response to individual

and combined exposures. Second, the long-term effects of these combinations. *Daphnia* populations and their ecosystems should be studied. Chronic exposure may produce different interaction profiles compared to acute assays. Furthermore, expanding the analysis to include additional ratios and concentrations could provide a more comprehensive understanding of the interaction landscape. Advanced modeling approaches, such as response surface methodology, could also be employed to predict interaction outcomes under various environmental conditions. These are the Recommendations for Future Research.

5. CONCLUSION

This study demonstrates that the combination of CHX and BP results in antagonistic interactions, reducing the overall efficacy of the combination in immobilizing *Daphnia* populations. The observed antagonism, characterized by CI values greater than 1 across all IC levels, suggests that the concurrent use of these agents may be detrimental rather than beneficial. The findings underscore the need for careful consideration when formulating combination therapies and highlight the importance of optimizing ratios, exploring sequential application strategies, and incorporating advanced delivery systems to mitigate antagonism.

Authors contribution: -

Conceptualization and design, Dharshini.P and Sri Sakthi.D; Literature review, Dharshini.P; Methodology and validation, Sri Sakthi.D; Investigation and Data collection, Taniya Mary Martin; Resources, Dharshini.P; Data Analysis and Interpretation, Meenakshi Sundaram Kishore Kumar; Writing - Original Draft Preparation, Dharshini.P; Writing - Review & Editing, Sri Sakthi.D; Supervision - Sri Sakthi.D;

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