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Synergistic and Counter Effect of Biocides, Amines and Emulsifier in the Combinatorial Toxicity Study

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Abstract

Pollution is one of the major environmental issues that affect human beings. Metalworking fluids are widely used in many industries. There are many chemical components such as amines and biocides in the metalworking fluids, which cannot be biologically treated, and disposal is still a problem. Often chemicals are tested for toxicity individually, however, there are interactions between combinations of chemicals. Hence, in this research, chemicals that are commonly used in metalworking fluids, are tested in combination as part of a factorial experimental design. Three types of commercially available biocides (A14, AEF, AOX - coded due to commercial rights), two amines (Monoethanolamide - MEA, Triethylolamine - TEA), and an emulsifier (blinded because of commercial rights) were tested. A bacterial biosensor *E.coli* HB101 was used to assess toxicity. A total of 63 tests were carried out. It was found that the toxic responses do not align with predictions based on the sum of the responses to individual compounds. Instead, there are interactions that cause synergistic or counter effects. For example, biocides A14 and AEF were found to be lethally toxic; biocide AOX and MEA were found to be slightly toxic. The combination of MEA, AEF, A14 was found to be the most toxic of the 63 possible combinations. However, when AOX was added, the toxicity level decreased in indication that toxicity was mitigated. This study shows that understanding the combinatorial toxicity could help to inform eco-design and promote sustainable biological treatment at the end of product life.

Introduction

Environmental issues such as air pollution, water scarcity, and waste management are a global problem. This has many impacts on lives today such as physical and mental health, resource degradation such as water and soil. The main source of those problems is human activity, such as polluting the environment, with a wide variety of chemicals including biocides. Biocides are widely employed in sectors such as agriculture, the food

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industry, manufacturing. It is used for anti-microbial applications in many industries to control the growth of microorganisms (Health and Safety Executive, 2012). They are used as antiseptics to treat infections in mucous membranes and damaged skin (Russell, 2003; Hussain et al., 2020). They are also used to control microbial contamination (Jones & Joshi, 2021) in industrial-scale applications. Because of the diversity of applications, biocides can contaminate the environment on a wide scale and result in damaged ecosystems since biocides are toxic to both microorganisms and nontarget species (Massei et al., 2018; Malhotra et al., 2021).

Current and safety regulations, require manufacturers to provide a material safety data sheet (MSDS) for each individual chemical. Chemicals are not tested in combination. Hence, a key issue is that there have been a few studies of the combined additive influence of two or more biocides in the same product, and where it has been studied the impact has been over-estimated (Liess et al., 2020). Predicting the combined effect of toxicant mixtures with distinct modes of action has yielded mixed results (Belden et al., 2007). A vast number of studies have suggested that the combined impacts of toxicants might reveal much higher effects than those expected by the Effect Addition (EA) or Concentration Addition (CA) approaches. Synergistic effects are the term for such unanticipated outcomes (Ralf & Schäfer, 2016). The counter effect is when the effect is canceled out and has a lower expected outcome. Therefore, the interaction between chemicals and the environment may lead to a synergistic or counter effect (Singh et al., 2017). For example, two or more benign compounds may result in a highly toxic reaction when comes together. Conversely, two or more toxic compounds could be benign to the environment. This approach can be applied as pollutant prevention for many industries.

Metalworking fluids are widely used in many industries for grinding, milling, drilling, and metal cutting (BP-Castrol Limited, 2012; Cheng et al., 2005; Gauthier, 2003). Metalworking fluids are composed of water, amine, emulsifier, and biocide (Canadian Centre for Occupational Health & Safety, 2005; Cheng et al., 2005; Byers, 1994; McCoy, 1994). Water sources and atmosphere deposited microorganisms cause contamination, which deterioration of metalworking fluids is caused by microbial action, this lowers the quality of metalworking fluids performance and necessitates premature disposal. Carbon, nitrogen, sulfur, and organic compounds produced from waste are nutrient-rich for the bacteria (*Escherichia coli*) to grow (Willing, 2001). Biocide or antimicrobial agent was introduced in the mid-20th century to prevent bacterial, viruses, yeasts, fungi, and protozoa growth and prolong life in use (Cheng et al., 2005). This work focuses on the bacterial growth because they are the dominant taxa in the metalworking fluids.

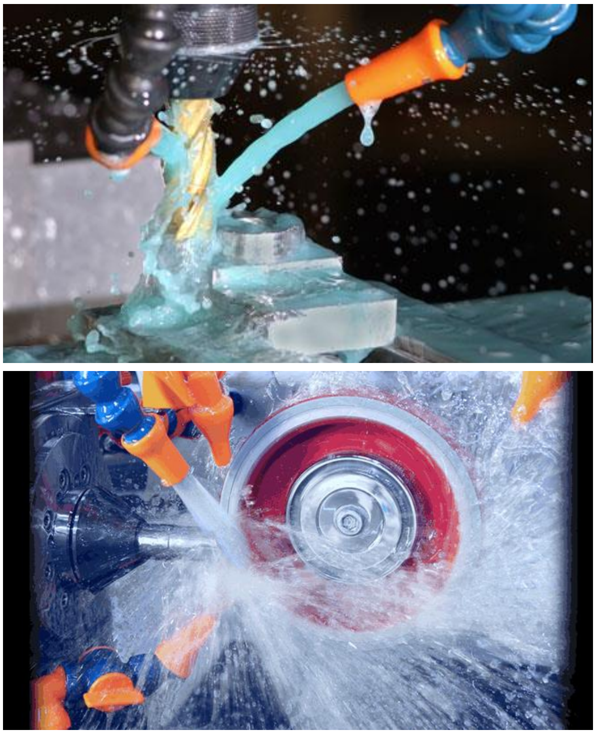


Fig. 1 a). Metalworking fluids employed in drilling process, b). in grinding process

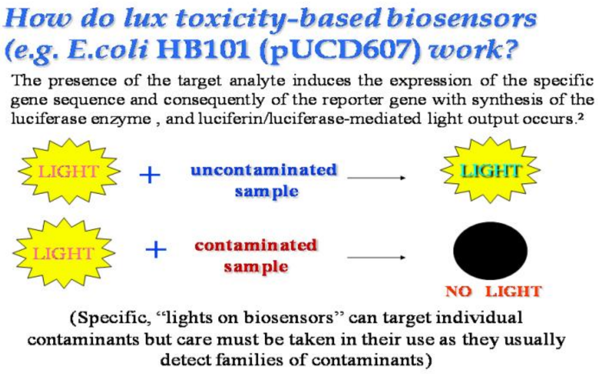
Source: Health and Safety Executive (2015); Northwest Aerospace Alliance (2013)

Biocides are an important component to prevent bacterial growth in MWFs and prolong product life in the machine. However, the biocide is toxic to the environment and an eco-design approach could be implemented to understand the reaction of the chemicals both individually and in combinations. This information then applies to the product development to prevent or prepare the solution for the foreseen challenges at the design stage. Eco-design can reduce the amount of waste, dangerous chemical uses, waste disposal, and resource costs (Uapipatanakul, 2020). This research is aimed to explore the interactive toxicity effect of the biocides in different combinations. Whereby the three types of biocides, two amines, and an emulsifier, which are the common components in the metalworking fluids were tested for combinatorial toxicity effect (Table 1).

Eco toxicity is described as the department of toxicology concerned with the consideration of poisonous impacts, caused by natural or manufactured toxins, to the constituents of biological systems, creature (counting human), vegetable and microbial, in an intrinsic context (Truhaut, 1977). Subsequently, it is critical to comprehend the concentration of chemicals which influence the environment and ecosystem recognizing that if one life form is influenced, other life forms within the web may also be impacted (Koeman, 1998; Bardgett, 2005; Procter & Gamble, 2005; Cole et al., 2006). Well-established bioassay strategies were utilized for this work. The bacterial bioassay approach used in this work is rapid reproducible, cost effective, and appropriate to a wide extent of toxins (Koskella & Stotzky, 1997; Tiensing et al., 2002).

Microscopic organisms in aqueous solutions react quickly to changes in natural conditions in their normal habitats due to their large surface to volume ratios. Whole cell bacterial biosensors, such as *E.coli* HB101 and *E.coli* dH5α strain can be utilized as a surrogate for higher living beings in harmfulness testing (Madigan et al., 2000; White et al., 1998; Kelly & Harwell, 1989). Biosensors report the aggregate impact of blended chemicals and are valuable in measuring the poisonous quality of dissolved chemicals at environmentally relevant concentrations. To perform as a biosensor the *E.coli* was transformed with a plasmid containing bacterial bioluminescence (*lux*) qualities taken from *Vibrio fischerii.* The *lux* operon was adjusted by deletion of the regulatory I and R genes so that CDABE conferring constitutive light output. The light response is directly related to intracellular adenosine triphosphate levels, the cells' medium of exchange. Lower vitality levels are in this way reflected by diminished light yield (Fig. 2). The light yield is measured in 96 well in a micro-titer plate spectrophotometer allowing high throughput screening.

Eco-design and sustainability are of interest to many researchers and industries. For instance, in northwest Europe, manufactured base stock MWF now substitute mineral oils with vegetable oils, thus reducing environmental impact and sustainability (Glenn & van Antwerpen, 1998). Understanding the relevant chemical interactions and optimizing them for in-use shelf life and natural treatment advancement. Producers plan most items without considering disposal at their end-of-life, which may be a boundary to accomplishing a closed circle economy. Many technologies require high carbon footprint treatment strategies, such as chemical and physical treatment because natural treatment isn't conceivable.


How do lux toxicity-based biosensors (e.g. E.coli HB101 (pUCD607) work?

The presence of the target analyte induces the expression of the specific gene sequence and consequently of the reporter gene with synthesis of the luciferase enzyme , and luciferin/luciferase-mediated light output occurs.²

(Specific, "lights on biosensors" can target individual contaminants but care must be taken in their use as they usually detect families of contaminants)

Fig. 2 Illustration of how lux based biosensors work
Source: Mwinyihija (2011)

Organic treatment is more energy and resource intensive than current innovations. Additionally, at the conclusion of treatment, materials are potentially reusable and recyclable (Uapipatanakul, 2020). Much waste requires high carbon footprint treatment methods, such as chemical and physical treatment because biological treatment is not available. Hence, understanding the interaction can help in considering disposal and recycling at end-of-life of products, which will challenge biological treatment development and promote the closed-loop economy (Uapipatanakul, 2020).

The overall aim of this research is to understand the toxicity of bacterial cells of individual compounds and combination mixtures employing biosensor *E.coli* HB101. The thinking was that by assembling metalworking fluids formulations based on the prior knowledge of the relative toxicity, degradability, and recalcitrance of individual components and when in combination, puts the industry in an improved position to be able to predict the durability and sustainability of end-of-life bio-treatment. Single compound responses were used to predict the fate of compounds when in combination. Combinations that were more or less toxic or biodegradable than predicted were studied in further detail. Such detailed analysis identified the pinch-point chemical components, which determine susceptibility to biodegradation and resistance to bio-deterioration.

Information can be exploited for the possible selection of biocide use in the formulation that will act as a bio-softening agent at the end-of-life biological treatment.

Materials and methods

This experiment examined combinations of common chemical components that are used in the formulation of metalworking fluids. Formulation with the choices of commonly used biocides, amines, and emulsifiers (Uapipatanakul, 2015; Azimi et al., 2017; Byers, 2006; Samuel et al., 2011). Metalworking fluid was selected as a model product and representative of many commercial products which are formulated to contain mixtures of chemical components including biocides. There are six generic chemical compounds used in formulating metalworking fluids which were studied in this experiment. These consisted of two amines (corrosion inhibitors), one emulsifier, and 3 biocides (antimicrobial agents) (Table 1).

Table 1 Chemical compound abbreviation

Type of chemical components	Code use in this research	Function
Isothiazolinone based biocide	A14	Antimicrobial
Oxazolidine biocide	AOX	Antimicrobial
Fungicide	AEF	Antimicrobial
Emulsifier	E	Emulsifier
Methanolamine	MEA	Surfactant/Emulsifier/Corrosion inhibitor
Triethanolamine	TEA	Surfactant/Emulsifier/Corrosion inhibitor

1. Preparation of biosensor

E. coli HB101, which contained *lux* genes of the plasmid vector pUCD607 (Microbial Solution Co.Ltd., Oxford, United Kingdom), was used as the biosensor in this study. The miniprep technique was used to extract plasmid pUCD607. Plasmid confirmation was done by gel electrophoresis. This strain is highly competent in terms of its ability to take up extracellular DNA. The cells energy status is reflected by the light emitted by the biosensor. The dose-dependent toxicity/light output relationship is exploited in this work as a measure of the aggregate toxicity of biocide solutions.

2. Determination of mid-exponential phase

The biosensor *E. coli* HB101 was grown to mid-exponential phase in the growth media; LB Broth (growth media) was prepared by autoclaving a mixture of 10 g/L Tryptone (OXOID), 5 g/L yeast extract (Fisher Scientific), 5 g/L sodium chloride (Fisher Scientific), 1 g/L glucose in deionised water. Ampicillin stock (25 µg/L) was also added after the solution had cooled down to prevent thermal degradation of the antibiotic. *E.coli* HB101 biosensor was inoculated into 5mL sterile LB Broth and kept overnight in a shaking incubator Innova 44, (New Brunswick Scientific, UK). at 35ºC and 120 rpm After 16 hours, the inoculum was transferred into 100 mL sterile LB Broth. Optical density (OD) and luminescence were recorded every 30 minutes using a UV spectrophotometer(UV-1800, Shimadzu, Japan) and a micro-titre plate spectrophotometer (Synergy HT, BioTek, USA). All processes were performed under aseptic conditions. Mid-exponential phase is when the membrane is at the most sensitive phase of growth, therefore, it is optimal phase to observe the effects that samples have on the cells. To identify the mid-exponential phase, typical growth and luminescence growth curves were produced. Growth and luminescence curves were obtained by incubating *E. coli* biosensor in the growth media and plot readings against time. The experiments were carried out in triplicate.

3. Toxicity testing

Toxicity was assayed using a bioluminescent bacterial sensor, which is an analytical detector of biological responses expressed as luminescence. Commercially available biocides (A14, AEF, AOX), amines (MEA, TEA), and emulsifier (E) (See Table 1) were prepared at 1.0% v/v at room temperature. With the factorial design, a total of 63 possible combinations (Equation 1, Table 2) were tested in different number of combinations from one to six as shown in Table 2.

Calculation for number of combinations

$$\binom{n}{k} = \frac{n!}{k!\,(n-k)!} = \frac{n(n-1)\ldots(n-k+1)}{1\cdot 2\ldots k}$$

n = total number of combinations; k = number of selected combinations

Source: Kreyszig (2011)

Table 2 Number of possible combinations in each complex level

No. of compounds in the combination	No. of possible combinations
1	6
2	15
3	20
4	15
5	6
6	1
Total	**63**

180 µL of test sample solutions were dispensed in a 96 well micro-titre plate (Nunc brand). *E. coli* HB101 biosensor was inoculated (20 µL) in the test samples and exposed for 15 minutes before luminescence (relative light units) were measured and recorded using a micro-titre plate spectrophotometer (Synergy HT, BioTek, USA). The chemical compounds were tested individually and in combinations employing a factorial design approach; started from two combinations and increased progressively in complexity to six combinations in 96-well microtiter plate format. This experiment was carried out at room temperature. All processes were performed under aseptic conditions. All experiments were carried out in triplicates.

Combinatorial studies were carried out to identify component interactions that produce bop "hardening" or "softening" effects, which could facilitate extended product life or effective bio-treatment, respectively. To be specific, a "hardening agent" or constituent in this respect is one, which when present on its own or in combination with another chemical constituent inhibits or slows down biodegradation activity and thus contributes to extending the life of the chemical mixtures. In contrast, a "softening agent" is defined here as one whose presence to is making the total combination more susceptible to premature biodegradation/bio-deterioration.

Results and discussion

From Fig. 3 and Fig. 4, it can be seen that the optimal time to conduct the experiment was 8 hours after inoculation, when *E. coli* HB101 biosensor was at the mid-exponential phase and was producing light.

In this toxicity testing employing an *E. coli* HB101 biosensor, deionized water was used as a control with the percentage luminescence of 100. Six samples were tested for toxicity individually and it was found that biocide A14 was the most toxic (lethal) and AOF, AEF, E, TEA, and MEA, respectively.

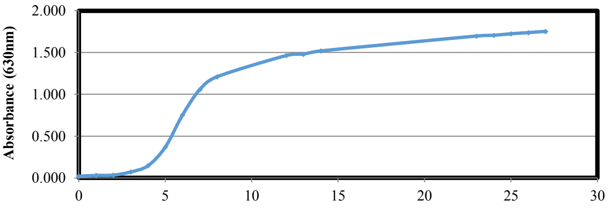


Fig. 3 Growth curve of *E.coli* HB101 biosensor

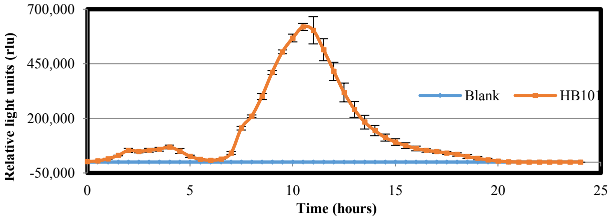


Fig. 4 Luminescence curve of *E.coli* HB101 biosensor

The experiment then further tested for toxicity in 63 combinations. Fig. 5 shows four factorial data, which is 56 possible combinations. Overall results suggests that 48% of combinations were very toxic. Interestingly, it can be seen that a combination of Methanolamine (MEA), A14 biocide, and AEF biocide was the most toxic in terms of light emitted by biosensor *E. coli* HB101 (left end of the graph), but once AOX was added into the mixture; MEA, AOX, A14, and AEF, the combination was found to be in top 20% most benign. This indicated that AOX has counter toxicity effect to this combinatorial interaction, *i.e* when present with the other two components the aggregate toxicity is decreased relative to single compound toxicities.

The experiment was designed to identify these unexpected interactions using a combinatorial approach with a small panel of biocides. It aims to inform the exploitation of their combined effects to create awareness of synergistic and counter effect of the chemical release to the environment. Furthermore, it can be used to formulate future products, such as metalworking fluids, either for making them more recalcitrant or biodegradable. An understanding of the effects of interactions between specific compound combinations on bacterial growth provided the basis for designing a new generation of metalworking fluids with more predictable performance in terms of interaction of microbial cells. This includes resistance to biodeterioration of in-use fluids and biological treatment at end-of-life.

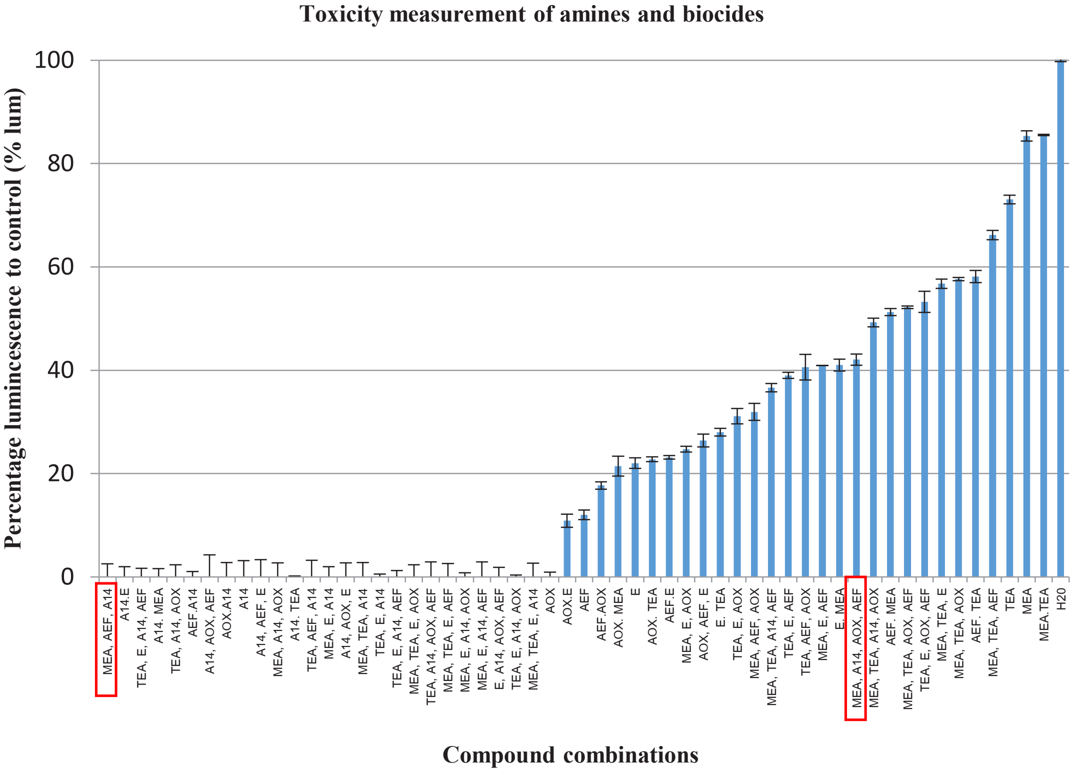


Fig. 5 Toxicity assessments of chemical combinations of increasing complexity (2, 3, and 4 compounds) composed of the following components: Methanolamine (MEA), Triethanolamine (TEA), Emulsifier (E), A14 biocide, AEF biocide, and AOX biocide. Reduce light output relative to the control is interpreted as a toxic response. Red boxes highlight one example where an additional biocide, AOX, was added to a toxic mixture resulting in a non-toxic response from the biosensor

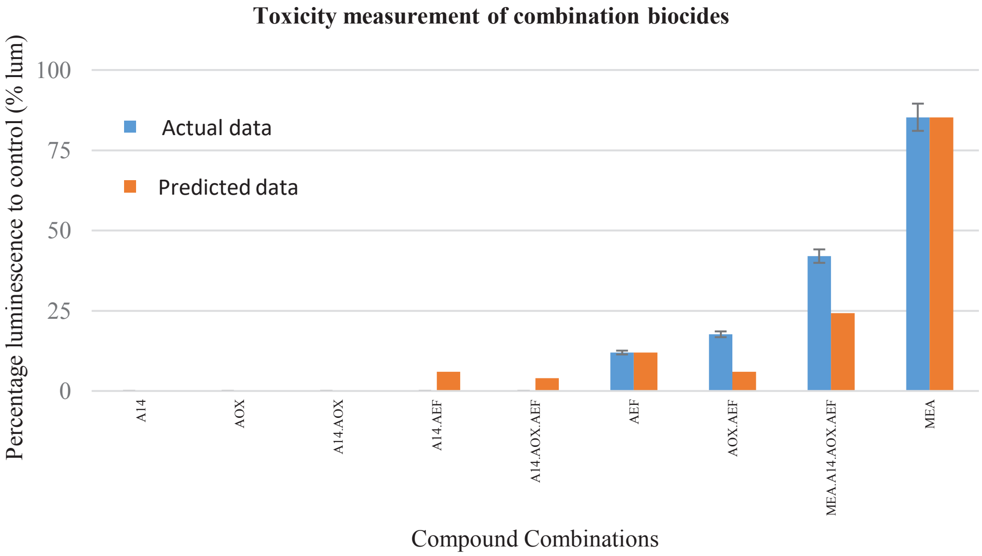


Fig.6 Toxicity measurement of full factorial combination mixture of MEA, A14, AOX, AEF employing biosensor *E. coli* HB101. Bars show the standard error for triplicate samples

From Fig. 6, it can be seen that biocide A14 and AOX are both toxic from the individual toxicity test. As predicted the combination of A14 and AOX is still toxic. However, there are cases where it does not align with the prediction. For example, biocide A14 is toxic from the individual toxicity test, while biocide AEF is mildly toxic from the individual toxicity test. When biocide A14 and AEF were combined, the toxic response to the combination was found to have a synergic effect, while the actual result is lower than the prediction. On the other hands, for combination MEA, A14, AOX, AOF, the toxic response to the combination was found to have a counter effect, where the actual result is less toxic than expected.

Toxic responses to combinations of biocides revealed that two or more toxic compounds sometime mitigated the toxic response from the biosensor to the individual compounds, such that the combination was much less toxic than when present as the single compound. Thus, in some cases observed and predicted toxicities from single compounds responses diverged for mixtures (Fig.5 and Fig 6). There is a considerable body of research that examines the toxicity and effectiveness of biocides. Usually, bacteria are killed in less than 12 hours in metal cutting fluids containing biocides. Although, biocides have been demonstrated to inhibit microbial growth in the metalworking fluids (Chazal, 1995) at lower concentration biocides have been shown to sustain growth of bacteria such as *Pseudomonas putida* and *Psuedomonas fluoresens* (BASF Agricultural Solutions – Global Website, 2022).

Conclusion

Metalworking fluids need to be designed to be safe to untimely biodegradable, arranged to draw out their working life (Cheng et al., 2005) and disposal, respectively. To attain this metalworking fluids are defined to be poisonous to microbial cells when in use. To date, the design of metalworking fluids has been directed, ease of generation, and usefulness with no thought of end-of-life treatment. This unavoidably leads to product formulations that are recalcitrant to biological treatment at the end of the product life. Consequently, until 2006, depleted metalworking fluids were disposed to landfill after energy intensive treatment by ultra-filtration or vacuum evaporation. However, chemical and physical technologies are accessible, and are widely used despite being costly in terms of running and capital costs (Chipasa, 2011; Thompson & van der Gast, 2010: Tchnobanoglous et al., 2004). Also, they create residual sludge that require incineration. Therefore, the biological solution is attractive as it is able to scale up from laboratory to business scale, and additionally water may be recycled on-site (Jiang et al., 2012; Luostarinen et al., 2009). However, the modern MWF formulations make this difficult considering they may be formulated to be proof against microbial deterioration. In order to embody those contradictory needs, it is crucial to reformulate metalworking fluids in order that they may be proof against biodeterioration while in use however they are made, preferably via way of means of a few easy chemical manipulations wherein the chemical interplay has counterintuitive responses and transfer from poisonous to benign property.

Current legislation (EU Directive 67/548/EEC) requires that toxicity and environmental assessment to be based on the single compounds and material safety data sheet. “Unless there is evidence to the contrary, authorities generally enforce regulations that assume that acceptable concentrations for pollutants can be treated independently, even when they are present in mixtures” (Walker et al., 1996; Walker et al., 1998; Strachan et al., 2001). This emphasizes the importance of individual compound and factorial toxicity screening. Furthermore, a key point raised in ecotoxicological legislation is that the particular trophic level of the organisms is of great importance, as certain pollutants interact specifically with organisms of a particular trophic level. This is not the case in a mixed system such as environmental systems. (Strachan et al., 2001). As the results in this research demonstrated, interactions between components in mixtures mean that responses to complex formulations do not necessarily correlate with the single compound assays and so an accurate picture of toxicity is not gained. Hence, the toxicology of compound mixtures is not predictive and often counter intuitive when based on single compound responses.

This work employed a factorial design to show that interactions between chemical compounds commonly found in industrial scale use can increase or decrease depending on the concentrations of other toxic components in mixtures. This knowledge can be exploited to identify combinations of effective biocides and can also be used to inform product disposal development at the end of the product life. Further work is needed to assess the toxicity across trophic levels using a broad panel of biosensors which includes eukaryotic cells and higher trophic levels. This should be accompanied by a detailed study of the underlying chemistry to identify key component interactions which could be exploited as switches for manipulating product life and subsequent sustainable disposal. This pre-emptive design approach would allow an end-of-life product to be softened and avoid releasing pollution to the environment.

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