Data Analysis of Lake Albert BGA Treatment using Waterzyme

Submitted by

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ABSTRACT

Waterzyme was used to treat harmful algal blooms (HAB's) at Lake Albert and this report analyses the data before, during and after treatment. Waterzyme demonstrated it was effective in decreasing cyanobacteria cell counts, however, further research is required to demonstrate the product can achieve 95% reduction levels seen in smaller systems.

Note: data used in this report on cell counts are sourced from Wagga Wagga City Council.

METHODOLOGY

Overview:

- Weekly sampling undertaken by Wagga Wagga City Council (WWCC)
- Waterzyme Australia (WZA) applied product as per application strategy in early January and through February
- Application volumes, dates and methods were recorded
- Analysis of results and discussion and collaboration between EPIC and Waterzyme Australia on reporting.

Sampling:

There was significant agglomeration of algae species in the southern (south-west and south-east) of Lake Albert due to prevailing northerly (west and east) winds. Whilst blooms were prevalent in these sections of the lake, cyanophyta were present across the water body, with some agglomeration of blooms in the northern part of the lake after southerly wind changes.

Three priority treatment areas were established – see Figure 1 below:

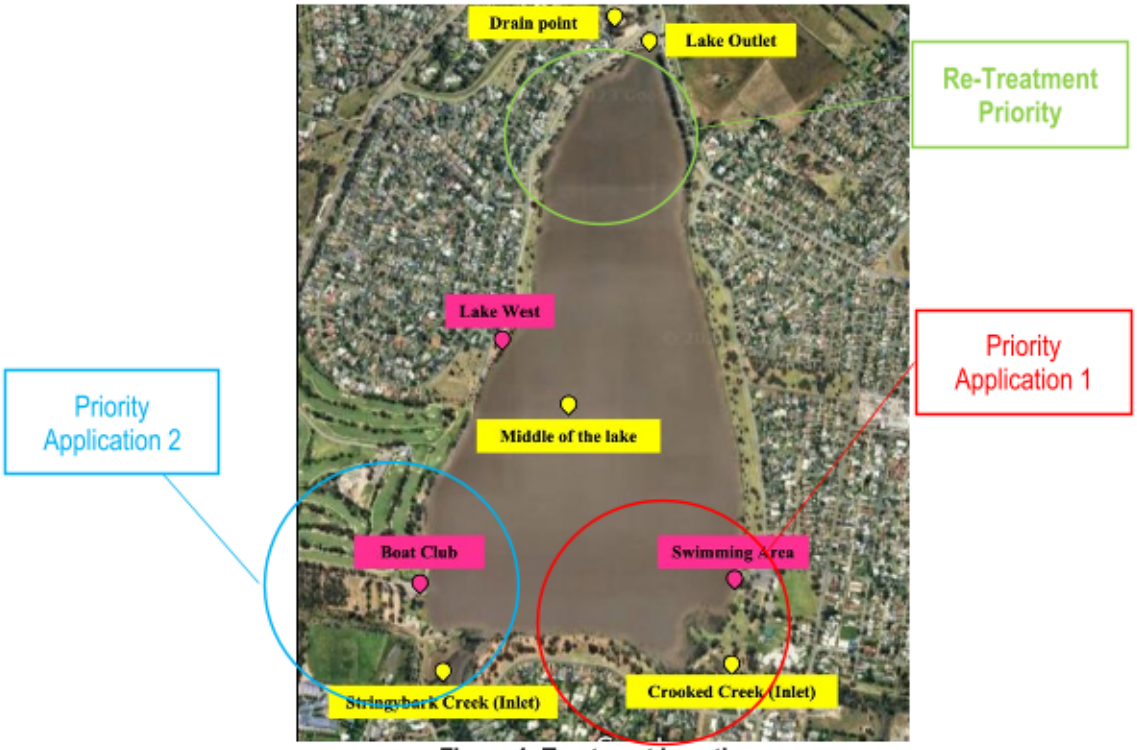

Figure 1: Treatment Locations

Sampling was undertaken by Council on a weekly basis (some variability in timing depending on context factors) during the summer months at three sample points (see Figure 1 above):

- Lake West
- Boat Club
- Swimming Area

Sampling has historically been undertaken in line with Lake Albert Water Sampling Protocols 2019 and samples taken by Council staff. More recently (February 2024), Council outsourced this sampling function to EnviroScience.

RESULTS:

Samples taken on 10 January triggered a 'red alert' and required that the Council issue health advice as provided by the relevant guidelines. Given that the time between the application of the product and the first sample was between 24-48 hours after the application of the product, these data should not be used to assess the product's effectiveness in managing HABs. These data are useful, however, in establishing a baseline against which to measure product effectiveness in subsequent weeks.

Samples taken on 10 January, and used as the baseline cell count for the research trial, showed the following results:

Table 1: Baseline Cell Count – 10 Jan 24

Date	Sample Point	Cell Count (cells/ml)
10-Jan-2024	Lake West	2,176
	Boat Club	87,318
	Swimming Club	437

Waterzyme Australia used the 87,318 cells/ml from the Boat Club sample on 10 January as the high benchmark for the trial.

Sampling for December 2023 to April 2024 showed the following results:

Table 2: Lake Albert Sampling Results – Dec 2023 to Apr 2024

Date	Lake West	Boat Club	Swim Area	Total Cells Sample Points
7-Dec-23	3713	4812	2309	10834
14-Dec	1026	1482	11534	14042
3-Jan-24	1093	17325	1330	19748
10-Jan-24	2176	87318	437	89931
18-Jan-24	1558	10474	1169	13201
22-Jan-24	24264	14533	30828	69625
30-Jan-24	24750	42750	2280	69780
7-Feb-24	40594	8188	2290	51072
14-Feb-24	19110	853	30510	50473
21-Feb-24	23940	53900	20160	98000
29-Feb-24	4500	1266	36633	42399
7-Mar-24	40155	30000	<5	70160
14-Mar-24	5250	12500	<5	17755
23-Mar-24	<5	10000	<5	10010

These results, and treatment interventions, are represented in Figure 2 below:

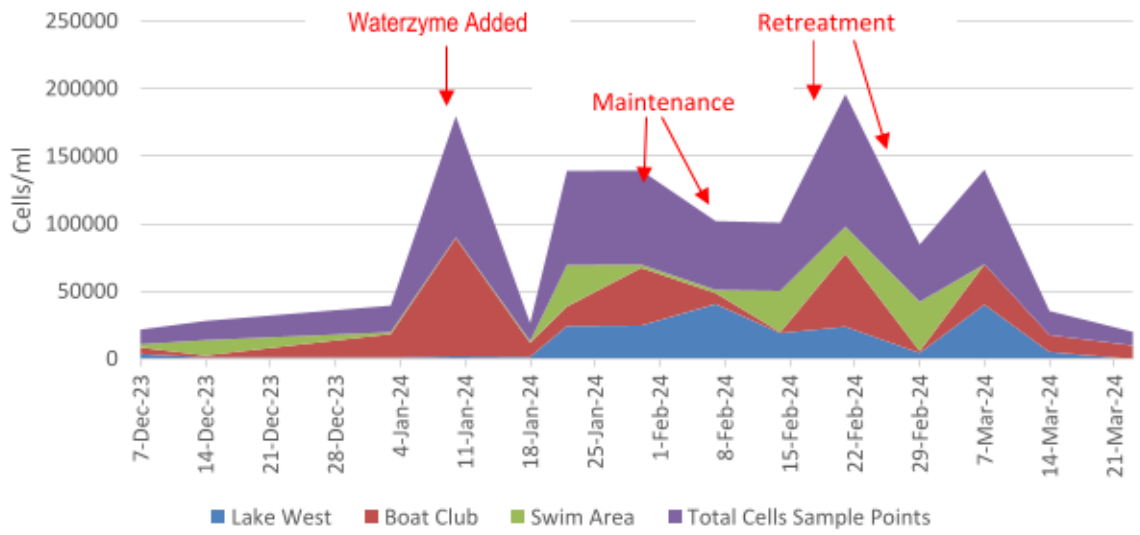

Figure 2: Lake Albert Sampling Results Dec23-Apr24

Key observations from the above results include:

- The initial large (12,000L) dose of Waterzyme on 10-11 January 2024 aligns with a sharp decline in cell counts.
- Waterzyme Australia informed EPIC that a decision was made to not maintenance dose through the rest of January but to await further trends from the data.
- Late January sees a jump back in cell counts across the lake.
- Maintenance dosing at lower volumes begins in early February. The data demonstrate some initial improvement in cell counts.
- Waterzyme Australia, working with EPIC scientists to determine the best way forward for the trial, made a decision in late February to isolate some of the bloom and dose at a higher rate (Retreatment). This corresponds with a decline in cell counts into early March.

A year-on-year comparison of total cell counts at the sampling points between 2022/23 and 2023/24 over the same period December to April show the following results:

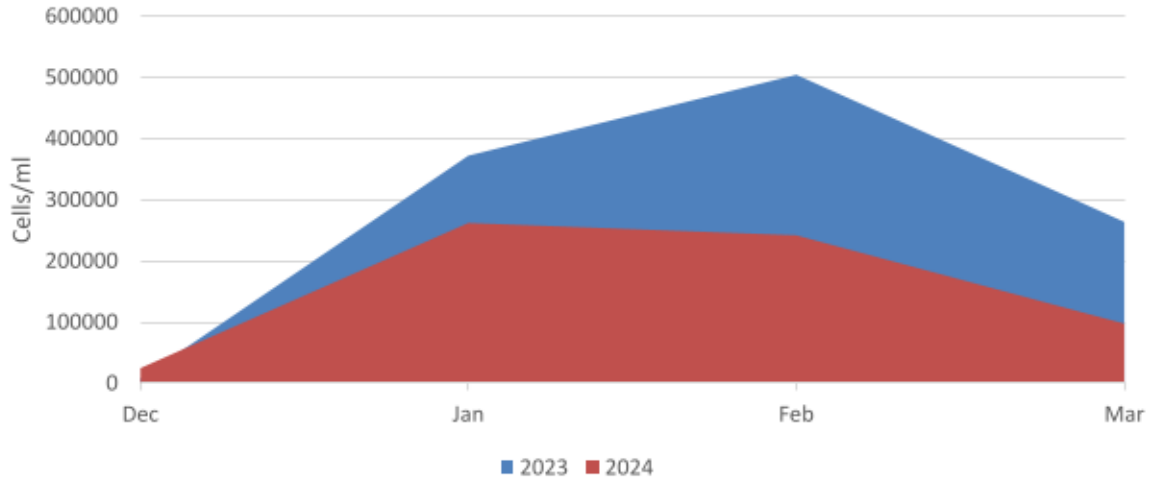

Figure 3: Lake Albert Comparative Total Cell Counts 2022/23 v 2023/24

Key observations from the above results include:

- Similar Cyanobacteria cell counts between 2023 and 2024 into early January
- In 2023, cell counts continue to rise significantly throughout January, before slow decline in February and March with high total counts
- In 2024, with the application of Waterzyme, we see a corresponding decline in cell counts in January, continuing into February and March with significantly lower total volumes.
- Data for 2023 shows cell counts at individual sampling points exceeded 50,000 cells/ml on five separate occasions. In 2024, results from individual sample points only exceeded 50,000 cells/ml once over the same sample period. Results over 50,000 cells/ml trigger requirements to issue health advice to close the water body to recreational use.

DISCUSSION

- Application of Waterzyme in sufficient volumes corresponds with reductions in cyanobacteria cell counts (for general information on Ecotoxicity and Waterzyme see Appendix A; for more information on Waterzyme function see Appendix B).
- Maintenance dosing was not implemented for three weeks to determine whether cell counts would remain low.
- In retrospect, it is the advice of EPIC that Waterzyme Australia should have consistently applied product throughout January – as the 3-weeks between doses aligns with an uplift in cell count.
- Smaller maintenance doses in early February would appear to have had some impact on cell counts, but higher dosage was required.
- Higher maintenance dosing of 12,000L over 2-3 weeks in late February, combined with isolation of the bloom and in-flow reductions through changes in the golf course water return, showed a decline into March.
- It is likely that catchment management issues have adversely impacted the Lake Albert trial and that the extraction, filtration and discharge of water by the golf club appears to be a significant factor in disrupting nutrient loads in sediment and concentrating those nutrients for HAB formation.

RECOMMENDATIONS

1. **Application**: Future trials commence in mid-late November to allow the product to demonstrate effectiveness in maintaining low bio-volume levels (preventative treatment). This will reduce the amount of product that is required. Maintenance dosing should become standard practice to keep cell counts at manageable levels;
2. **Sampling**: testing and sampling regimes be standardised for consistency; and,
3. **Catchment Management**: catchment management strategies be implemented to ensure water extraction by community stakeholders does not create highly concentrated biovolume discharge into Lake Albert. Techniques may include quarantining stormwater flows through artificial or naturalisation techniques, and discrete treatment of concentrated discharge from the golf club's extraction prior to release into the lake.

Appendix A

Ecotoxicity Assessment of Waterzyme to non-target organisms – *Allium cepa* L.

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ABSTRACT

In this study, we assessed commercial algaecides' cyto- and genotoxicity potential compared to Waterzyme products. In terms of cytotoxicity, the incidences of chromosomal aberrations (CA) and the reduction in mitotic index (MI) were higher in commercial algaecides over Waterzyme. On the other hand, metabolomics analysis using 1H-NMR showed, commercial algicides induced stress and stress-related metabolites in *A. cepa*. However, exposure to Waterzyme at 0.1%, the growth promoting compounds were upregulated in the root meristem of *A. cepa*.

THE CHALLENGE: Is Waterzyme a safe option over commercial algaecides?

The natural solutions represent a safer alternative to conventional commercial chemical-based algaecides. Unlike traditional chemical treatments that often pose risks to aquatic ecosystems and non-target organisms, the natural enzyme solution prioritizes environmental safety and sustainability.

This natural product-based approach harnesses the power of naturally occurring biological processes to mitigate harmful algal blooms. The natural solution disrupts their growth without introducing harmful chemicals into the ecosystem by targeting specific algae life cycle pathways. This method minimizes the risk of unintended consequences, such as toxicity to aquatic organisms, persistence in the environment, or the development of resistance in algal populations. The natural HAB control solution is biodegradable and environmentally friendly, ensuring its application does not leave a lasting ecological footprint. This contrasts with chemical algaecides that can accumulate in water bodies and potentially lead to long-term detrimental effects on aquatic habitats.

Generally, higher plants are often used extensively to monitor the toxicity of various harmful environmental pollutants (Rank et al. 2002; Iqbal & Nisar, 2015; Jiang et al. 2019). In this study, we used *Allium cepa* L. as the test organism to assess the cytotoxicity of 5 commercial algaecides compared with Waterzyme, since the use of *A. cepa* is a sensitive, cost-effective, and reliable test system for environmental biomonitoring (Fiskesjo, 1985) of several classes of environmental pollutants, including nanoparticles and emerging priority pollutants and complex mixtures in contaminated soil and water ecosystems (Leme & Marin-Morales, 2009). In addition, *A. cepa* assay has the additional advantages of offering the measurements at the microscopic and macroscopic level (Herrero et al. 2012). In addition, the *A. cepa* test systems positively correlate with the mammalian test systems (Rank and Nielsen, 1994; Teixeira et al. 2003). In addition, genotoxicity assessment of environmental pollutants by *A. cepa* root meristem chromosomal aberrations assay is an established plant bioassay validated by the

International Programme on Chemical Safety (IPCS, WHO) and the United Nations Environment Programme (UNEP) (Cabrera et al. 1999).

METHODOLOGY

Test organism Healthy onion bulbs were grown for a week in the dark in a cylindrical glass beaker containing deionised water at room temperature (25 °C) to facilitate root growth. The deionised water was renewed every 24 hours to avoid mould formation. When the roots reached 2-3 cm in length, they were exposed to 5 commercial algaecides (active ingredients: Diuron 5g L^{-1}, Simazine 3g L^{-1}, Poly(oxyethylene(dimethylliminio)ethylene (dimethylliminio)ethylene dichloride) 45g L^{-1}, copper as cupric ammonium complex 40g L^{-1}, Benzalkonium chloride 150g L^{-1}) at recommended dose as per the manufacturers' instructions and waterzyme at 0.1%. A positive control (Hexavalent Chromium @ 10 mg L^{-1}) was included. All the treatments and controls were in triplicate. The roots (2.0 - 3.0 cm) were harvested during their second mitotic cycle and were used for cytological analysis.

Cytotoxicity assay The harvested roots were fixed immediately in Carnoy's reagent (ethanol and acetic acid @ 3:1 ratio) for 24 hours at ambient temperature. The roots were washed with distilled water twice to remove traces of acetic acid. Then, the roots were hydrolysed with 1N HCl in a water bath at 60 °C for 10 min. The hydrolysed roots were again washed with distilled water. The roots were stained with basic Fuchsin dye and kept in the dark overnight to facilitate staining. About 2 mm-stained root tips were squashed over a microscopic glass slide using a sterile scalpel with 45% glacial acetic acid using coverslips and observed for chromosomal changes using an Olympus CX31 microscope at 10 X and 100 X magnifications. The microscopic analysis included recording the mitotic index (MI), the number of micronuclei (MN) in the interphase cells, and aberrant cells during metaphase, anaphase, and telophase. MI was calculated by the total number of dividing cells per 500 observed cells (Fiskesjö 1985; Smaka-Kincl et al. 1996). Chromosomal aberrations (CA) at each stage of the division were scored by examining cells in treatments and control (Bakare et al. 2000; Kumari et al. 2009). MN in the daughter cells appears to have a similar structure but is size-reduced compared to the central nucleus (Leme and Marin-Morales 2009). Five slides per sample were analysed (Fiskesjo 1997). Chromosomal aberrations were presented with micrographs.

Oxidative stress Briefly, 100 mg root samples (post 48 h exposure) in 100 mM PBS buffer were homogenised and centrifuged at 8000 g for 15 min. Negative control using deionised water was maintained. The supernatant was used for all the assays. Lipid Peroxidation was measured by adding 5 µl C11-BODIPY™ 581/591 (ThermoFisher Scientific, Australia) to the supernatant and incubated for 20 min in the dark, and the fluorescence was measured at 581 (excitation) and 600 (emission) nm. All the fluorescence measurements were carried out in an EnSight™ Multimode plate reader (PerkinElmer® Inc.) equipped with Kaleido 2.0 software.

NMR Metabolomics Onion roots were harvested and freeze-dried (John Morris Scientific, Osterode, Germany) and powdered. Around 100 mg of root samples (5 replicates per sample) were used to extract polar and non-polar metabolites with a solvent mixture of methanol and (1:2) chloroform for 15 min Ultrasonic Water Bath. The aqueous and organic layers were separated carefully, and the solvent phase was evaporated in a vacuum concentrator (Eppendorf, Hamburg, Germany). The residues containing polar metabolites were dissolved in a 1.0 mL mixture of an internal chemical shift standard composed of 0.05% (w/v) 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP) sodium salt in deuterated water (Sigma-Aldrich, St Louis, MO) and deuterated methanol (1:1, v/v). Twenty mg of residues containing non-polar metabolites were dissolved in 1.0 mL of an internal chemical shift standard consisting of 0.05% (w/v) tetramethylsilane (TMS) in deuterated chloroform (Sigma-Aldrich, St Louis, MO). The contents were then sonicated at 20 Hz for 10 min, vortexed and transferred to NMR vials (5 mm) for further spectral analysis.

The 1H-NMR spectra of the metabolites were acquired in Bruker BioSpin Avance III (600 MHz) NMR spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) by collecting 16 scans. The parameters used include the size of free-induction decay (FID), 65 K; spectral width, 20 ppm; acquisition time, 2.73 s; FID resolution, 0.37 Hz; and changing relaxation delay to 2.0 s for polar extracts (Kim et al. 2010). Each spectrum was manually calibrated at 0.0 ppm per the reference standards (TSP for aqueous extract and TMS for non-aqueous extract). All the 1H-NMR spectra were processed using TopSpin 4.0.5 (Bruker, Rheinstetten, Germany). The metabolites were identified using the Biological Magnetic Resonance (BMR) and Human Metabolome Database (HMDB). A heatmap was obtained using MetaboAnalyst 6.0 software (Pang et al. 2024), and the datasets were normalised by reference feature, log transformation and autoscaling. The Ward clustering algorithm and Euclidean distance measure were used for hierarchical clustering in the heatmap. The regulated pathways of putative metabolites were identified by considering the pathway enrichment and topology analysis. Pathways available at the Kyoto Encyclopedia of Genes and Genomes (KEGG) were used as references.

THE RESULTS:

For assessing the toxicity of groundwater, chromosomal aberrations, and micronuclei tests in *A. cepa* are considered an efficient short-term assay (Leme & Marin-Morales,2008; Leme et al., 2008; Silveira et al., 2017). In addition, *A. cepa*, as a test organism, has an 82% correlation to rodent assays in terms of sensitivity and effectiveness (Rank & Nielsen, 1994). *A. cepa*. has been used to assess the toxicity of metals, nanoparticles, firefighting foams, perfluoroalkyl substances, pharmaceutical drugs, herbicides, and insecticides (Achary et al. 2008; Ahmed et al. 2017 & 2018; Patnaik et al. 2013; Kumari et al. 2009; Sivaram et al. 2020; Prasath et al. 2016; Sharma and Vig. 2012). In the present study, we utilised *A. cepa* root meristem to assess the cytotoxic and cellular effects of five commercial algicides, positive control, tap water, and Waterzyme (0.1%) (Fig. 1). The positive control onion meristem root cells exhibited the lowest mitotic index (MI) of 23.67% at 48 h. MI is an efficient method to assess the cytotoxic effects of pollutants on cell division. The MI of commercial algicides was significantly lower than

that of tap water, followed by the Waterzyme treatment. Algicide D showed a lower MI than positive control among all the algicides.

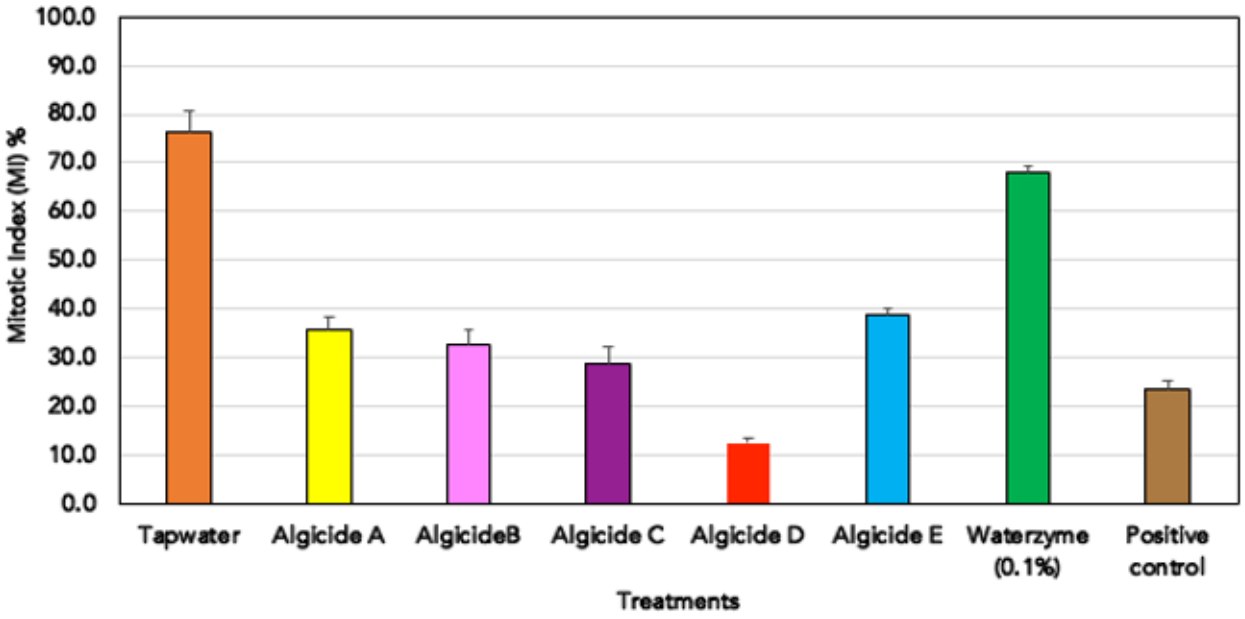

Figure 1. Mitotic index of A. cepa meristem root cells exposed to commercial algicides and waterzyme (0.1%)

The chromosomal aberrations such as chromatin condensation, spindle disturbances, micronuclei, bi-vacuole cells, sticky metaphase, spindle formation, abnormal anaphase, chromosome bridges, laggards, lagging chromosomes, nuclear bud, C0mitosis, C-metaphase, vagrants, multipolar anaphase, and disrupted prophase were observed in root cells exposed to Algicides (Fig 2). In Waterzyme (0.1%) treatment, sticky metaphase was observed. Interestingly, Algicide D produced all the aberrant type in 48 h of exposure when compared to the positive control. Due to the breakage and fusion of chromosomes, bridges are formed, whereas laggards result from weak c-mitosis, which increases the risks of aneuploidy (Lemme & Marin-Morales, 2009). When acentric fragments or laggards fail to incorporate into the telophasic daughter nucleus, micronuclei are formed, which may further lead to cellular death (Kirsch-Volders et al. 2002). Interestingly, the incidence of micronuclei in waterzyme was not recorded. The total chromosomal aberrations followed the following order: Algicide D > Algicide C > Algicide A > Algicide B > Algicide E > Waterzyme (0.1%) (Fig. 3).

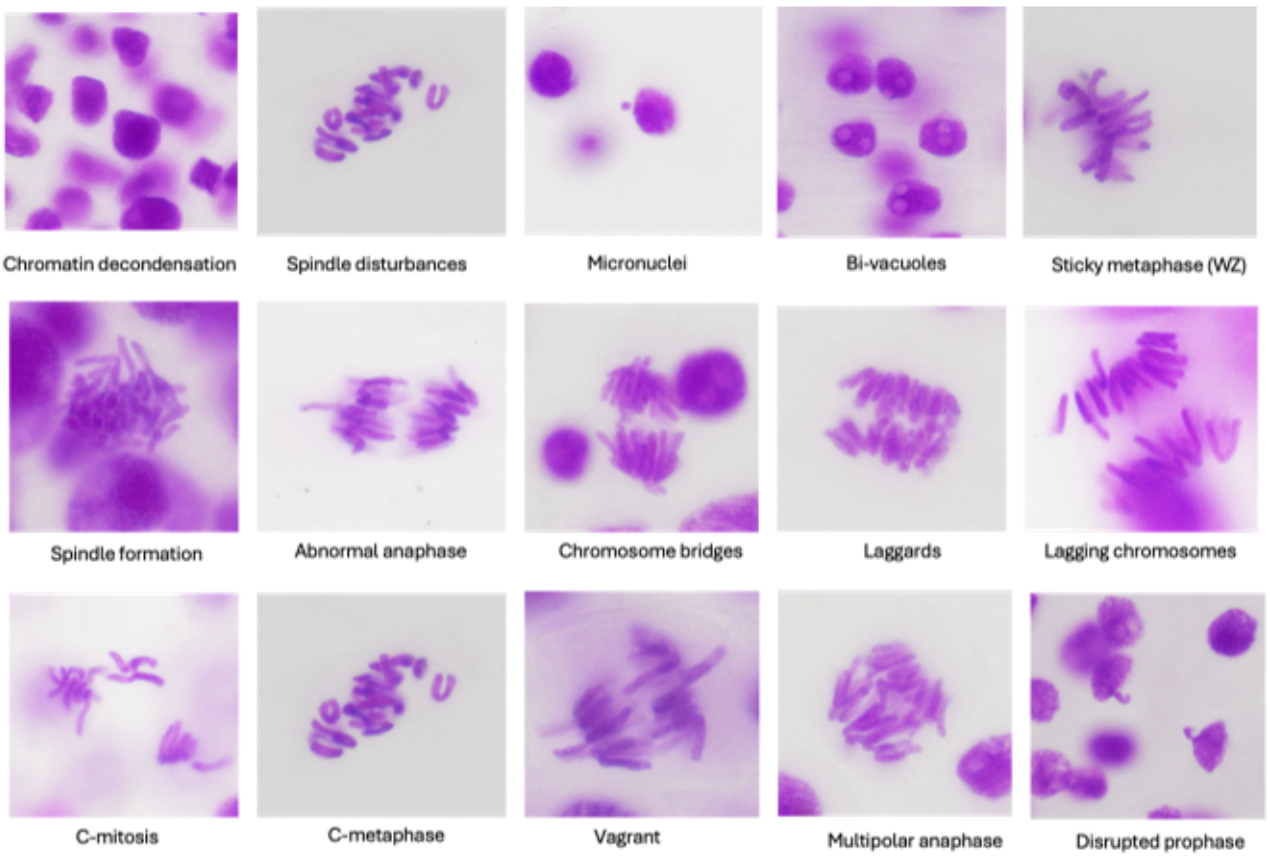

Figure 2. Chromosomal aberrations observed in algicides and waterzyme treatment.

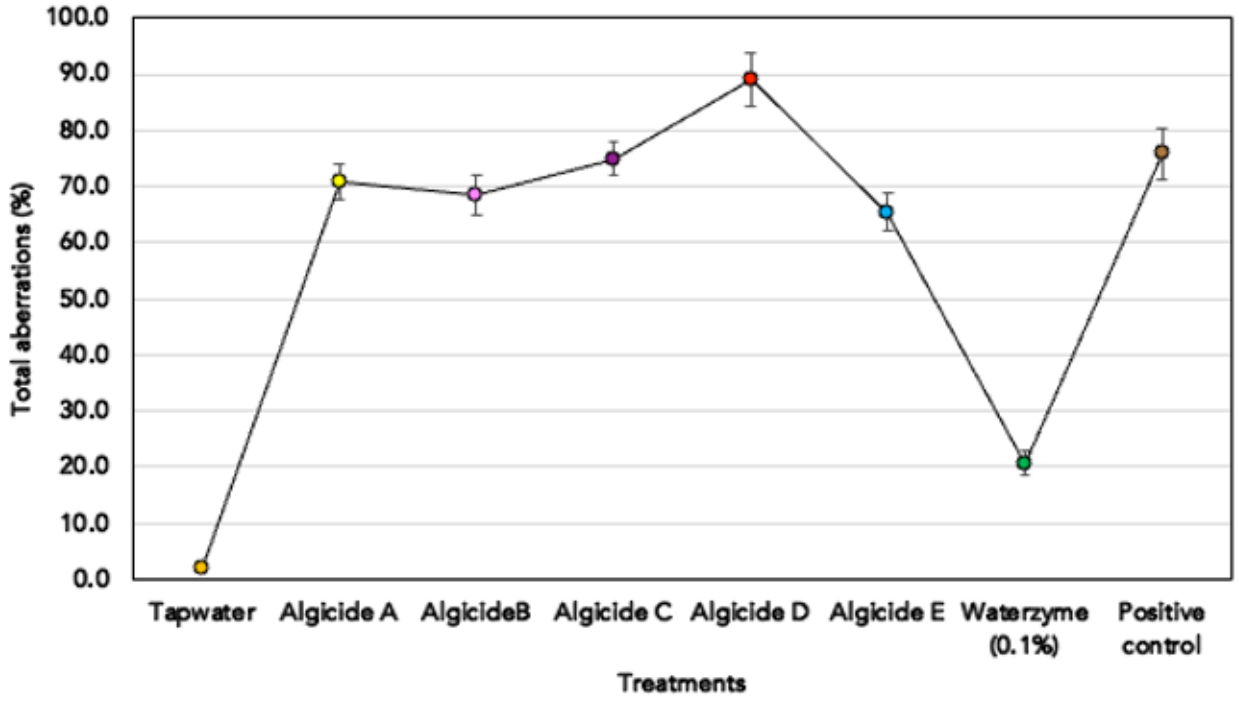

Figure 3. Chromosomal aberrations of *A. cepa* meristem root cells exposed to commercial algicides and waterzyme.

Generally oxidative stress is a commonly observed secondary stress due to a diverse primary abiotic and biotic stresses. Among the biochemical responses to stress, changes in reactive oxygen species (ROS), damage to the lipids, and membrane dysfunctions are well-established. The oxidative stress parameter, lipid peroxidation was measured in the *A. cepa* roots exposed to chemical algicides and waterzyme with positive and negative controls (Fig. 4). Generally, ROS is essential in plant signaling, controls plant growth and development, and mainly responds to biotic and abiotic environmental stimuli. A significant increase in lipid peroxidation in the chemical algicides over negative control was recorded however there was an insignificant effect recorded in Waterzyme (0.1%) over the negative control was also recorded. BODIPY 581/591 C11 fluorescent probe pointed out an increase in lipid oxidative damages of treated samples.

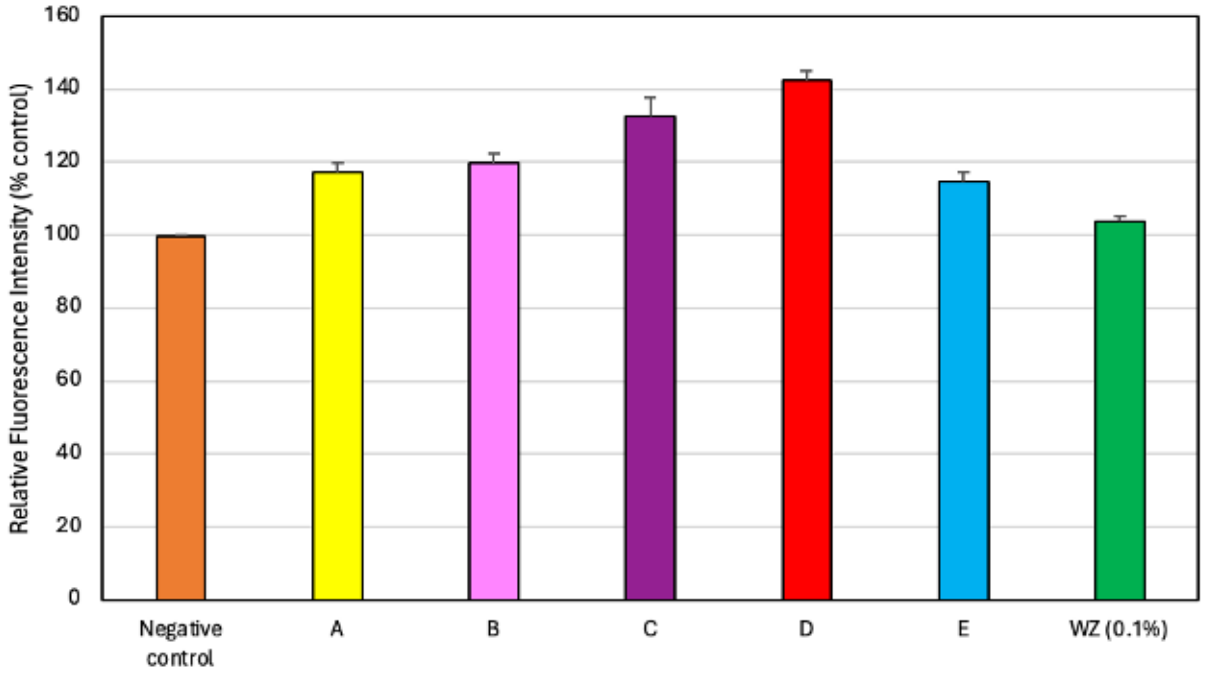

Figure 4. Lipid peroxidation in A. cepa treated with chemical algicides and Waterzyme (0.1%).

The polar extracts obtained from *A. cepa* roots exposed to commercial algicides, waterzyme, tap water and positive control analysed by ^{1}H-NMR showed both common and distinct signals with varied peak areas at low (δ 0.7 – 4.7 ppm) and high (δ 5 - 9.6 ppm) frequency regions. The peaks at these frequency regions indicated the presence of amino acids (aromatic, branched), organic acids (aliphatic and aromatic), vitamins, peptides, and energy storage compounds. Linear discriminant analysis (OPLS-DA) plotting projections to latent structure by combining first and second component showed a 42.8% variance among the treatments (Fig. 5). The treatments were significant ($P < 0.05$) and formed clear distinct and clustered groups. The treatments of Algicides A, B, C, D, and negative control showed an overlap of metabolites suggesting the upregulation of metabolites, Algicide E and positive control had slight overlap of metabolites possibly due to similar effects caused by them to the *A. cepa* root cells. Interestingly, treatment with Waterzyme (0.1%) formed a separate cluster without any overlap with other treatments suggesting the metabolites formed during this treatment is completely different to other algicides and treatments (Fig. 6). A significant change in the biosynthesis of metabolites is presented in the heat map (Fig. 7). A majority of metabolites related to chemical defense against abiotic stress, growth retardants, and stress responses were upregulated in all chemical algicides and positive control treatment and on the other hand negative (tap water) control and Waterzyme (0.1%) had majority of the metabolites were downregulated. Interestingly, three metabolites, namely pyridoxal, phenyl acetic acid, and deoxyguanosine were significantly upregulated in waterzyme treatment. These metabolites are related to growth promotion, and stress tolerance.

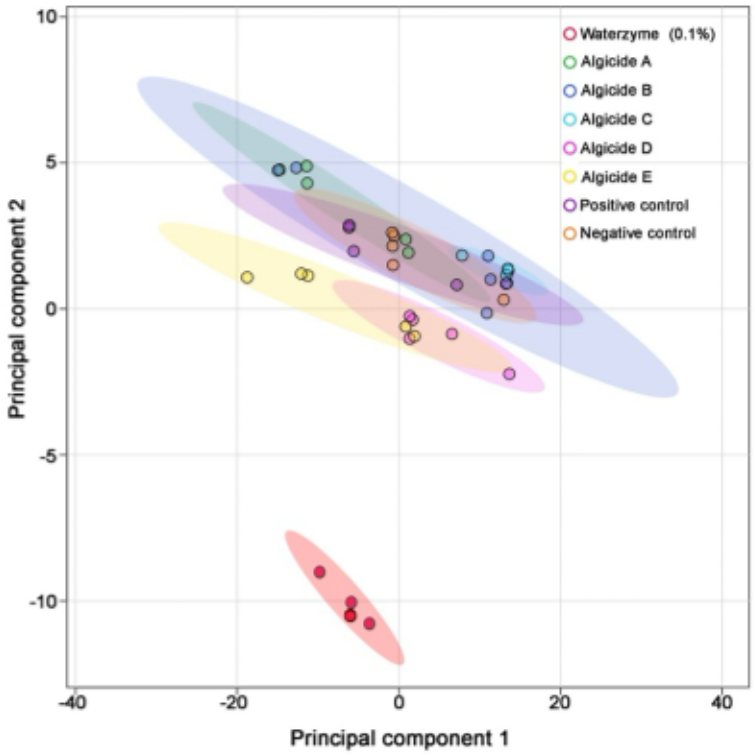

Figure 5. Principal component analysis of metabolites from ^{1}H-NMR analysis

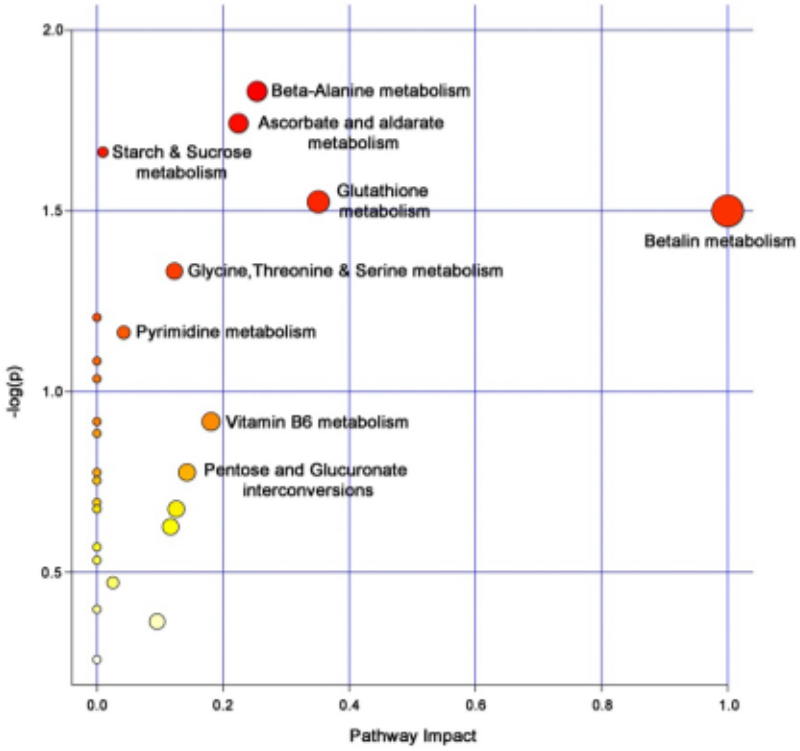

Figure 6. Pathway impact analysis of metabolites from ^{1}H-NMR analysis

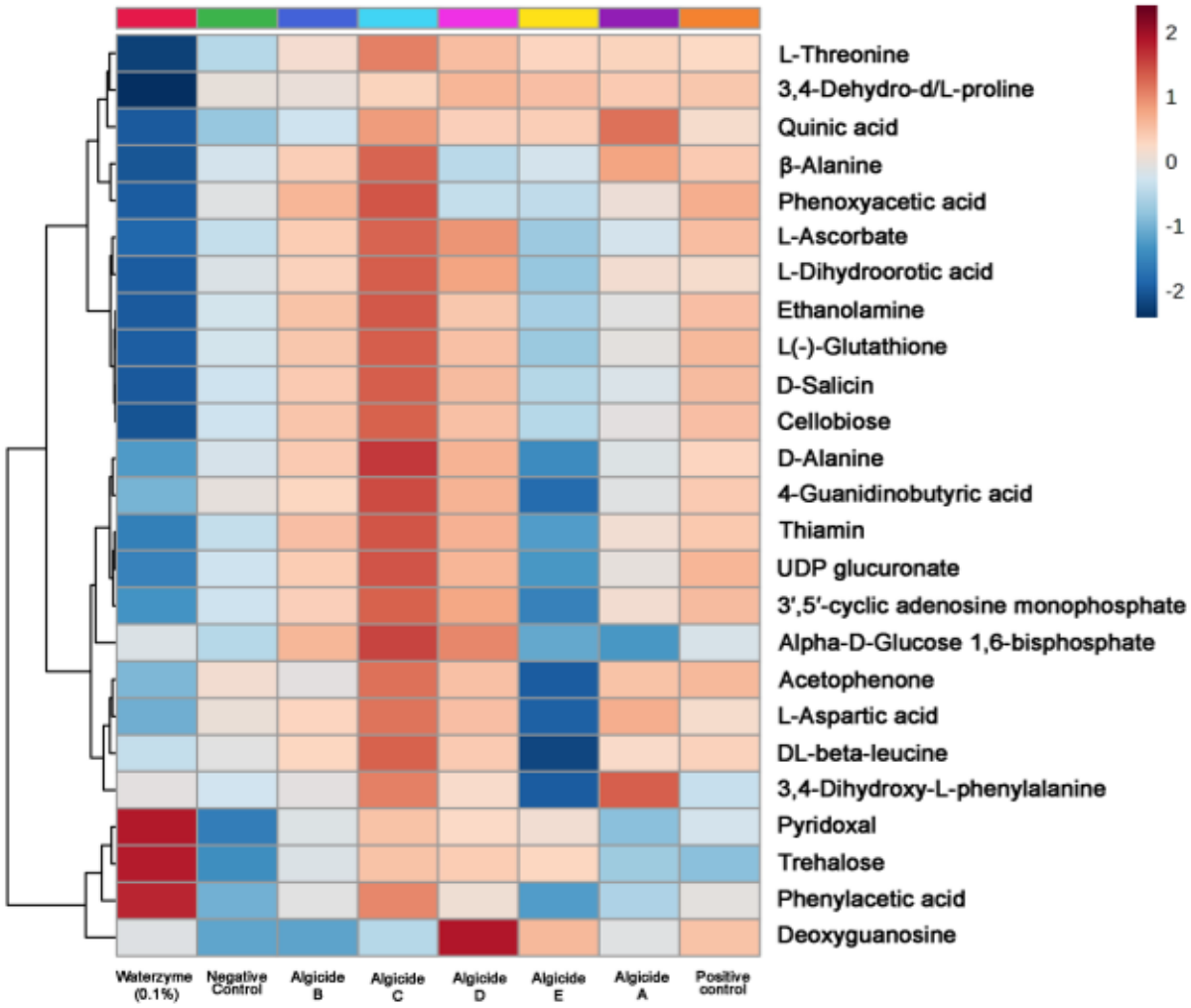

Figure 7. Heat map of 25 significant ($P \leq 0.05$) metabolites.

DISCUSSION

The results obtained from the ecotoxicity assessment of commercial algaecides and Waterzyme (a natural solution) on *Allium cepa* root cells provide valuable insights into their cytotoxic and genotoxic potential. This discussion interprets the findings considering environmental safety, effectiveness, and potential implications for ecotoxicological risk assessment.

Cytotoxicity Assessment The mitotic index (MI) serves as a reliable indicator of cytotoxic effects, reflecting the percentage of actively dividing cells. Commercial algaecides exhibited significantly lower MI compared to tap water, indicating their detrimental impact on cell division. Among the algicides, Algicide D displayed the most pronounced cytotoxicity, surpassing even the positive control. Conversely, Waterzyme demonstrated a relatively higher MI, suggesting lesser cytotoxicity compared to chemical algaecides. This aligns with the hypothesis that natural solutions like Waterzyme present safer alternatives to conventional chemical treatments, prioritizing environmental safety without compromising efficacy.

Genotoxicity Assessment Chromosomal aberrations observed in root cells exposed to commercial algaecides signify their genotoxic potential. Various aberrations such as chromatin condensation, micronuclei formation, and abnormal spindle structures indicate DNA damage and impaired chromosomal integrity. Algicide D induced the highest frequency of chromosomal aberrations, highlighting its severe genotoxic effects. In contrast, Waterzyme treatment resulted in fewer aberrations, with sticky metaphase being the only observed abnormality. This suggests a milder genotoxic impact compared to chemical algaecides.

Oxidative Stress Lipid peroxidation, a hallmark of oxidative stress, was significantly elevated in root cells exposed to chemical algaecides compared to the negative control. This indicates the induction of oxidative damage to cellular membranes, likely attributed to the reactive oxygen species (ROS) generated by algaecide exposure. In contrast, Waterzyme treatment showed insignificant effects on lipid peroxidation, implying lower oxidative stress induction. This underscores the eco-friendly and less harmful nature of Waterzyme compared to chemical alternatives.

Metabolomics Analysis Metabolomics profiling revealed distinct metabolic shifts induced by commercial algaecides and Waterzyme. Chemical algaecides elicited upregulation of metabolites associated with stress responses and biochemical defense mechanisms, indicative of cellular distress and adaptive responses to toxicity. In contrast, Waterzyme treatment resulted in downregulation of stress-related metabolites, suggesting minimal metabolic perturbations and possibly enhanced stress tolerance. Notably, specific metabolites related to growth promotion and stress tolerance were significantly upregulated in Waterzyme-treated samples, emphasizing its potential benefits beyond mere algal control.

Implications The findings underscore the superior safety profile of Waterzyme compared to chemical algaecides, highlighting its potential as a sustainable and environmentally friendly solution for harmful algal bloom management. The reduced cytotoxic and genotoxic effects, coupled with minimal oxidative stress induction and distinct metabolic responses, position Waterzyme as a promising alternative for mitigating algal proliferation without adverse ecological consequences. Further validation through field studies and long-term monitoring is warranted to assess its efficacy under real-world conditions and its potential for widespread adoption in aquatic ecosystems management.

CONCLUSION

In conclusion, the results emphasize the importance of considering both effectiveness and environmental safety in the selection of algaecidal agents. Waterzyme emerges as a safer and more sustainable option, offering effective algal control while minimizing ecological risks. Continued research and development of natural solutions like Waterzyme are essential for promoting environmentally responsible approaches to aquatic ecosystem management and safeguarding biodiversity.

This discussion underscores the significance of integrating multidisciplinary approaches, including cytotoxicity assays, genotoxicity assessments, oxidative stress analyses, and metabolomics profiling, to comprehensively evaluate the ecological implications of algaecidal agents and inform evidence-based decision-making in environmental management strategies.

RECOMMENDED FURTHER ANALYSES

Metabolomics studies emerge as invaluable tools in unravelling the sublethal effects of commercial algaecides and waterzyme products, providing a comprehensive and nuanced understanding of the metabolic shifts induced by these substances. By scrutinizing the intricate web of biochemical processes within organisms exposed to algaecides, metabolomics allows us to discern subtle alterations in cellular pathways, signaling cascades, and metabolic profiles that may not be apparent through traditional toxicity assessments.

REFERENCES

Achary, V.M.M., Jena, S., Panda, K.K. and Panda, B.B., 2008. Aluminium induced oxidative stress and DNA damage in root cells of Allium cepa L. Ecotoxicology and environmental safety, 70(2), pp.300-310.

Ahmed, B., Dwivedi, S., Abdin, M.Z., Azam, A., Al-Shaeri, M., Khan, M.S., Saquib, Q., Al-Khedhairy, A.A. and Musarrat, J., 2017. Mitochondrial and chromosomal damage induced by oxidative stress in Zn 2+ ions, ZnO-bulk and ZnO-NPs treated Allium cepa roots. Scientific reports, 7(1), pp.1-14.

Ahmed, B., Shahid, M., Khan, M.S. and Musarrat, J., 2018. Chromosomal aberrations, cell suppression and oxidative stress generation induced by metal oxide nanoparticles in onion (Allium cepa) bulb. Metallomics, 10(9), pp.1315-1327.

Bakare, A.A., Mosuro, A.A. and Osibanjo, O., 2000. Effect of simulated leachate on chromosomes and mitosis in roots of Allium cepa(L.). Journal of Environmental Biology, 21(3), pp.263-271.

Bakare, A.A., Mosuro, A.A. and Osibanjo, O., 2000. Effect of simulated leachate on chromosomes and mitosis in roots of Allium cepa(L.). Journal of Environmental Biology, 21(3), pp.263-271.

Cabrera, G.L. and Rodriguez, D.M.G., 1999. Genotoxicity of soil from farmland irrigated with wastewater using three plant bioassays. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 426(2), pp.211-214.

Fiskesjö, G. 1985. The Allium test as a standard in environmental monitoring. Hereditas 102(1): 99-112.

Fiskesjo, G., 1997. Allium test for screening chemicals; evaluation of cytological parameters. Plants for environmental studies, 11, pp.307-333.

Herrero, O., Martín, J.P., Freire, P.F., López, L.C., Peropadre, A. and Hazen, M.J., 2012. Toxicological evaluation of three contaminants of emerging concern by use of the Allium cepa test. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 743(1-2), pp.20-24.

Iqbal, M. and J. Nisar (2015). "Cytotoxicity and mutagenicity evaluation of gamma radiation and hydrogen peroxide treated textile effluents using bioassays." Journal of Environmental Chemical Engineering 3(3): 1912-1917.

Jiang, X., Chen, H., Liao, Y., Ye, Z., Li, M. and Klobučar, G., 2019. Ecotoxicity and genotoxicity of polystyrene microplastics on higher plant Vicia faba. Environmental Pollution, 250, pp.831-838.

Kim, H.K., Choi, Y.H. and Verpoorte, R., 2010. NMR-based metabolomic analysis of plants. *Nature protocols*, *5*(3), pp.536-549.

Kumari, M., Mukherjee, A. and Chandrasekaran, N., 2009. Genotoxicity of silver nanoparticles in Allium cepa. Science of the total environment, 407(19), pp.5243-5246.

Kumari, M., Mukherjee, A. and Chandrasekaran, N., 2009. Genotoxicity of silver nanoparticles in Allium cepa. Science of the total environment, 407(19), pp.5243-5246.

Kumari, M., Mukherjee, A. and Chandrasekaran, N., 2009. Genotoxicity of silver nanoparticles in Allium cepa. Science of the total environment, 407(19), pp.5243-5246.

Leme, D.M., Angelis, D.F., and Marin-Morales, M.A., 2008. Action mechanisms of petroleum hydrocarbons present in waters impacted by na oil spill on the genetic material of Allium cepa root cells. Aquatic Toxicol. 88, 214–219.

Pang, Z., Lu, Y., Zhou, G., Hui, F., Xu, L., Viau, C., Spigelman, A.F., MacDonald, P.E., Wishart, D.S., Li, S. and Xia, J., 2024. MetaboAnalyst 6.0: towards a unified platform for metabolomics data processing, analysis and interpretation. Nucleic Acids Research, p.gkae253.

Patnaik, A.R., Achary, V.M.M. and Panda, B.B., 2013. Chromium (VI)-induced hormesis and genotoxicity are mediated through oxidative stress in root cells of Allium cepa L. Plant growth regulation, 71(2), pp.157-170.

Rank, J. and M. Nielsen (1994). "Evaluation of the Allium anaphase-telophase test in relation to genotoxicity screening of industrial wastewater." Mutation Research/Environmental Mutagenesis and Related Subjects 312(1): 17-24.

Sharma, S. and A. P. Vig (2012). "Genotoxicity of atrazine, avenoxan, diuron and quizalofop-P-ethyl herbicides using the Allium cepa root chromosomal aberration assay." Terrestrial and Aquatic Environmental Toxicology 6(2): 90-95.

Sivaram, A.K., Logeshwaran, P., Surapaneni, A., Shah, K., Crosbie, N., Rogers, Z., Lee, E., Venkatraman, K., Kannan, K., Naidu, R. and Megharaj, M., 2021. Evaluation of Cyto-genotoxicity of Perfluorooctane Sulfonate (PFOS) to Allium cepa. Environmental Toxicology and Chemistry, 40(3), pp.792-798.

Smaka-Kincl, V., Stegnar, P., Lovka, M. and Toman, M.J., 1996. The evaluation of waste, surface and ground water quality using the Allium test procedure. Mutation Research/Genetic Toxicology, 368(3-4), pp.171-179.

Smaka-Kincl, V., Stegnar, P., Lovka, M. and Toman, M.J., 1996. The evaluation of waste, surface and ground water quality using the Allium test procedure. Mutation Research/Genetic Toxicology, 368(3-4), pp.171-179.

Teixeira, R.D.O., Camparoto, M.L., Mantovani, M.S. and Vicentini, V.E.P., 2003. Assessment of two medicinal plants, Psidium guajava L. and Achillea millefolium L., in in vitro and in vivo assays. Genetics and Molecular Biology, 26(4), pp.551-555.

Appendix B

UNLOCKING THE POWER OF ENZYMES TO ELIMINATE POTENTIALLY TOXIC CYANOBACTERIA

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KEYWORDS

Enzymes, Cyanobacteria, Cyanotoxins, Microcystis, Water Quality, Physiochemical, Biological, Ecosystems, Wastewater Treatment, Waterzyme

EXECUTIVE SUMMARY

The application of a novel enzymatic aqueous solution called 'Waterzyme' was assessed at Singleton Wastewater Treatment Plant (SWWTP) to estimate the reduction of Total Cyanophyta and Microcystis. The secondary objective was to monitor water quality and aquatic species parameters in the pond and receiving environment to determine the effect of this application. Total Cyanophyta and Microcystis had a 95% and 96% overall removal rate respectively, after application of Waterzyme. Analysis of water quality and aquatic species data revealed Waterzyme only targets the toxic algae species, not affecting the other beneficial organisms.

INTRODUCTION

Cyanobacteria originated around 3 billion years ago and they are known as blue green algae, but strictly speaking they are not algae. The name comes from the distinct cyan (blue- green) hue of their accessory pigment phycocyanin. They are oxygen-producing bacteria that use sunlight as an energy source to convert carbon dioxide into biomass. Cyanobacteria can form dense blooms and common bloom-forming genera include: Aphanizomenon, Cylindrospermopsis, Dolichospermum, Microcystis, Nodularia, Planktothrix, and Trichodesmium.

Cyanobacteria affect water quality by depleting oxygen, resulting in hypoxia and anoxia. They produce a variety of cyanotoxins, which are the secondary metabolites produced by Cyanobacteria. The toxins produced by cyanobacterial blooms is highly variable in space and time. Cyanobacterial blooms often consist of mixtures of toxic and non-toxic strains. Changes in strain composition can therefore cause major alterations in toxin content and composition.

Cyanobacterial blooms constitute a major global environmental problem because of significant risk to public health and aquatic ecological systems. Current physicochemical treatments of toxic cyanobacteria cause the significant release of cyanotoxin from damaged cells. Biological control is a promising eco-friendly technology to manage harmful cyanobacteria and cyanotoxins. Waterzyme is an efficient nature-based enzymatic solution developed using 100% natural ingredients.

METHODOLOGY

The tertiary wastewater treatment plant at Singleton has been experiencing harmful algae blooms (HAB), routinely monitored through EPA sample point 3. The following methodology was completed to determine the effectiveness of Waterzyme on this HAB and other key water quality and aquatic species parameters in the receiving environment.

- The tertiary pond is around 30ML in capacity, 9 days of travel time, constant inflow and outflow.
- Recommended dose of Waterzyme is 1L per 250,000L of contaminated water, with dosing adjusted to account for constant outflow.
- Around 150L of Waterzyme applied at the front end, in line with the water-flow.
- Singleton Council has a weekly sampling and analysis program with the help of AECOM and ALS Pty Ltd.

- A pre-treatment sample was taken on 11th April, Waterzyme applied on 12th April, followed by weekly sampling and analysis as per Singleton Council routine analysis program.
- Independent testing by ALS on 17th April, 26th April and 1st May 2023

RESULTS

Initial testing by ALS at SWWTP on 11th April 2023 determined Total Cyanophyta at 143,000 cells/ml, dominated by Microcystis at 138,000 cells/ml.

Waterzyme was applied on the 12th April 2023 and independent monitoring results on 17th April 2023 presented the Total Cyanophyta and Microcystis dropped to 20,800 cells/ml and 18,400 cells/ml, respectively. Results on 26th April 2023 displayed the toxic cyanophytes dropped to 5,070 cells/ml (Microcystis spp.). Results on 1st May 2023 showed the toxic cyanophytes dropped further to 4,950 cells/ml (Microcystis spp.). Figure 1 displays the full results showing Total Cyanophyte and Microcystis was significantly reduced following the application of the product.

Water quality parameters as well as physicochemical and biological parameters improved or were not affected, as per Table 1. Diatoms increased to compete with the cyanoblooms and supported other non-toxic microbes, as shown in Figure 2.

CONCLUSION

It is known that the lysis of cyanobacteria during the bloom kill results in the significant release of cyanotoxins into the surrounding water. When applied, Waterzyme, eliminated both the toxic algae by directly attacking the algae cells and inhibited further blooms by encouraging the beneficial organisms to compete for the nutrients or excreting algicidal compounds.

The overall removal rate for Total Cyanophyta and Microcystis at SWWTP was 95% and 96% respectively. The real-field validation that Waterzyme is destroying Microcystis is proven using a tertiary pond at Singleton WTP. Waterzyme demonstrates consistent performance in various conditions, with validation by independent monitoring and analysis.

Water quality parameters as well as physicochemical and biological parameters revealed Waterzyme only targets the toxic algae species and does not affect the other beneficial organisms.

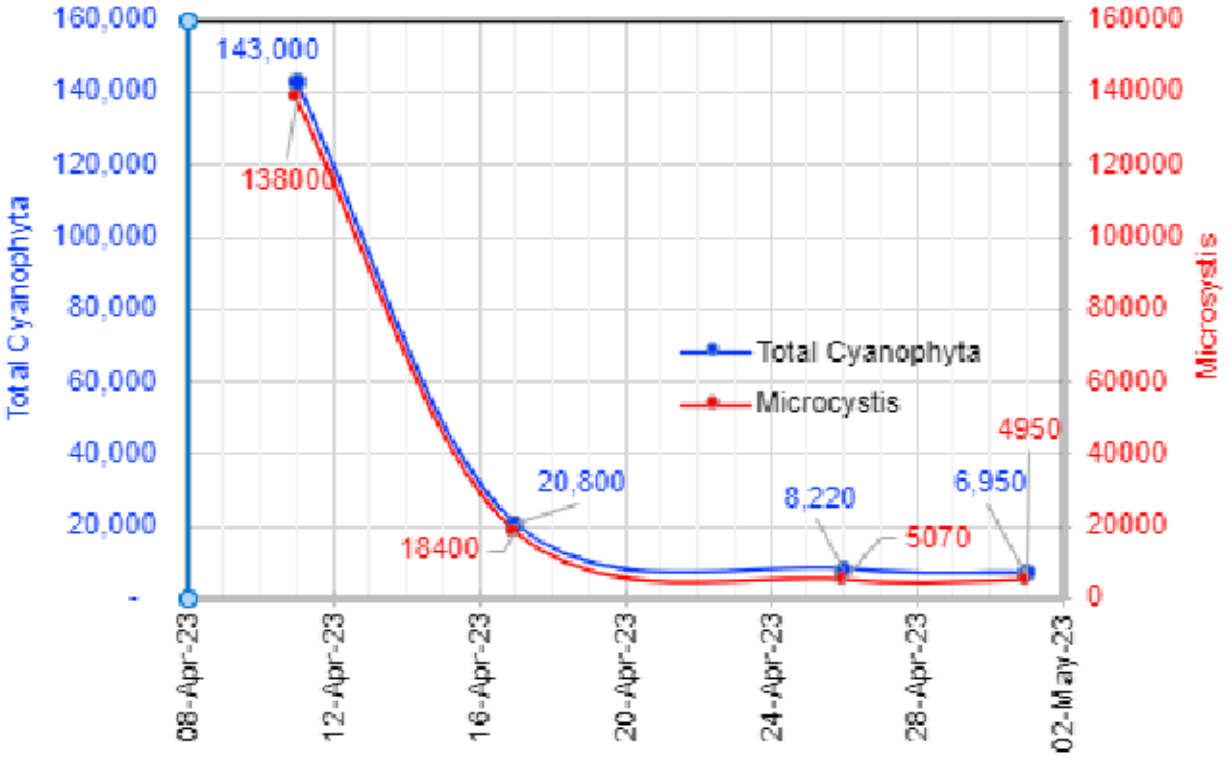

Figure 1 – Effect of Waterzyme on the Total Cyanophyta and Microcystis at SWWTP April/May 2023

Table 1 – Water Quality Parameters including Physicochemical and Biological Parameters.

			Untreated	Week 1	Week 2	Week 3	Week 6
Parameter	LOR	Unit	11-Apr-2	17-Apr-2	26-Apr-2	1-May-2	26-May-2
pH	0.01	pH Unit	8.08	7.81	7.64	7.58	8.08
Suspended solids	5	mg/L	17	8	9	10	24
UV transmission	0.01	%	47.5	50.6	53.9	53.9	49.8
Turbidity	0.1	NTU	8.9	6.5	5	6	9.6
Ammonia	0.01	mg/L	4.21	2.97	5.51	4.21	0.39
Nitrite	0.01	mg/L	0.4	0.72	1.86	1.72	0.22
Nitrate	0.01	mg/L	0.7	1.74	0.79	1.56	4.17
Total N	0.1	mg/L	9.3	8.4	9.8	9.5	6.9
Total P	0.01	mg/L	4.21	3.73	3.53	3.63	4.63
Chlorophyll a	1	mg/m3	116	58	28	0	0
Total Oil and Grease	2	mg/L	2	<2	<2	<2	<2
BOD	2	mg/L	6	3	9	12	2
Faecal Coliforms	1	CFU/100ml	110	76	87	340	<20
Escherichia coli	1	CFU/100ml	18	25	13	60	<20
Coliforms	1	CFU/100ml	2300	4000	1500	6000	5500
Enterococcus	1	CFU/100ml	<2	<2	<2	31	<2
Total Bacillariophytes	5	cells/ml	9120	1650	2300	4560	52800
Chlorophytes (Green Algae)							
Actinastrum spp.	5	cells/ml	6200	1300	175	325	75
Ankistrodesmus spp.	5	cells/ml	NA	NA	NA	NA	NA
Chlamydomonas spp.	5	cells/ml	NA	NA	NA	NA	25
Closterium spp.	5	cells/ml	NA	NA	NA	NA	25
Crucigenia spp.	5	cells/ml	3650	350	400	100	1680
Desmodesmus spp.	5	cells/ml	300	200	100	25	75
Dictyosphaerium spp.	5	cells/ml	11500	4650	2780	6050	1250
Elakatothrix spp.	5	cells/ml	125	175	50	550	NA
Eudorina spp.	5	cells/ml	325	NA	675	NA	NA
Kirchneriella spp.	5	cells/ml	6330	2800	2230	2820	825
Micractinium spp.	5	cells/ml	1500	1440	NA	NA	NA
Monoraphidium spp.	5	cells/ml	550	300	550	175	1440
Oocystis spp.	5	cells/ml	2840	100	1050	875	125
Other green cells	5	cells/ml	12300	6200	6750	7500	NA
Pediastrum spp.	5	cells/ml	400	NA	NA	NA	NA
Scenedesmus spp.	5	cells/ml	850	550	NA	50	250
Sphaerocystis spp.	5	cells/ml	800	200	25	200	1980
Total Chlorophytes	5	cells/ml	47700	19200	14800	19000	7780
Cyanophytes							
Arthrospira spp.	5	cells/ml	4630	1750	1400	750	NA
Merismopedia spp.	5	cells/ml	NA	NA	NA	NA	NA
Microcystis spp. (PTP)	5	cells/mL	138000	18400	5070	4950	40
Planktothrix spp. >5 µm	5	cells/ml	400	NA	NA	NA	NA
Pseudanabaena spp.	5	cells/ml	NA	NA	NA	90	200
Total Chroococcales	5	cells/ml	138000	19000	5070	4950	1040
Total Oscillatoriales	5	cells/ml	5030	1750	3150	200	200
Total Cyanophytes	5	cells/ml	143000	20800	8220	6950	1240
Total Potentially Toxic C	5	cells/ml	138000	18400	5070	4950	40
Flagellates-Cryptophytes							
Chroomonas spp.	5	cells/ml	1590	550	350	275	325
Cryptomonas spp.	5	cells/ml	5400	3500	1170	175	200
Flagellates-Euglenophytes							
Euglena spp.	5	cells/ml	75	50	0	125	25
Phacus spp.	5	cells/ml	0	20	0	0	0
Flagellates-Pyrrophytes							
Other dinoflagellates	5	cells/ml	10		0	0	
Peridinium spp.	5	cells/ml	5	50	100	50	0
Total Flagellates	5	cells/ml	7080	4170	1620	625	550
Centritractus spp.	5	cells/ml	5	0	0	0	0
Mallomonas spp.	5	cells/ml	275	50	0	0	25
Total Chrysophytes	5	cells/ml	280	50	0	0	25
Total Algae Count	5	cells/ml	207000	45800	26900	31100	62400

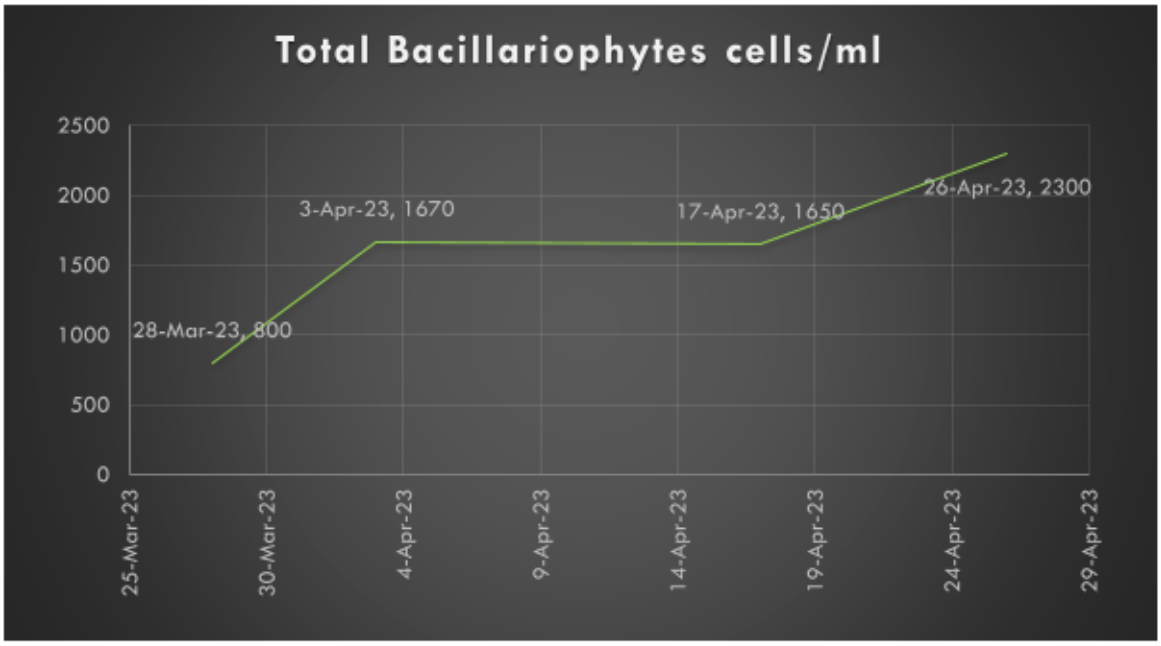

Figure 2 – Total Bacillariophytes (cells/ml)