Toxicity of Thiobencarb on Green Freshwater Alga and the Mitigating Role of Ozone

Amira Ali [1]*, Nabila S. Ahmed[1], Salwa M. Abdallah[2], Maher E. Saleh[3], Khaled A. Osman[1]

ABSTRACT

Toxicity of the formulated thiobencarb (50%, EC) on the growth of green freshwater alga, *Pseudokirchneriella subcapitata* was evaluated after 96 hours, both individually and in combination with a concentration of 100 ppm of ozone. The effective median concentrations (EC_{50}) were determined to be 0.004 for thiobencarb and 0.9 ppm for thiobencarb in combination with ozone (O_3). Algal biomass, growth rate, growth inhibition percentage, algal growth response, and the rate of division/day were decreased, while the generation time was increased in a concentration-dependent manner. Biochemical analysis of EC_{50} concentration of thiobencarb alone on microalga showed a reduction in protein, carbohydrate, and pigments (chlorophyll-a, chlorophyll-b, and carotenoids). In contrast, there was an increase in the activity of catalase, ascorbate peroxidase, superoxide dismutase and reduced glutathione content, lipid peroxidation, sucrose and free proline. However, when EC_{50} concentration of thiobencarb was in combination with 100 ppm of O_3, most of these biomarkers showed improvement, indicating that the ozone treatment can mitigate the adverse effects of thiobencarb. Microalga can serve as bioindicators for this herbicide toxicity in water, while the measured biochemical parameters may be candidates for biomarkers for thiobencarb exposure in microalga. Additionally, the use of O_3 as an eco-friendly technology is recommended for reducing thiobencarb contamination in water bodies.

Key words: bioindicator; biomarkers; degradation; *Pseudokirchneriella subcapitata*.

INTRODUCTION

Rapid industrialization and the intensive use of pesticides in agriculture have led to the release of significant amounts of pollutants into aquatic environments (Narayanan *et al.,* 2024). Currently, over 4,000,000 tons of pesticides are used globally each year, resulting in concentrations that exceed threshold limits in water bodies due to agricultural runoff (Rad *et al.*, 2022). Moreover, application of synthetic pesticides in various tropical rice fields has negatively impacted soil flora and fauna (Dutta and Baruah, 2020). The presence of multiple pesticide residues in aquatic ecosystems can have harmful effects on aquatic organisms and ultimately on human health (Eissa *et al*., 2021).

Currently, there are over 17,000 different herbicide products available in the global market, with annual consumption exceeding $30 billion (Gonzalez-Rey *et al*., 2015 and Smedbol *et al*., 2018). However, only 10-30% of these herbicides can be absorbed by target plants or soil particles. The majority of these chemicals find their way into groundwater or surface water, including streams and lakes (Buma *et al*., 2009 and Dupraz *et al*., 2018).

The microalga *Pseudokirchneriella subcapitata*, currently named *Raphidocelis subcapitata* and formerly known as *Selenastrum capricornutum*, is a planktonic species found in freshwater ponds, lakes and rivers (Machado and Soares, 2024). It is frequently included among the species used in bioassay batteries for hazard assessment of chemically contaminated waste, as recommended by several international organizations (Pablos *et al*., 2009). Additionally, moreover, monitoring biomarkers in organisms living within ecosystems can reflect environmental stressors that may impact the aquatic phytoplankton community (Huschek & Hansen, 2006 and Abd-Allah *et al.*, 2012). These stressors can induce the production of reactive oxygen species (ROS) within the cells (Marshall & Newman, 2002; Dröge, 2003 and Halliwell & Gutteridge, 2007) leading to alterations in the structure and function of organs, systems, specialized transport mechanisms, and gene expression (Ames *et al*., 1993 and Apel & Hirt, 2004).

Thiobencarb (S-4-chlorobenzyl diethyl thiocarbamate) is widely used in modern agricultural practices to control barnyard grass in paddy rice fields. The recommended field application rate, in terms of active ingredients, is approximately 40 mg/l for a 10-cm deep paddy (Beste *et al*., 1983). Unfortunately, Egypt is currently experiencing an annual water deficit of around seven billion cubic meters, with many agricultural areas dedicated to rice farming (UNICEF, 2021). The use of

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DOI: 10.21608/asejaiqjsae.2024.392380
[1]Department of Pesticide Chemistry & Technology, Faculty of Agriculture, Alexandria University, Alexandria, P.O 21545, Egypt.
[2] Department of Mammalian and Aquatic Toxicology.
Central Agricultural Pesticide Laboratory (CAPL),
Agricultural Research Center (ARC), Doki 12168, Giza, Egypt.
[3]Department of Soil and Water Sciences, Faculty of Agriculture,
Alexandria University, Alexandria, P.O 21545, Egypt.
*Email: amiraali551992@gmail.com
Received, October 15, 2024, Accepted November 18, 2024.

herbicides like thiobencarb in rice fields facilitates their entry into river water, as they can easily be released through direct application over ponded water, rainfall, runoff, or discharges during water depth management (Matsui *et al*., 2006). Thiobencarb functions as a selective herbicide, being absorbed by the coleoptile, mesocotyl, roots, and leaves to provide early post-emergence control of *Echinochloa*, *Leptochloa*, and *Cyperus* spp., and other monocotyledonous and annual broadleaf weeds in both direct-seeded and transplanted rice (Tomlin, 2002). In Egypt, it is commonly used in rice fields to control broadleaf weeds, grasses, and sedges (Abbas *et al*., 2007 and El-Shahway *et al*., 2015).

Various methods have been employed for the degradation of pesticides, including ultrasonic waves (Zhang *et al*., 2011), biodegradation (Cycoń *et al*., 2009), oxidation via anodic Fenton (Wang and Lemley, 2002), UV/H_2O_2 (Shemer and Linden, 2006), photocatalytic degradation (Daneshvar *et al*., 2007 and Merabet *et al*., 2009), and ozonation (Kouloumbos *et al*., 2003; Maldonado *et al*., 2006; Wu *et al*., 2009 and Tansu *et al*., 2021). The application of thiobencarb leads to its residues in surface water, making it crucial to evaluate the adverse effects this herbicide may have on non-target organisms in aquatic ecosystems (Peterson *et al*., 1994). Consequently, the present study aimed to assess the toxicity and biochemical effects of thiobencarb on the green alga, *Pseudokirchneriella subcapitata* as bioindicators due to its significance as a primary producer in freshwater systems. Additionally, the study explored the effectiveness of ozone treatment (O_3) as a simple, safe, and environmentally friendly method to mitigate the side effects of thiobencarb on these green alga.

MATERIAL AND METHODS

The green alga species, *Pseudokirchneriella subcapitata*, were sourced from the Central Agricultural Pesticides Lab (CAPL), Egypt, and cultured according to the recommend guidelines (EPA, 2002 and OECD, 2006). The formulated herbicide, thiobencarb (50%, EC, Saturn®), was acquired from Kafr El-Zayat Pesticides & Chemicals (KZ), Egypt. In accordance with OECD and EPA guidelines, the toxicity of thiobencarb to green alga was evaluated (EPA, 2002 and OECD, 2006). An algal culture with a specific density of 1 x 10^4 cells/mL was exposed for 96 hours to ozonated thiobencarb concentrations ranging from 0.001 to 10 ppm, (100 ppm at an air flow rate of 2.5 L/min and an ozone output of 32 mg/h) and non-ozonated thiobencarb concentrations ranging from 0.001 to 10 ppm. Ozone was generated using a corona discharge system (Xetin Ozone Air & Water Purifier, Model XT 301, Taiwan). Algal biomass/mL, the percentage of growth rate inhibition, the algal growth response, the rate of division/day, and the generation time after exposure were all measured as indicators of thiobencarb toxicity on the alga. Based on probit analysis, statistical parameters were determined with 95% confidence limits (Finney, 1971). A thiobencarb EC_{50} concentration was compared to the same concentration treated with O_3 to evaluate its effects on specific biomarkers in the microalga. Following 96 hours of exposure, the biomass was centrifuged using a CU-5000 centrifuge (Damon/IEC division) at 2500 rpm for 10 minutes and the obtained pellets were suspended in distilled water (1:10 w/v) for the determination of proteins, superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase. For the lipid peroxidation assay, 400 mg of fresh algal biomass was homogenized in 1.5 mL of trichloroacetic acid (TCA). For the determination of algal pigments, including chlorophyll-a, chlorophyll-b, and carotenoids, 1 gram of algal biomass was homogenized in 50 mL of methanol. For the sucrose assay, 10 mg of fresh algal biomass was homogenized in 10 mL of ethanol. In case of free proline determination, 500 mg of fresh algal biomass was homogenized in 10 ml of sulphosalicylic acid. The reduced glutathione (GSH) content was assessed by homogenizing 1 gram of fresh algal biomass in five volumes of 5% TCA-1 mM. For carbohydrate determination, 1 mg of algal biomass was homogenized in 1.25 mL of distilled water and 4 mL of anthrone reagent (0.2% w/v). Algal pigments (chlorophyll-a, chlorophyll-b, and carotenoids) were measured according to Dere *et al*. (1998), while carbohydrate content was estimated following the method in Stainer *et al*. (1971). The contents of osmolytes, such as sucrose and free proline, were determined using the procedures described in Victor *et al*. (2011) and Bates *et al*. (1973), respectively. Total protein in of alga was assayed using bovine serum albumin as standard (Lowry *et al*. 1951). Enzyme activities of catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were determined following the methods in Beers & Sizer (1952), Nakano & Asada (1981) and Winterbourn *et al*. (1975), respectively. Reduced glutathione content (GSH) in the alga was measured using the method outlined in Sedlak and Lindsay (1968).

The Costat statistics package (Costat, 1986) was used for all chemometric calculations. Three replicates of each treatment were administered. The data was analyzed using ANOVA and presented as the mean ± standard deviation (SD). The statistical significance level was set at 0.05 or less for the probability value.

RESULTS AND DISCUSSION

1. Toxicity of thiobencarb alone and in combination with ozone on the growth of green fresh water alga:

The percentages of algal growth rate inhibition and generation time/day increased, while algal biomass, growth rate, algal growth response, and the rate of division/day decreased in a dose-dependent manner following exposure to thiobencarb concentrations ranging from 0.001-0.007 ppm for 96 hours (Table 1), indicating its toxicity to alga in water. These findings align with those of several researchers who have reported that thiobencarb in water reduces cell count, growth rates, and algal biomass yield (Battah *et al*., 2001and Eladel, 2010).

In Table (2), the alga treated with ozone exhibited greater tolerance to thiobencarb, with concentrations ranging from 0.001 ppm to 10 ppm applied to assess its effects. The calculated EC_{50} values after 96 hours were 0.004 ppm for thiobencarb alone and 0.9 ppm when in combination with ozone, indicating that ozone reduced the toxicity of thiobencarb by 225-fold. This suggests that ozone can be utilized to treat water contaminated with thiobencarb, as it may degrade the compound. Ozonation has been shown to effectively degrade 60-99% of diazinon, parathion, methyl-parathion, and cypermethrin within 30 minutes, with the degradation rate being highly dependent on the amount of dissolved ozone (Wu *et al*., 2007). Ozone has therefore been widely used for water treatment due to its efficacy as a disinfectant and its ability to eliminate harmful pesticides (Aidoo *et al*., 2023). This study emphasizes that ozone treatment could mitigate thiobencarb toxicity, making it a viable option for cleanup efforts following pesticide application in aquatic environments.

Table 1. Dose-response of thiobencarb on the growth of green freshwater alga, *P. subcapitata* after 96 hours of exposure

Concentration (ppm)	Algal biomass/ mL	Growth rate (µ)	Growth inhibition (%)	Algal growth response	Rate of division/ day	Generation time/day
Control	4.51×10^6	1.53	0.0	6.65	2.21	0.45
0.001	1.25×10^6	1.21	20.91	6.10	1.75	0.57
0.003	0.76×10^6	1.08	29.41	5.88	1.56	0.64
0.005	0.42×10^6	0.93	39.21	5.62	1.34	0.75
0.006	0.10×10^6	0.57	62.74	5.00	0.82	1.22
0.007	0.05×10^6	0.40	73.86	4.70	0.58	1.72
0.01	0	0	100	0	0	0
EC_{50} = 0.004 ppm						

Table 2. Dose-response of thiobencarb in combination with ozone on the growth of freshwater alga, P. *subcapitata* after 96 hours of exposure

Concentration (ppm)	Algal biomass/ mL	Growth rate (µ)	Growth inhibition (%)	Algal growth response	Rate of division/day	Generation time/day
Control	3.03×10^6	1.43	0.00	6.48	2.06	0.48
0.001	2.63×10^6	1.39	2.80	6.42	2.01	0.50
0.005	2.44×10^6	1.37	4.19	6.39	1.98	0.51
0.01	1.98×10^6	1.32	7.70	6.30	1.90	0.53
0.05	0.78×10^6	1.09	23.78	5.89	1.57	0.64
0.1	0.25×10^6	0.80	44.06	5.40	1.15	0.87
0.5	0.19×10^6	0.74	48.25	5.28	1.07	0.93
1	0.15×10^6	0.68	52.45	5.18	0.98	1.02
5	0.11×10^6	0.60	58.04	5.04	0.87	1.15
10	0.05×10^6	0.40	72.03	4.70	0.58	1.72
EC_{50} = 0.90 ppm						

2. *In vivo* effects of thiobencarb alone and in combination with ozone on *P. subcapitata biomarkers*:

Data in Table (3) illustrate the effect of EC_{50} concentration for thiobencarb alone and thiobencarb in combination with ozone, on various biochemical parameters in green alga exposed for 96 hours. After exposure to thiobencarb alone or thiobencarb in combination with ozone, the alga had significantly reduced protein, carbohydrates, chlorophyll-a, chlorophyll-b, and carotenoids concentrations. The percentage reductions in these biomarkers for alga exposed to thiobencarb alone were greater than those for alga treated with thiobencarb in combination with ozone. These findings indicate that untreated thiobencarb has more pronounced biochemical toxic effects compared to thiobencarb in combination with ozone, suggesting that ozone treatment may mitigate the toxic effects of thiobencarb on microalga. This aligns with numerous studies that have shown those herbicides, including thiobencarb, diuron, neburon, monuron, fenuron, and S-metolachlor, decrease protein content in alga (Battah et al., 2001); carbohydrates (Gerald and Thomas, 1971); Chlorophyll-a (Wong & Chang, 1988 and Wang et al., 2019). Chlorophyll-b (Maronic et al., 2018) and carotenoids (Maoka, 2020). The chlorophyll found in green alga is known to be essential for absorbing solar energy for the photochemical reactions of photosynthesis (Edarous, 2011; Salem, 2016 and Shymanska et al., 2017). Carotenoids function as antioxidants, protecting chlorophyll from photo-oxidation. When carotenoids are affected, chlorophyll can be indirectly damaged as well (Viljanen et al., 2002). Exposure to herbicides can disrupt these reaction centers, leading to imbalances in chlorophyll and an increase in reactive oxygen species (ROS) production, which triggers oxidative stress in cellular macromolecules (Dyer et al., 2008). In this study, exposure to thiobencarb negatively impacted photolysis processes, resulting in a reduction in chlorophyll content. Therefore, both chlorophyll and pigment levels can serve as effective biomarkers for thiobencarb exposure.

Data in Table (3) demonstrate that when microalga is continuously exposed to abiotic stress conditions, such as thiobencarb alone and thiobencarb in combination with ozone, the levels of sucrose and proline significantly increase as a defensive response to hyperosmotic stress. Under such conditions, cells often accumulate various metabolites, including sugars and proline (Mattioli et al., 2009; Bremauntz, 2011 and Barera & Forlani, 2023). Proline levels rose by 1.87-fold with thiobencarb alone and by 1.84-fold when in combination with ozone, while sucrose content increased to a lesser extent, showing a rise of 1.19-fold with thiobencarb alone and 1.07-fold with ozone. This indicates that the treated alga exhibited heightened proline and sucrose responsiveness, serving as protective osmolytes and potential biomarkers of thiobencarb toxicity. The increase in osmolytes in the presence of thiobencarb suggests their role in scavenging free radicals (Habib et al., 2011). Conversely, the reduction in either proline or sucrose levels may be attributed to the ozone treatment, which can decrease thiobencarb concentration through degradation and alleviate its toxic effects on alga, highlighting ozone's potential as a tool for water treatment.

Table 3. *In vivo*, effect of EC_{50} thiobencarb alone and in combination with ozone on some biomarkers of microalga

Parameter	Control	Thiobencarb alone	Thiobencarb in combination with ozone
Protein (mg/g fresh weight)	168.49±1.17c	148.73±0.84a	156.67±0.95b
Carbohydrates (mg/g fresh weight)	33.84±0.08c	26.96±0.11a	29.56±0.09b
Chlorophyll-a (µg/g fresh weight)	5.60±0.03c	4.31±0.11a	4.72±0.06b
Chlorophyll-b (µg/g fresh weight)	13.97±0.11c	11.07± 0.29a	12.29±0.30b
Carotenoids (µg/g fresh weight)	0.96±0.08b	0.63±0.06a	0.72±0.06a
Sucrose (mg/g fresh weight)	35.10±0.08a	41.73±0.21c	37.58±0.58b
Free proline (mg/g fresh weight)	0.68±0.002a	1.29±0.007c	1.27±0.007b

Data are expressed as mean ± S.D (n= 3). Means within the same raw and having the same letter are not significantly different from each other, $p \leq 0.05$.

Table 4. *In vivo*, effect of EC_{50} thiobencarb alone and in combination with ozone on some antioxidant enzymes and components

Parameter	Control	Thiobencarb alone	Thiobencarb with ozone
CAT (U/mg protein)	2.49±0.08a	4.76±0.14b	4.63±0.08b
APX (U/mg protein)	24.73±1.22a	48.68±1.30b	47.87±2.28b
SOD (U/mg protein)	1.8 ±0.11a	3.06±0.13b	2.76±0.21b
GSH (µmole/mg protein)	49.05±0.43a	56.36±0.62c	50.22±0.66a
Lipid peroxidation (mM/g fresh weight)	0.84±0.005a	1.02±0.004c	0.95±0.008b

Data are expressed as mean ± S.D (n= 3). Means within the same raw and having the same letter are not significantly different from each other, $p \leq 0.05$.

3. *In vivo*, effect of thiobencarb alone and in combination with ozone on biomarkers of oxidative stress in alga, *P. subcapitata*:

Data presented in Table (4) illustrate the effects of exposure to the EC_{50} concentration of thiobencarb and subsequent treatment with ozone over 96 hours on various antioxidant enzymes. The activities of CAT, APX, and SOD in the alga *P. subcapitata* was significantly elevated, showing increases of 191, 197, 116% of control for thiobencarb alone, and 186, 194, and 150 % of control for thiobencarb in combination with ozone, respectively. In regard to the non-enzymatic components of antioxidative defense system, GSH levels significantly increased following exposure to thiobencarb alone, with a non-significant increase noted when thiobencarb in combination with ozone. Lipid peroxidation levels also rose significantly in response to thiobencarb exposure, whether alone or with ozone. However, in all cases, the levels observed in the presence of ozone were relatively lower compared to those with thiobencarb alone, indicating that ozone treatment ameliorated these effects. The increase in GSH content and ROS levels indicates that these molecules were activated in response to thiobencarb exposure. SOD, a metalloprotein, serves as the first line of defense against oxidative stress by catalyzing the dismutation of superoxide radicals into O_2 and H_2O_2 (Kangralkar *et al*., 2010). While CAT and APX enzymes are essential enzymes that primarily function to convert H_2O_2 into H_2O and O_2 (Nandi *et al*., 2019). Also, GSH plays a crucial role in adjusting the redox potential of amino acids and proteins, scavenging oxidative damage, acting as a non-specific reductant, serving as a substrate or cofactor for enzyme-catalyzed reactions, reconstructing protein disulfide bonds, and suppressing H_2O_2 and organic peroxides (Tan and Spivack, 2009). Research has documented the impact of xenobiotics, such as herbicides, on lipid metabolism and the sensitivity of fatty acid profiles to alterations in the homeostasis of organisms (Ana *et al*., 2021). The current study aligns with findings from Wang *et al*. (2019), which reported that levels of protein adducts with the reactive aldehyde 4-hydroxy-2-nonenal (HNE), the end-product of lipid peroxidation, were significantly elevated in cells of unicellular green microalga *Parachlorella kessleri* treated with the herbicide S-metolachlor (S-MET). This suggests that the antioxidant mechanisms of the unicellular green microalga *Parachlorella kessleri* are insufficient against persistent lipid damage. Additionally, the results are consistent with another study Mohanty and Jena (2019) that highlighted how ozone treatment can oxidize the molecular structures of the Chloroacetanilide class of herbicides, such as acetochlor, alachlor, and butachlor, enhancing their biodegradability in aqueous environments and reducing their toxicity to aquatic organisms.

CONCLUSION

Thiobencarb demonstrates toxicity to the tested microalga across various concentrations, resulting in decreased algal biomass, growth rate, percentage of growth inhibition, algal growth response, and rate of division/day. Microalga can serve as effective bioindicators for assessing the toxicity of this herbicide in aquatic environments. When alga was exposed to 0.0004 mg/L of thiobencarb, protein, carbohydrate, and pigments (chlorophyll-a, b, and carotenoids) levels were decreased, whereas antioxidants enzymes and components were induced. These biochemical parameters may serve as valuable biomarkers for monitoring thiobencarb exposure in microalga. On the contrary, when thiobencarb was in combination with 100 ppm of O_3, there was a notable improvement in most of the biochemical biomarkers, suggesting that the ozonation process can effectively reduce the toxic effects of thiobencarb. This highlights the potential of ozone treatment as a green, eco-friendly technology for mitigating thiobencarb toxicity in contaminated water bodies.

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الملخص العربي

سمية الثيوبنكارب على طحالب المياه العذبة الخضراء ودور الأوزون في خفض سمية المبيد

أميرة علي عثمان علي سعد، نبيلة صابر أحمد ، سلوي مصطفي عبدالله، ماهر السيد صالح ، خالد أحمد عثمان

تم تقييم سمية مركب الثيوبنكارب على نمو الطحالب العذبة، *سودو كيركينيلا سابكابتاتا* بعد ٩٦ ساعة، سواءً بمنفرده أو معامل بالأوزون بتركيز ١٠٠ جزء في المليون. وتم تحديد التركيزات التي تثبط نمو الطحالب بنسبة خمسون في المائة بقيمة ٠,٠٠٤ جزء في المليون للثيوبنكارب و ٠,٩ جزء في المليون للثيوبنكارب المعامل بالأوزون. انخفضت الكتلة الحيوية للطحالب، معدل النمو، نسبة تثبيط النمو، استجابة النمو للطحالب، ومعدل الانقسام/اليوم، بينما زاد الزمن اللازم لإنتاج الجيل من الطحالب وذلك بدرجة تعتمد على تركيز الثيوبنكارب. أظهر التحليل البيوكيميائي لتركيز الثيوبنكارب بمفرده الذي يثبط نمو الطحالب بنسبة خمسون في المائة انخفاضًا في البروتين، الكربوهيدرات، والأصباغ (الكلوروفيل-أ، الكلوروفيل-ب، والكاروتينات)، وعلى النقيض من ذلك، زاد نشاط الكاتالاز، الأسكوربات بيروكسيداز، سوبر أوكسيد ديسميوتاز، محتوى الجلوتاثيون المختزل، بيروكسيد الدهون، السكروز، والبرولين الحر. وبالرغم من ذلك، عند معاملته بتركيز الثيوبنكارب المعامل بالأوزون الذي يثبط نمو الطحالب بنسبة خمسون في المائة، أظهرت معظم هذه المؤشرات الحيوية تحسنًا، مما يشير إلى أن المعاملة بالأوزن يمكن أن تخفف من الآثار السلبية للثيوبنكارب. يمكن أن تُستخدم الطحالب الدقيقة كمؤشرات حيوية لهذه السمية في الماء، وقد تكون المعايير البيوكيميائية المقاسة مرشحة كمؤشرات حيوية للتعرض للثيوبنكارب في الطحالب الدقيقة. بالإضافة إلى ذلك، يوصى باستخدام الأوزون كوسيلة صديقة للبيئة للحد من تلوث المياه بالثيوبنكارب.

الكلمات المفتاحية: مؤشرات حيوية، علامات بيولوجية، تحطم المبيد، سودوكيركرينلا سابكابيتاتا.