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A bioassay on tissue cholinesterase activity of *Rutilus kutum* (Kamensky, 1901) exposed to some common pesticides in Iran

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Abstract

The toxicological effects of three commonly used pesticides in Iran on the fries of Caspian kutum (*Rutilus kutum*, Kamensky, 1901) were investigated through determining 50.00% lethal concentration (LC_{50}) 96-hr and cholinesterase (ChE) inhibition. The LC_{50} 96-hr of carbaryl, glyphosate, and malathion were equal to 11.69, 6.64, and 0.97 mg L^{-1}, respectively, that were considered as harmful (10.00 - 100 mg L^{-1}), toxic (1.00 - 10.00 mg L^{-1}) and very toxic (< 1.00 mg L^{-1}) compounds for this species. The exposure of fries to sub-lethal concentrations of the pesticides over 15 days indicated that the average of ChE activity in the head and trunk were 1086.89 ± 124.34 and 627.36 ± 99.60 mU min^{-1} per mg protein, respectively, with a significant difference relative to each other. There was a significant difference between fry exposed to all three pesticides and the control group in cholinesterase inhibition. The fries exposed to carbaryl (890.12 ± 28.08 mU min^{-1} per mg protein) and glyphosate (891.77 ± 31.61 mU min^{-1} per mg protein) showed lower ChE inhibition than those exposed to malathion (790.00 ± 58.14 mU min^{-1} per mg protein).

Introduction

The southern Caspian region is one of the mega centers of agriculture in Iran where pesticides are commonly overused. Surplus pesticides reach the rivers and finally the Caspian Sea through run-off.[1] Pesticides are accumulated in the body of aquatic organisms causing biological responses, transfer to the higher trophic levels, and finally humans.[2,3] Malathion and carbaryl are two widely-used pesticides that are employed in orchards and paddy fields.[4-6] On the other hand. Glyphosate is an herbicide that is extensively used in the developing countries,[7,8] and it has a half-life of about 1-2 weeks and has been detected in many rivers.[9]

In toxicological studies, the lethal effects of compounds are investigated using bioassay and 50% lethal concentration (LC50) determination and help the researchers to study the effects of sub-lethal dosages.[10,11] Changes of cholinesterase (ChE) activity in various animal tissues are among the most important effects of organophosphate and carbamate pesticides, which have been used as a biomarker,[12,13] because they inhibit the enzyme irreversibly. The pesticides act as the enzyme's substrate.[13] The organophosphate pesticide detaches from cholinesterase so slowly that the enzyme is unable to hydrolyze the neural messenger, acetylcholine accumulates in the neural groove and neural transfer slows down.[2,14] The ChE as a biomarker of organophosphates, carbamates, and some herbicides have been used in numerous studies.[6,15-17] However, recently some evidence has demonstrated the influence of tissue and blood ChE to ion metals especially copper and mercury even up to 50.00% inhibition.[18]

The Caspian kutum (*Rutilus kutum*, Kamensky, 1901) is one of the most economic species of the southern Caspian. This anadromous species migrate into rivers in spring for reproduction. The Iranian fishery organization is being released Caspian kutum fries to restock its natural populations.[19] However, the decrease of annual catch hints at a high mortality rate of the fries in estuaries where they are released stemming from pollution originated in urbanization and agriculture.

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The present study aimed to find lethal (LC_{10}, LC_{50}, and LC_{90}) and sub-lethal dosages (based on cholinesterase activity) of malathion, carbaryl, and glyphosate in Caspian kutum fries. The findings of this research may estimate the success of releasing fry concerning the concentration of those pesticides in the environment.

Materials and Methods

Specimens and experimental conditions. One thousand fries of Caspian kutum (1.10 ± 0.40 g) were purchased from Shahid Rajaee aquaculture center (Sari, Iran), and were transferred to the aquaculture laboratory in Department of Fisheries and Environment Tehran University (Karaj, Iran). Then they were kept in two 1000 L round fiberglass tanks for 2-3 weeks for acclimation. Water temperature, dissolved oxygen, acidity, and total hardness were 20.00 ± 2.00 ˚C, > 7.00 ppm, 7.00, and 175 mg L^{-1}, respectively, during the experiment. The fries were fed at a rate of 2.00% of the body weight.

Lethal concentration toxicity test. Malathion (Arysta lifescience, Villebon-sur-Yvette, France), carbaryl (Hualong Chemical, Hangzhou, China), and glyphosate (Monsanto Co., St. Louis, USA) were used in the experiments. Preliminary experiments were used to determine the range of each pesticide for *R. kutum*. As an example, the specimens exposed to 9.00 ppm carbaryl showed no mortality, however, those exposed to 21.00 pm carbaryl all died. The following concentrations were applied: Malathion: 0.50, 1, 1.50, 2.00 and 2.50 ppm, Carbaryl: 9.00, 12.00, 15.00, 18.00 and 21.00 ppm and glyphosate: 4.00, 5.50, 7.00, 8.50 and 9.00 ppm. Effects and mean lethal concentration of the pesticides were determined using the organization of economic cooperation development's direction.[20] The bioassay of each pesticide was performed on 150 fries that were randomly distributed into 15 100-L fiberglass tanks (three replicates with 10 specimens per tank). The fries were not fed during the experiment. Each tank was controlled two times a day to record dead specimens at 24, 48, 72 and 96 hr after starting of the study.[21] "Maximum allowable toxicant concentration" (MATC) was calculated according to the value of 10.00% LC_{50} 96-hr and "lowest observed effect concentration" (LOEC) was equal to LC_{10} 96-hr.[22,23]

Sub-lethal concentration toxicity test. 360 fries were randomly allocated to nine 100-L fiberglass tanks (40 Fries tank-1). The experimental concentrations of the pesticides were determined based on 10.00% and 20.00% of 96-hr LC_{50},[24] i.e. Malathion: 0 (control), 0.15 and 0.30 ppm), Carbaryl: 0 (control), 1.20 and 2.40 ppm and glyphosate: 0 (control), 0.60 and 1.20 ppm). Each concentration had three replicates (triplicate). The fries were fed at a rate of 2.00% of the body weight. Feeding was stopped 24 hr before the start of the toxicity test. Ten percent of water was renewed every day with pesticides concentrations being set after the water exchange.

Sampling and preparation of supernatant. The experiments were performed for 15 days. On days 5, 10, and 15 after the start of the experiment, some specimens were selected randomly. The specimens after anesthesia with high doses (30 mg L^{-1}) of tricaine methanesulfonate (Sigma-Aldrich) complied with animal research rights described in ARRIVE guidelines,[25] were frozen in –70.00 ˚C and homogenized later using phosphate buffer solution (0.10 M, pH = 7.00 containing 1.00% TritonTM X-100). The homogenized mixture was centrifuged at 10,000 *g* for 15 min at 4.00 ˚C and the supernatant was used as the repository of the enzyme.[26]

Total protein and acetylcholinesterase activity. Total protein and specific activity of acetylcholinesterase of the fish head (from the snout to the back of operculum) and trunk (from the back of operculum to the tail), separately, were measured colorimetrically using an ELISA microplate reader (ELx808; BioTek, Winooski, USA) at 540 and 420 nm, based on Lowry *et al.*[27] and Ellman *et al.*[26] methods, respectively. In brief, for total protein, the stock bovine serum albumin (BSA) solution and Folin (Sigma-Aldrich) were used as standard curve and color reagent, respectively. For the enzyme measurement, the supernatant, the phosphate buffer solution, a color reagent, and acetylthiocholine iodide (Merck, Darmstadt, Germany), as the substrate was added to an Eppendorf tube and 100 µL of the mixture was transferred to the wells of microplate reader with the absorption per minute (OD min^{-1}).

Data analysis. Lethal concentrations were calculated using the Probit analysis[28] performed by POLO-PC 2002 software (version 1.0; Tehran University, Karaj, Iran). A comparison between overall the head and trunk-specific activity of the enzyme (ChE) has done by an independent *t*-test. The effects of pesticide concentration and exposure times on specific activity of the enzyme were examined using a two-way ANOVA followed by Duncan's multiple range test ($\alpha = 0.05$). All statistical analyses related to enzymatic testing data were accomplished in SPSS software (version 20.0, IBM, Chicago, USA).

Results

Lethal concentration toxicity tests. Mean LC_{10}, LC_{50}, and LC_{90} of malathion, carbaryl, and glyphosate for 24-96 hr have been presented in Table 1. With an increase in exposure time, lethal concentration (LC) was decreased. From LC_{10} to LC_{90}, the mean LC of the three pesticides were increased at all exposure times. With the increase in pesticides concentration, the mortality rate of fries was increased (Fig. 1).

According to the globally harmonized system of classification and labeling of chemicals for the aquatic environment,[29] malathion, glyphosate, and carbaryl are

Table 1. Mean LC10, LC50, and LC90 of malathion, carbaryl, and glyphosate for 24-96 hr measured on Rutilus kutum fries.

Toxicant	Lethal concentration	Exposure time (hr)			
		24	48	72	96
Malathion	LC_{10}	0.73	0.64	0.57	0.52
	LC_{50}	1.79	1.32	1.02	0.97
	LC_{90}	4.48	2.74	1.82	1.79
Carbaryl	LC_{10}	10.66	9.67	9.28	8.69
	LC_{50}	13.68	12.80	12.56	11.69
	LC_{90}	17.56	16.95	16.00	15.74
Glyphosate	LC_{10}	4.60	4.50	4.33	4.39
	LC_{50}	7.07	7.02	6.74	6.64
	LC_{90}	10.88	10.95	10.48	10.06

very toxic (<1.00 ppm), toxic (<10.00 ppm), and harmful (<100 ppm) for fries of *R. kutum*, respectively. Results of MATC, LOEC, and toxicity degree of each toxicant were shown in Table 2.

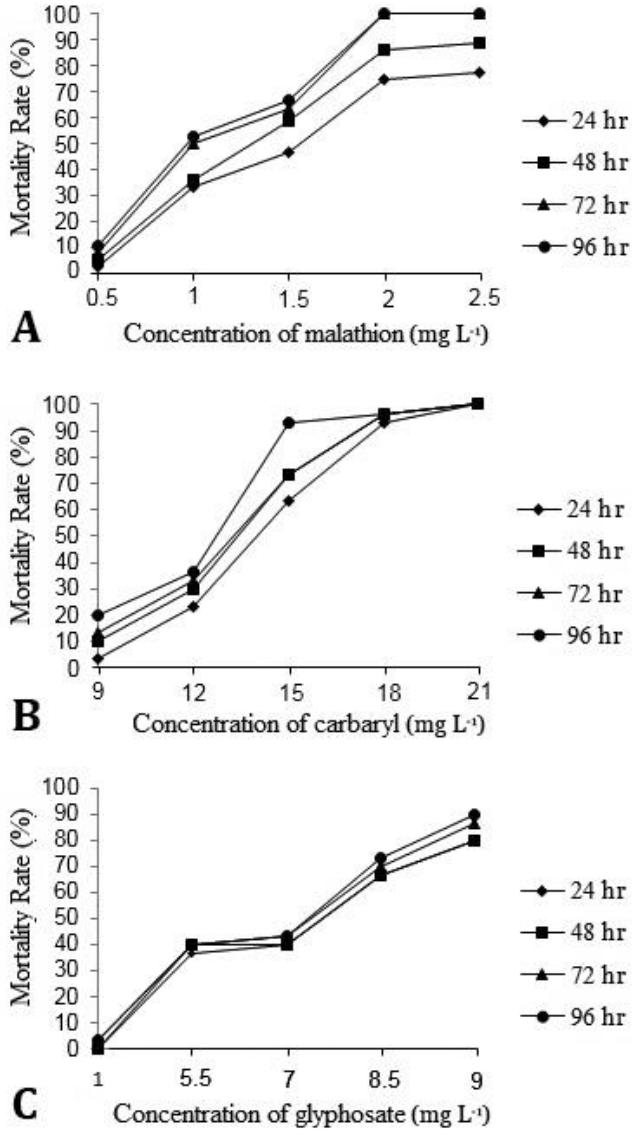

Fig. 1. The mortality rate of *Rutilus kutum* fries exposed to malathion (A), carbaryl (B), and glyphosate (C).

Table 2. The LC_{50} 96-hr, maximum allowable toxicant concentration (MATC), lowest observed effect concentration (LOEC) and the degree of toxicity calculated for malathion, carbaryl, and glyphosate of the Caspian kutum fries.

Toxicant	LC_{50} 96-hr (mg L^{-1})	MATC (mg L^{-1})	LOEC (mg L^{-1})	Toxicity class
Malathion	0.97	0.09	0.52	Very toxic
Carbaryl	11.69	1.16	8.69	Toxic
Glyphosate	6.64	0.66	4.39	Harmful

LC50: 50% lethal concentration.

Sub-lethal concentration toxicity test. The mean activity of cholinesterase was measured equal to 1086.89 ± 124.34 and 627.36 ± 99.60 mU min^{-1} per mg protein in the head and trunk, respectively. Based on independent *t*-test results, a statistical difference was seen between the head and the trunk ChE activity (T = 17.30, $p < 0.001$).

Effects of concentrations of malathion and exposure time on the activity of cholinesterase in the head and trunk have been shown in Figure 2A. Both concentrations of malathion and exposure time had significant effects on activity of cholinesterase in the head ($F_{2,45} = 1335.31$, $p < 0.001$; $F_{2,45} = 23.42$, $p < 0.001$) and the trunk ($F_{2,45} = 1011.34$, $p < 0.001$; $F_{2,45} = 137.52$, $p < 0.001$). There was a significant interaction between pesticide concentration and exposure time in activity of cholinesterase in the head ($F_{4,45} = 27.41$, $p < 0.001$) and trunk ($F_{4,45} = 14.78$, $p < 0.001$). On days 10 and 15, with an increase of malathion concentration, the cholinesterase activity was decreased significantly in the head (average = 1024.56 ± 67.44 mU min^{-1} per mg protein) and trunk (average = 555.43 ± 48.84 mU min^{-1} per mg protein).

Effects of concentrations of carbaryl and exposure time on the activity of cholinesterase in the head and trunk have been shown in Figure 2B. Both concentrations of malathion and exposure time had significant effects on activity of cholinesterase in the head ($F_{2,45} = 230.73$, $p < 0.001$; $F_{2,45} = 6.79$, $p = 0.001$) and the trunk ($F_{2,45} = 41.05$, $p < 0.001$; $F_{2,45} = 15.17$, $p < 0.001$). There was a significant interaction between carbaryl concentration and exposure time in activity of cholinesterase in the head ($F_{4,45} = 14.76$, $p < 0.001$) and trunk ($F_{4,45} = 4.51$, $p = 0.001$). Duncan test indicated that the activity of cholinesterase in the head (average = 1096.68 ± 42.07 mU min^{-1} per mg protein) and

trunk (average = 683.56 ± 14.10 mU min^{-1} per mg protein) of the specimens exposed to carbaryl was significantly different compared to control specimens.

Effects of concentrations of glyphosate and exposure time on the activity of cholinesterase in the head and trunk have been shown in Figure 2C. Both concentrations of glyphosate and exposure time had significant effects on activity of cholinesterase in the head ($F_{2,45}$ = 262.57, p < 0.001; $F_{2,45}$ = 196.42, p = 0.001) and the trunk ($F_{2,45}$ = 265.31, p < 0.001; $F_{2,45}$ = 67.34, p < 0.001). There was a significant interaction between glyphosate concentration and exposure time in activity of cholinesterase in the head ($F_{4,45}$ = 51.49, p < 0.001) and trunk ($F_{4,45}$ = 3.72, p = 0.001).

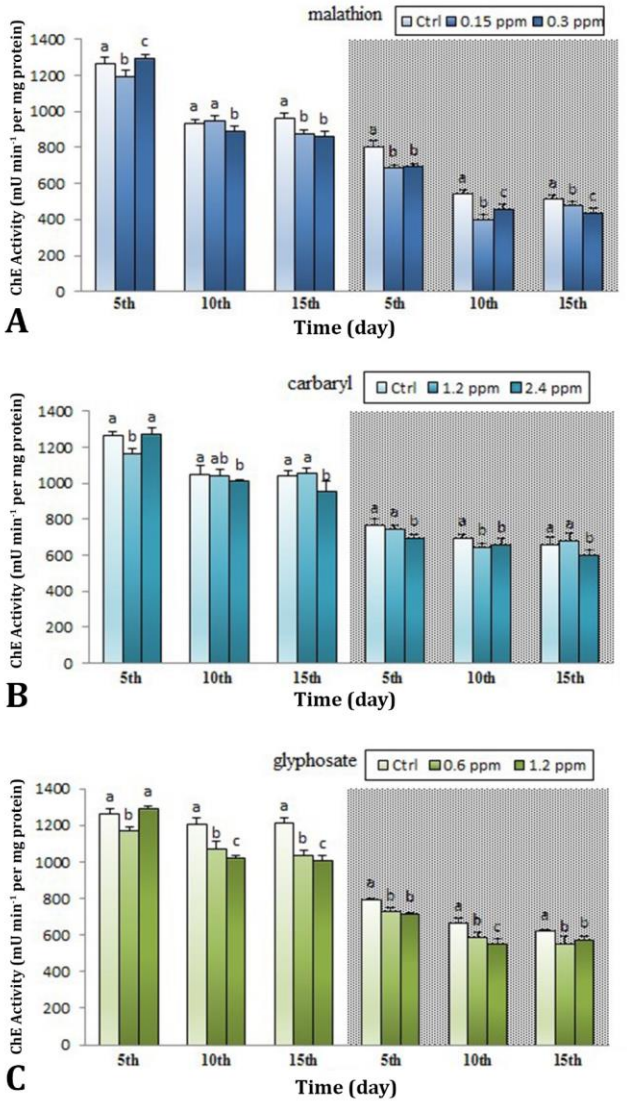

Fig. 2. Effects of concentrations of malathion (A), carbaryl (B), and glyphosate (C) during experiments on the Caspian kutum fries' cholinesterase activity of the head (bright background area) and trunk (shaded area).
[abc] Different letters indicate statistical significant differences among trial groups (p < 0.05).

Duncan test indicated that the activity of cholinesterase in the head (average = 1140.44 ± 35.45 mU min^{-1} per mg protein) and trunk (average = 643.11 ± 27.78 mU min^{-1} per mg protein) of the specimens exposed to glyphosate was significantly different compared to control specimens.

Discussion

In this study, the lethal (bioassay) and sub-lethal (ChE assay) effects of three agricultural compounds on *R. kutum* were evaluated. Any mortality was seen in the acclimation stage and the control group (96-hr test). Therefore, it could be concluded that treated fish mortality during the lethal experiment was caused by experimentally adding toxicants to the water. The LC50 96-hr of carbaryl to *R. kutum* was found 11.69 ppm which was relatively higher than *Ptychocheilus Lucius*,[30] *Cyprinus carpio*,[31] *Colisa fasciatus*,[32] and *Channa punctatus*[33] with the amounts of 0.293, 0.38, 7.85, 8.00, 8.50 ppm, respectively. However, this value was relatively lower when compared with *Carassius auratus* (13.90 ppm)[12] and *Clarias batrachus* (13.24 ppm),[34] against the carbamate chemical. In the case of malathion, the median lethal concentration for 96 h was 0.97 ppm that was lower than *C. batrachus* (1.00 ppm),[35] *C. fasciatus* (2.20 ppm),[32] and *P. Lucius* (3.71 ppm).[30] Furthermore, the lethal toxicity of malathion was much less for blue catfish equal to 17.00 ppm, however, the toxicity of Malaoxon for these fish (LC50 96-hr = 3.10) was higher than parent compound malathion.[16] Lethal toxicity of this organophosphate pesticide for *R. kutum* was nearly the same as the toxicity for *C. punctatus* equal to 0.90 ppm.[5] These values of lethal experiments dramatically were higher than *Labeo rohita* equal to 9.00 ppb,[36] and compared to *C. auratus* (0.79 ppm).[37] For the herbicide in our study, the LC50 96 h was calculated 6.64 ppm. A similar 96-hr test had been performed on *Oreochromis niloticus* exposed to glyphosate that the LC50 96 hr for Nile tilapia juveniles was 1.05 ppm.[38] Despite this report, these values were determined for *O. niloticus* adults to be equal to 36.80 ppm[39] that demonstrated the dramatic difference. In comparison with other research that had been studied on *C. carpio* (620 ppm),[40] Caspian kutum was more sensitive to this herbicide. The kutum's LC50-96-hr was relatively lower than *Ruditapes decussatus*[41] and *Utterbackia imbecillis*[42] amount of 32.17 and 18.30 ppm, respectively. The 96-hr LC50 value of *R. rutilus* exposed to tribenuron-methyl (an herbicide) was measured 152.74 ppm[43] that showed lethal toxicity of glyphosate was higher than tribenuron-methyl for this gender (Cyprinidae: Rutilus).

Numerous authors reported that the most common poisoning response to pesticide toxicity is the inhibition of AChE activity. This inhibition by insecticides was also found in the brain of *P. Lucius*,[30] *C. carpio*,[44] *C. auratus*,[12] and *Ictalurus furcatus*.[16] There was a significant difference

between the fries exposed to the three pesticides and the control group in cholinesterase inhibition. The fries exposed to carbaryl and glyphosate showed lower cholinesterase inhibition than those exposed to malathion. Head and trunk AChE of specimens exposed to both the pesticides results obtained in this study were in agreement with the results of carbosulfan (3.00-30.00 µg L^{-1}) and carbaryl (39.00 - 390 µg L^{-1}) insecticide (carbamates) inhibited brain AChE activity as compared to control.[31] In a toxicity experiment on *L. rohita* exposed to malathion (0.90 µg L^{-1}) for 15 days, an organophosphate compound, abnormal swimming, and respiratory distress which might be due to inactivation of AChE activity, has been observed.[36] It has been stated that inhibition of AChE resulted in the accumulation of acetylcholine, which causes twitching of muscle leading to tetanus and eventual paralysis of the muscles.[32] In addition to neurotic effects in the fish,[6] malathion can impact on the hematological and biochemical parameters,[35] as well as, behavioral changes such as irregular, erratic and darting swimming, hyper-excitability, bruise in the caudal section and finally loss of the equilibrium and sinking the bottom.[12] Also, paralysis of respiratory muscle may lead to death in fishes.[45] Likewise, the results concerning the activity of head and trunk AChE that exposed to glyphosate were significantly different compared to control specimens. These results were relatively in agreement with some findings that showed glyphosate exposure in fishes showed AChE inhibition and reduction of activity, however, no effects on AChE activity were observed in muscle tissue.[15] However, some researchers detected an inhibition in brain and muscle tissue after exposure to glyphosate.[8,9,46] Nevertheless, there is a report that indicated some herbicides like quinclorac and metsulfuron-methyl increased brain AChE activity in *Rhamdia quelen*.[47] Substantially, the effects of insecticides and herbicides on cholinergic system and AChE inhibition is due to oxidative stress situation and related enzymes like superoxide dismutase and glutathione peroxidase.[8] These changes caused abundant alteration in transcripts in related genes on the aquatics influenced by discharged toxicants to the rivers, lagoons, and estuaries.[48]

There was a significant difference between exposure times of all three pesticides compared to the control group in AChE inhibition. Reduction of head and trunk AChE was observed 10 days after compounds exposure. Our results were according to glyphosate herbicide inhibited brain AChE activity of *Leporinus obtusidens* as compared with control.[15] In comparison with the F-value (statistics) effect of exposure time with concentration (three compounds) results indicated that exposure time had a stronger effect on cholinesterase inhibition than the pesticides concentrations (F time > F concentration). Research demonstrated that exposure times (eight days) of glyphosate on *L. obtusidens* were important than the herbicide concentrations (1 to 5.00 mg L^{-1}),[8] and in agreement with this issue, some researchers emphasized the dominance of exposure time.[3,9,41] The exposure of animals to sub-lethal concentrations of the three pesticides over 15 days indicated that the average of cholinesterase activity in the head and trunk were 1086.89 and 627.36 mU min^{-1} per mg protein, respectively. It seems that the muscle and liver have both forms of ChE, acetylcholinesterase, and butyrylcholinesterase.[49] In some researches, two forms of ChE in the homogenized head[50] and muscle[2] were referred.

In conclusion, the toxicity test findings derived in this study showed that Caspian kutum fries were significantly more tolerant to carbaryl exposure than malathion and glyphosate after their lethal concentration exposures. Based on World Health Organization pesticide dictionary,[28] malathion, glyphosate, and carbaryl had a high, moderate, and low degree of toxicity for *R. kutum*, respectively. Despite low concentrations of these compounds recognized as the permissible concentrations, they could cause enzymatic reactions.

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Conflicts of interest

The authors of the paper do not declare a competitive financial interest.

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