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Antioxidant Responses and Bioaccumulation in Green-lipped Mussels (*Perna Viridis*) Under Acute Tributyltin Chloride Exposure

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Abstract Green-lipped mussels (*Perna viridis*) were exposed to waterborne tributyltin chloride (TBTCl) at different concentrations (0.2, 0.4 and 0.8 μg L^{-1}) for up to 72 h. Accumulated TBTCl in *Perna viridis* correlated linearly with the exposure concentrations of 0.2 μg L^{-1}(R^2 = 0.772), 0.4 μg L^{-1}(R^2 = 0.952), and 0.8 μg L^{-1}(R^2 = 0.909). The results of superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) all decreased in gill tissues after 24 h of exposure, but the hepatic SOD and the hepatic GPx showed either little or no effect on exposure of TBTCl solutions. Analysis using the Spearman rank correlation coefficient showed the hepatic GPx activity appeared to have a significant negative correlativity (R_s = −0.42) with the exposed TBTCl concentrations, and the hepatic MDA was significantly negatively correlated (R_s = −0.33) with the tissue TBTCl concentrations. Conversely, a significant positive correlation (R_s = 0.60) was shown between the gill MDA contents and exposure time. This study illustrates oxyradical scavenger GPx best correlated with stress level of pollutants among the various antioxidant parameters.

Keywords Antioxidant response · Bioaccumulation · Tributyltin chloride (TBTCl) · Marine mussels (*Perna viridis*)

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Organotin compounds, particularly Tributyltin (TBT) are widely used as biocides in antifouling paints for both recreational and commercial watercraft, which are nontarget toxic contaminant of marine and freshwater ecosystems. Besides the highly acute toxicity of TBT, some studies show that TBT have embryotoxicity and genotoxicity (Antizar-Ladislao 2008), TBT even can produce possible endocrine disrupting effects (Lagadic et al. 2007). In awareness of the undesired impacts of TBT, some worldwide and detailed organotin regulatory strategies have been taken to protect the aquatic environment. However, present and future restrictions will unfortunately not immediately remove TBT and its degradation products from the marine environment, since these compounds are retained in the sediments where they persist (Antizar-Ladislao 2008). In recent years many reviews have reported on high TBT environmental levels in some coastal waters and TBT residues in some seafood (Jiang et al. 2001). Therefore, wide distribution, high hydrophobicity, and persistence of organotin compounds have showed their bioaccumulation, their potential biomagnification in the food webs, and their adverse effects to the human health and environment.

Antioxidant responses of living organisms are the important defense mechanisms to resist the exposure of toxicants. Numerous reports have demonstrated that these responses can be used as biomarkers for reflecting the pollution levels of these substances (Emmanouil et al. 2008). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) form antioxidant enzymes defense against reactive oxygen species (ROS) in the cells, as a result their increased activities have been widely adopted to indicate the presence of oxidative stress (Huang et al. 2005). Peroxidation by ROS can lead to the formation of lipid peroxides such as malondialdehyde (MDA) and its increase suggest a high cytotoxicity and inhibitory action on

protective enzymes. Variations in antioxidant enzymes levels and the associated biomarkers associated with organotin exposure have been studied, when antifouling paints were related to the worldwide decline of marine molluscs in coastal areas. Previous reports have indicated that TBT could inhibit the activities of GPx (Cheung et al. 2001; Wu et al. 2007), imbalance the activities of glutathione-S-transferases (GST) (Huang et al. 2005). In most cases, antioxidant responses of indigenous animals such as fish species and mussel species from polluted areas have been used to predict the relationship between toxicity and bioaccumulation levels of pollutants from polluted areas (Wu et al. 2007). However, no data is available correlating the relations between antioxidant responses and bioaccumulation under experimental TBTCl exposure to date. Among all kinds of aquatic organisms, green-lipped mussels (*Perna viridis*) have been widely used in the evaluation of the quality of aquatic environments. This study chose *Perna viridis* as the test animal, and aimed to investigate the influence of TBT on bioaccumulation, activities of antioxidant enzymes and their correlation during 72 h of exposure. The result from this study would be useful to provide a better understanding on the mechanism of organotin toxicity to the marine mussel species and help assessing the sensitivity of the marine mussel species to TBT toxicity.

Materials and Methods

Tributyltin chloride (TBTCl) was obtained from Alfa Aesar, USA, with a purity of greater than 98%. TBTCl was diluted in 98% ethanol to concentrations of 1 mg L^{-1}. All other chemicals were of analytical grade and were obtained from commercial sources.

Perna viridis (average body weight 23.1 ± 5.9 g) were purchased from a local coast market (Xincun Port, Hainan Province, China), acclimated in tanks containing 500 L of aerated marine water, with natural photoperiod for 7 days. TBTCl stock solution was prepared by dissolving 0.1 g of TBTCl in 100 mL ethanol and stored under 4°C. Eighty-six mussels were placed into four tanks containing 50 L of TBTCl solutions at concentration of control, 0.2, 0.4, 0.8 μg L^{-1}, respectively, and then divided into four groups. No TBTCl stock solution was added to the control group, which received 1 mL solvent ethanol per 10 L of solutions. The seawater containing TBTCl was exchanged with the same concentration daily. During the experimental period, *Perna viridis* were fed on phytoplankton (*Chlorella* spp., 5 × 103 cells mL^{-1}) every morning, and water temperature and salinity were 20–25°C and 32, respectively. Eight individuals in each treatment group were sampled after exposure for 24, 48 and 72 h.

TBTCl concentrations in the soft body of *Perna viridis* were analyzed according to Inoue et al. (2006), with a slight modification. In short, three mussels in each group were washed with deionized water and dried with absorptive paper, and then about 5 g (wet weight) of total tissue sample was spiked with 2 μg of TBTCl-$_{d27}$ as an internal standard and homogenized with 12 mL of 0.1% Potassium hydroxide–methanol solution by incubating shaker for 45 min. The homogenate was extracted twice with 2 mL of hexane and 150 mg of sodium borohydride using an ultrasonicator for 10 min, then the extract was centrifuged at 4,000*g* for 10 min and the supernatant was used for chromatographic analysis. Further extraction and measurement of TBTCl were performed using the methods mentioned above. TBTCl concentration was expressed on a wet-weight basis in the present study.

For biochemical analyses, five mussels in each group were washed with deionized water and surface dried with absorptive paper, then gills and hepatopancreases were rapidly dissected from the mussels of each group, thoroughly washed with ice-cold physiological saltwater (0.9% NaCl), and then manually homogenized with a glass homogenizer in 10 mM of ice-cold Tris–HCl buffer (pH 7.4; for 0.1 g of sample, 0.9 mL of buffer was added) and then centrifuged at 4,000×*g* for 15 min at 4°C. The supernatant was immediately analyzed for SOD and GPx activity as well as MDA and protein content. Soft tissues of another three mussels washed with deionized water were dissected from the shell and stored at −20°C for biochemical analysis.

SOD activity was determined by measuring the inhibition of nitrite formation in reaction of oxidation of hydroxylammonium with superoxide anion radical (Oyanagui 1984), with a slight modification. The reaction mixture contained 0.2 mL of xanthine (7.5 mM), 0.1 mL of hydroxylammonium chloride (10 mM), 1 mL of phosphate buffer (65 mM, pH 7.8), 0.49 mL of distilled water, and 0.2 mL of xanthine oxidase (0.2 mg mL^{-1}). The inhibitory effect of inherent SOD was assayed at 37°C during 20 min of incubation with 40 μL of tissue extracts. Determination of the resulting nitrite was performed on the reaction (20 min at room temperature) with 2.0 mL of sulfanilic acid (3.3 mg mL^{-1}) and 2.0 mL of a-naphthylamine (1 mg mL^{-1}). Optical absorbance at 530 nm was measured with visible spectrophotometer (Model 722, Shanghai Spectrum Instruments, China). One unit of enzyme activity is defined as 50% inhibition of by nitrite formation in this condition.

GPx activity was measured by the method described by Rotruck et al. (1973). Briefly, the reaction mixture contained 0.2 mL of Tris–HCl buffer (0.4 M, pH 7.0), 0.1 mL of sodium azide (10 mM), 0.2 mL of homogenate (homogenized in 0.4 M, Tris–HCl buffer, pH 7.0), 0.2 mL of glutathione, and 0.1 mL of H_2O_2 (0.2 mM). The

contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 mL of 10% TCA, and then centrifuged at 3,000*g* for 10 min. Finally a yellow product which had peak absorbance at 412 nm could be formed as glutathione reacted with Ellmans reagent (19.8 mg of 5,5′-dithiobis-nitro benzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate). One unit of GPx activity is defined as the amount that reduced the level of glutathione by 1 μmol/L in 1 min per milligram protein.

LPO level was assayed by measuring the content of MDA according to the method reported by Ohkawa et al. (1979). The reaction mixture contained 0.4 mL of supernatant, 0.2 mL of sodiumdodecyl sulfate (8.1%), 1.5 mL of acetic acid buffer (20%, pH 3.5), 1.5 mL of 2-thiobarbituric acid (1%) and 1 mL of distilledwater. Then the mixture solutions were heated at 95°C for 60 min in photophobic condition, cooled and centrifuged at 3,000*g* for 10 min. The supernatant was determined using a spectrophotometer at 532 nm. MDA content produced by polyunsaturated fatty acid hydroperoxides was expressed as nanomoles per milligram of protein.

Protein content was determined using bovine serum albumin as a standard (Bradford 1976). Absorbance was recorded at 595 nm.

All data were processed using SPSS 13.0 for Windows software (SPSS, Chicago, IL, USA). All parameters detected were statistically compared by one-way analysis of variance and LSD multiple comparison test amongst the means. The non-normal nature of these data required the use of nonparametric procedure. The relationships among the biochemical and chemical parameters were calculated by Spearman's rank correlation. Statistical analysis was expressed as means ± SD, with $p < 0.05$ being considered significant, and $p < 0.01$ being considered extremely significant.

Results and Discussion

All treatment groups showed an increasing accumulated TBTCl in *Perna viridis* tissues (Fig. 1). Before the exposure experiments started, TBTCl had been detected in *Perna viridis* with the concentrations of 0.093 mg kg^{-1} wet wt. After a 72 h exposure to TBTCl, the concentration of control group did not change significantly during the experiment (ANOVA, $p = 0.30$). The TBTCl accumulation was linear up to 72 h when mussels were exposed to TBTCl concentrations of 0.2 μg L^{-1} ($R^2 = 0.772$), 0.4 μg L^{-1} ($R^2 = 0.952$), and 0.8 μg L^{-1} ($R^2 = 0.909$). In the 72 h static renewal toxicity exposure, TBTCl was rapidly accumulated in *Perna viridis* tissue shortly after the start of the exposure and increased with the increasing of

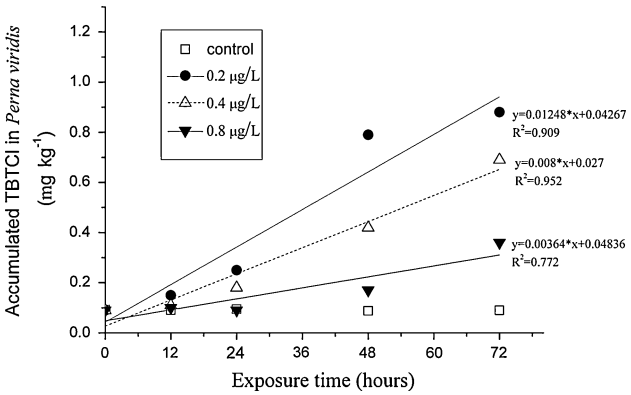

Fig. 1 Bioaccumulation of different concentrations TBTCl in green-lipped mussels *Perna viridis* during 72 h exposure

exposure concentration and exposure time. However, the concentration of 0.2 μg L^{-1} showed an overall decreasing trend compared with other group after a 72 h exposure, which may result from the trend of reaching an apparent steady state as reported by Vanssay et al. (1999). During the accumulation processes of TBT, the accumulation mechanism is an overall combination of uptake, metabolism, and excretion processes. Other marine mussel, *Mytilus edulis*, exposed to 25 and 49 ng L^{-1} of TBT in a daily renewal system reached a steady state within 14 days (Laughlin and French 1988). However, some unicellular green algae, Chlorella vulgaris and *Chlorella* sp., had been shown to be highly tolerant to TBTCl, and reached a steady state in the intracellular TBTCl levels within 6 h of incubation (Tsang et al. 1999). The apparent biological accumulation indicated that partitioning of this compound between seawater and mussels was an important accumulation mechanism, and a steady state was reached after a short treatment time.

The results of SOD and GPx of *Perna viridis* after TBTCl exposure are reported in Figs. 2 and 3. After 24 h of exposure to TBTCl, statistically significant inhibition was recorded for SOD and GPx enzymes. With the increase of exposure time, the SOD activity was significantly increased at all TBTCl concentrations in the gill at the time of 48 h, enzymatic activity of other groups recovered to the level of control group. At the exposure time of 72 h, the SOD and GPx activity in gill was inhibited significantly at the group of 0.2 μg L^{-1}, and the enzymatic activity in hepatopancreas was imbalanced at 0.4 μg L^{-1} group and 0.8 μg L^{-1} group, respectively. In general terms of overall tissue specificity, the activities of SOD and GPx were higher in gill tissues than in hepatopancreas. The lipid peroxidation status of gill and hepatopancreas tissues followed the similar specificity pattern as SOD and GPx, which was expressed as MDA content as shown in Fig. 4.

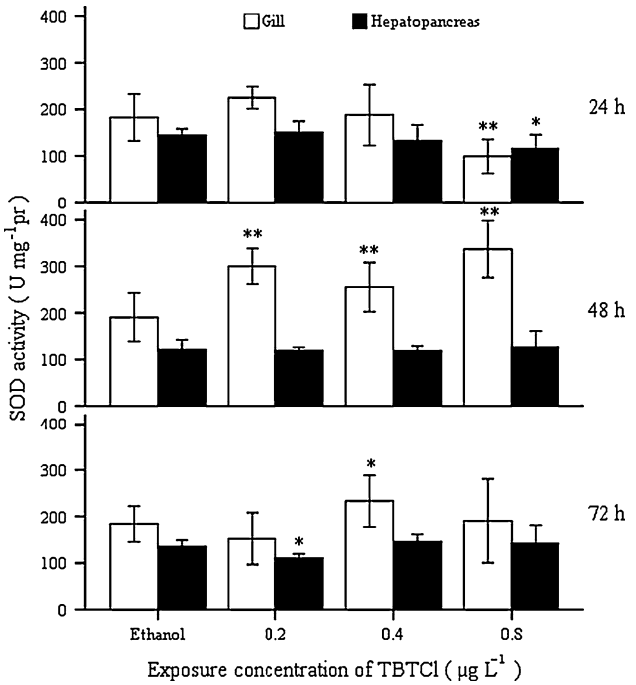

Fig. 2 Levels of SOD enzymes in *Perna viridis* exposed to TBTCl for 72 h. Values were means ± SD. $*p < 0.05$, $**p < 0.01$

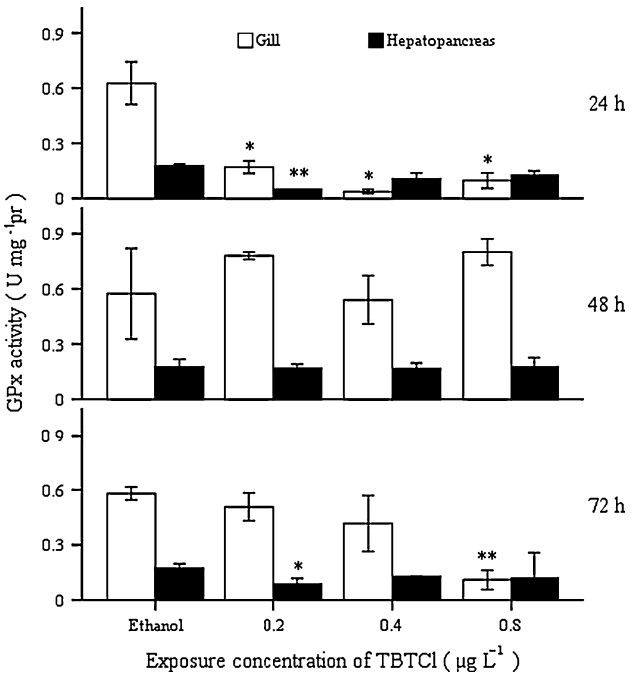

Fig. 3 Levels of GPx enzymes in *Perna viridis* exposed to TBTCl for 72 h. Values were means ± SD. $*p < 0.05$, $**p < 0.01$

Many environmental pollutants can cause oxidative damage to biological systems, however, there is limited information concerning antioxidant systems of different tissues in marine mussels exposed to TBTCl at different concentration levels. Research undertaken has shown that

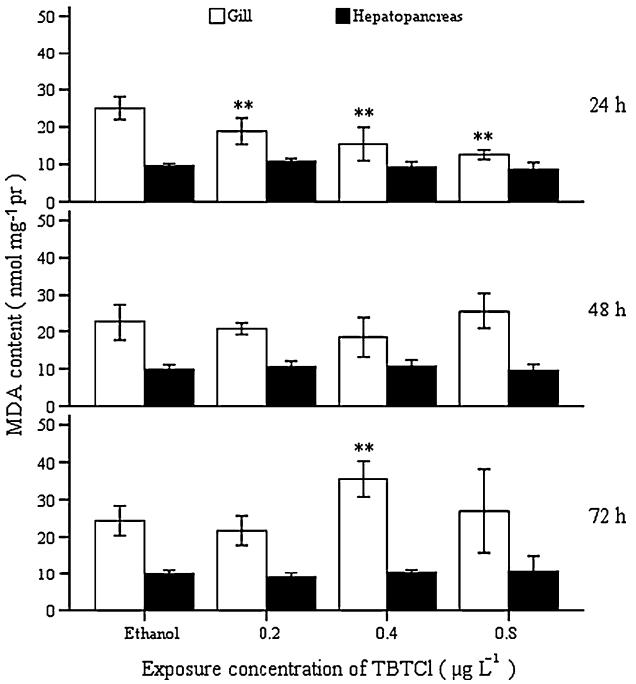

Fig. 4 Levels of LPO in *Perna viridis* exposed to TBTCl for 72 h. Values were means ± SD. $*p < 0.05$, $**p < 0.01$

TBT is very toxic to aquatic life, particularly to marine mollusks. The present results showed that the activities of SOD, GPx, and the content of MDA were affected during a 72 h exposure period compared to those of the control group. These changes indicated the difference and possible mechanism of oxidative stress between gill and hepatopancreas tissues induced by TBTCl on marine mussels. In gills of *Perna viridi* after TBTCl treatment, all the activity of antioxidant enzymes firstly had a significant decline after 24 h exposure, then showed an obvious induction trend with the increase of exposure time of TBTCl. Huang et al. (2005) also showed that the GPx activities in 10 ng L^{-1} TBTCl group were significantly inhibited after exposure for 2 days, and were mainly induced after exposure for 8 and 20 days, which indicated strong production of reactive oxygen species in the process of accumulation and metabolism of TBT in the organism. Conversely, the hepatic antioxidant responses were not obvious compared with the control group. This may be due to more TBT reach the hepatopancreas via the filtering system of gill in *Perna viridi*. Similar findings have shown that Glutathione peroxidase (GR) and GPx activity in gill were higher than in hepatopancreas (Cheung et al. 2001). It is conceivable that the higher activities in gills may represent a first line of defence against tissue exposure or damage. Recent researches have demonstrated that TBTCl may induce obviously production of reactive oxygen species in organisms, such as clam and scorpion fish et al. (Huang et al. 2005). Although several endpoints including acute

toxicity have been considered to evaluate TBT toxicity, but growth impairme has been observed as much more sensitive response to TBT exposure than mortality (Meador and Rice 2001). Antioxidant defense strategies play a protective role for organisms inhabiting environments contaminated with xenobiotic, which can be induced by a slight oxidative stress due to compensatory response. However, a severe oxidative stress suppresses the activities of these enzymes due to oxidative damage. Thus, Antioxidant responses on TBTCl integrated with multiple parameters may estimate the true response.

Spearsman's rank correlation analysis was used to test whether the levels of antioxidative responses from *Perna viridis* were related to the tissue concentrations, exposure concentration and exposure time of TBTCl (Table 1). The results showed the GPx in gill and hepatopancreas tissues appeared to have a negative correlativity with the tissue TBTCl concentrations, and the gill and hepatic MDA showed a positive correlativity with the tissue TBTCl concentrations. Such a different response might be due to the fact that the toxicant concentrations induced GSH converted to oxidized glutathione (GSSG) in the biotransformation of ROOH (hydroperoxide) to ROH. However, statistical results showed the GPx in gill tissue was significantly negatively correlated ($R_s = -0.42$) with the exposed TBTCl concentrations, and the hepatic MDA was significantly negatively correlated ($R_s = -0.33$) with the tissue TBTCl concentrations. It is also possible during the chronic exposure to toxicants, a different mechanism of gill and hepatopancreas in mussel was accommodated to the antioxidative stress of pollutants. Until recently, most risk assessments focused on the monitoring the accumulated concentration of environmental contaminants in various aquatic organisms. In this way, it is not enough to evaluate the combined effect of environmental contaminants and environmental parameters. In two recent studies, an integrated approach was proposed that chemical determination of environmental contaminants should be integrated with their effects of biomarkers providing a better picture of the stress situations (Porte et al. 2001). This approach has been proven to be an effective means of evaluating the impact of pollution in the aquatic environment, and the application in the field of such a combined approach using bivalves has been successfully applied in a number of environmental situations (Cheung et al. 2001). In aquatic invertebrates, such as mussels, a stimulation of antioxidant responses was evidenced after exposure to environmental pollutants. However, there is little information on the correlation with antioxidant enzymes and bioaccumulation in mussels under acute TBT experimental treatment.

In the aquatic environment, TBT is quickly removed from the water column and adheres to bed sediments because TBT has high specific gravity and octanol water partition coefficient (log Kow) (Landmeyer et al. 2004). An exceptional increase of TBT in bottom sediments have been reported as hot spots associated with ship channels, ports, harbours, and marinas in Japan (Harino et al. 2007). In newly industrializing countries, a high TBT pollution level has also been observed in old neglected deposits of toxic waste (Jiang et al. 2001). However, the adsorption of TBT to natural sediments is reversible for changes in the pH and salinity (Burton et al. 2004), therefore contaminated sediments can act as a long-term source of dissolved-phase contamination to the overlying water column. In most cases, the most obvious routes of organotin exposure to biota and consequently to human by food web is through the diet and accumulation from water and sedimentary surroundings (Lee et al. 2006). For example, the average concentrations of the three butyltins (TBT, DBT, MBT) species normally monitored in marine food range from 100 to 1,500 ng g^{-1} with highest concentrations present in cultured fish and molluscs in Asian and Oceanian countries (Kannan et al. 1995). Thus, the accumulation integrated with biomarker and eco-toxicological implications of organotins in benthic marine organisms is needed. This study has examined the antioxidant responses in *Perna viridis* exposed to TBTCl under laboratory condition. As shown in the results, not all the antioxidant parameters measured showed significant responses with increasing tissue TBTCl concentrations, and also affected by the treatment level. Such variations have also been reported in other studies in field studies (Cheung et al. 2001). In the

Table 1 Relationships between the different biochemical and experimental variables (Spearman rank correlation coefficient, Rs, n = 36)

Experimental variables	SOD		MDA		GPx	
	Gill	Hepatic	Gill	Hepatic	Gill	Hepatic
Accumulated in *Perna viridis*	0.15^{ns}	-0.08^{ns}	0.06^{ns}	0.28^{ns}	-0.05^{ns}	-0.33*
Exposure concentration	0.12^{ns}	-0.03^{ns}	-0.27^{ns}	-0.10^{ns}	-0.42*	-0.29^{ns}
Exposure time	0.08^{ns}	-0.07^{ns}	0.60**	0.21^{ns}	0.20^{ns}	0.08^{ns}

^{ns} Not significantly correlated

* Significantly correlated at $p < 0.05$

** Significantly correlated at $p < 0.01$

present study, the hepatic GPx showed significant negative correlation with tissue TBT concentrations, and may be attributed to inherent biological variations. Clearly, the toxic mechanisms of TBT will need to be conducted to further elucidate. Furthermore, there is a need to compare field investigations with laboratory conditions to further verify their correlation.

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