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Activities of antioxidant enzymes (SOD and CAT) in the coral *Galaxea fascicularis* against increased hydrogen peroxide concentrations in seawater

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Abstract. The activities of two antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were studied in a colony of *Galaxea fascicularis* corals with increased concentrations of hydrogen peroxide (H_2O_2) in seawater using an incubation chamber. Changes in enzyme activity were compared to those induced by increased seawater temperature. Three H_2O_2 concentrations (0, 0.3, and 3.0 μM) were tested at 27°C, and an elevated seawater temperature of 31°C was tested without added H_2O_2. CAT activities in both coral tissue and zooxanthellae increased with increased H_2O_2, but SOD activities remained relatively unchanged, suggesting that increased H_2O_2 in seawater affected coral cytosol but did not induce superoxide formation. In contrast, the elevated seawater temperature caused both SOD and CAT activities in coral tissue and zooxanthellae to increase. Coral bleaching was not observed at the levels of H_2O_2 tested during the 5-day exposure period. Although the long-term effects of H_2O_2 remain unknown, these results suggest that coral bleaching would likely not occur from short-term (5 days) exposure to increased H_2O_2 concentrations in seawater.

Key words: hydrogen peroxide, antioxidant enzyme, catalase, coral bleaching, *Galaxea fascicularis*

Introduction

In recent years, coral reefs have experienced extensive degradation throughout the world (Stone et al. 1999). This is the result of coral bleaching, whereby corals lose their symbiotic algal zooxanthellae or the photosynthetic pigments of the symbiotes are degraded. The causes of coral bleaching are thought to be abnormally high seawater temperatures, high levels of irradiance, strong ultraviolet (UV) radiation, changes in salinity, or a combination of these factors (Brown 1997; Fitt et al. 2001).

Environmental stress can induce increased production of reactive oxygen species (ROS), leading to significant oxidative damage to the coral-algae symbiotic system (Lesser et al. 1990; Dykens et al. 1992; Downs et al. 2002). The cellular response to the formation of oxygen radicals includes many defense mechanisms (Shick et al. 1995). For example, enzymes such as superoxide dismutase (SOD) and catalase (CAT) act in concert to inactivate superoxide radicals ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2). This prevents the formation of the most reactive form of ROS, the hydroxyl radical (•OH), and subsequent cellular damage (Fridovich 1986). SOD catalyzes the dismutation of superoxide into oxygen and H_2O_2, and CAT is responsible for inactivating H_2O_2 into water and oxygen. These enzymes are responsible for detoxifying ROS, and their elevated activities indirectly indicate increased production of ROS in corals as a result of environmental stresses such as temperature, irradiance, and UV radiation (Lesser et al. 1990).

Smith et al. (2005) hypothesized that H_2O_2 may be the most important ROS associated with coral bleaching because it can act as an important signaling molecule between *Symbiodinium*, i.e., zooxanthellae, and their symbiotic host. In addition, Suggett et al. (2008) reported that the thermal tolerance of *Symbiodinium* is related to adaptive constraints associated with photosynthesis and that sensitive phylotypes are more vulnerable to H_2O_2. Furthermore, Mydlarz and Jacobs (2004) indicated that H_2O_2 production occurred as an oxidative burst in a physically injured *Symbiodinium* sp. that was isolated from *Pseudopterogorgia elisabethae*. Finally, Ross et al. (2006) reported that micromolar concentrations of H_2O_2 (>10 μM) induced cell death in the cyanobacterium *Microcystis aeruginosa* as measured by a caspase protease assay. The production of H_2O_2 was involved in the inhibition of the chlorophyll *a*-chlorophyll c_2-peridinin-protein complexes (acpPC) mRNA translation in *Symbiodinium* (Takahashi et al. 2008). Although many studies have reported the effects of in-cell H_2O_2 on zooxanthellae, few studies have examined the effects of increased H_2O_2 in the seawater surrounding a coral colony.

Here we examined the changes in activities of antioxidant enzymes (SOD and CAT) with varied concentrations of H_2O_2 in seawater and with different seawater temperatures by using a well-controlled incubation chamber. We also compared the magnitudes of changes in enzyme levels induced by H_2O_2 to those induced by high seawater temperature.

Material and Methods

Coral specimens

Colonies of *Galaxea fascicularis* were collected from a coastal region of Okinawa Island, Japan, with permission from the Okinawa prefectural government (No. 18-11). The coral colonies were immediately transferred to an outdoor tank at the University of the Ryukyus Sesoko Station of the Tropical Biosphere Research Center (TBRC). Coral branch tips (ca. 4 cm long) from three large coral colonies were cut and attached to a polycarbonate resin bolt (ca. 1 cm in diameter and 2 cm long) using glue intended for underwater use. The branch tips were kept in the outdoor tank for 3 months, after which they were moved to an indoor tank at the University of the Ryukyus Nishihara campus (ca. 60 km from TBRC) for the enzyme experiments.

Experimental design

We studied changes in coral enzyme activities using a continuous-flow complete-mixing (CFCM) experimental system. The CFCM system consists of a water tank incubation system and a flow-through system, which can continuously supply seawater while maintaining the volume of the seawater in the incubation tank during the course of the experiments. A detailed description of the CFCM is provided by Fujimura et al. (2008).

We investigated the effects of H_2O_2 on the activities of SOD and CAT in coral by controlling H_2O_2 concentrations in the supplied seawater. Various concentrations of H_2O_2 were introduced into the coral incubation tank. The H_2O_2 stock solution was prepared with 0.7 M NaCl + 2 mM $NaHCO_3$ aqueous solution to minimize decomposition of H_2O_2 and changes in salinity and carbonate concentration of the supplied seawater during the experiments. The concentration of H_2O_2 in the stock solution was determined by measuring UV absorbance at 240 nm (ε_{240} = 38.1 M^{-1} cm^{-1}; Miller and Kester 1988). Immediately before the experiments, we prepared 6 and 60 μM H_2O_2 solutions that were diluted 20-fold in the incubation tank to 0.3 and 3.0 μM, respectively. It should be noted that dilution of the filtered seawater was always kept at 20-fold throughout the experiments using the 0.7 M NaCl + 2 mM $NaHCO_3$ aqueous solution even when no H_2O_2 was added.

Seawater temperature was held constant at 27°C, and three H_2O_2 concentrations (0, 0.3, 3.0 μM) were used for 1, 3, and 5-day periods, respectively. In an additional treatment, temperature was maintained at 31°C, and no H_2O_2 (0 μM) was added. Six coral branch tips were used in each treatment. Light was provided by a metal halide lamp, and photon flux density (200 or 0 μmol m^{-2} s^{-1} during a 12:12 h light/dark cycle) was maintained throughout the experiments.

Isolation of zooxanthellae and analyses of antioxidant enzyme activities

Enzyme assays were conducted according to Levy et al. (2006) and Yakovleva et al. (2004). Tissue homogenates were prepared using the air-pick method, in which an air jet connected to a scuba regulator is used to strip the tissue from the coral skeleton into approximately 10 ml of 100 mmol l^{-1} phosphate buffer (pH=7). The volume of the homogenate was measured, and 1 ml of each subsample was used for determination of zooxanthellae density using direct counts on a Neubauer hemocytometer. A 2-ml aliquot of each homogenate sample was filtered using a GF/A filter (Whatman) and used for chlorophyll *a* (Chl. *a*) analysis. Chlorophyll was extracted from the filter overnight with acetone in the dark at 4°C. The absorbance was measured at 630 and 663 nm. We used the Jeffrey and Humphrey (1975) equation to calculate the amount of Chl. *a* extracted from the filter.

The homogenate was further centrifuged twice at 1500 × *g* for 15 min to separate the supernatant and pellets. The supernatant was used to analyze protein and enzyme activities of the host coral. The pellets containing the zooxanthellae were suspended in 2 ml of 100 mmol l^{-1} phosphate buffer, and were then dissolved by sonication for 30 min in an ice bath. A 0.05% Triton X-100 solution was added to the sonicated suspension. After incubation for 10 min, the suspension was centrifuged at 14000 × *g* for 30 min and used as the algal solution for protein and enzyme assays.

SOD activity was assayed spectrophotometrically as described by Elstner and Heupel (1976) and Oyanagui (1984). Standards for activity were prepared using bovine erythrocytic SOD (Sigma) for each set of samples. CAT activity was measured by the depletion of H_2O_2 at 240 nm (Beers and Sizer 1952). All assays were conducted at 25°C, and enzyme activity was expressed as units (U) per mg protein. Protein content was determined by the Bradford assay (Bradford 1976). Dunnett's test was used for the statistical analyses for comparing the data under various conditions against the control data (JMP 6.0.2, SAS).

Results and Discussion

Effects on SOD activity

Figure 1 shows the effects of H_2O_2 and seawater temperature on the activity of SOD in the coral tissue and in the isolated zooxanthellae. With H_2O_2 exposure, there were no significant differences in SOD activity in either coral tissue or zooxanthellae during the 5-day exposure period. Similarly, with a high seawater temperature of 31°C and without added H_2O_2, SOD activity of the host coral was relatively unchanged on day 1 and day 5, even though SOD activity of coral tissue on day 3 was significantly high. Conversely, with high seawater temperature, SOD activity in the zooxanthellae increased significantly by approximately 2-fold on day 3 and day 5 (P<0.01). Increases in antioxidant enzyme activities are indicative of increased concentrations of ROS (Lesser et al. 1990). Given that changes in SOD activity reflect changes in $\bullet O_2^-$ concentrations, the results suggested that H_2O_2 exposure did not induce $\bullet O_2^-$ formation in the coral tissue or zooxanthellae during the incubation period.

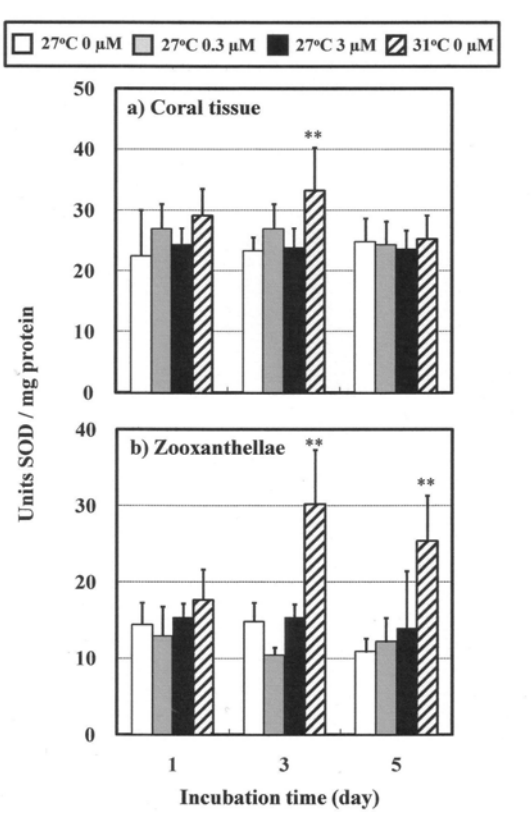

Figure 1: Effects of hydrogen peroxide and/or temperature on the activity of superoxide dismutase (SOD) in (a) coral tissue and (b) zooxanthellae. Means±S.D. (n=6).
**: P<0.01, *: P<0.05 significantly different with Dunnett's test for comparing treatments to a control.

Yakovleva et al. (2004) showed that SOD activity of coral tissue and the zooxanthellae in *Stylophora pistillata* and *Platygyra ryukyunensis* increased under high seawater temperatures, while SOD activity of the coral tissue in *P. ryukyunensis* showed little change. Dykens et al. (1992) suggested that the SOD activity in host coral changed in response to the amount of photosynthetically generated O_2 by its algal symbiotes in the sea anemone *Anthopleura elegantissima*. Our results show a greater increase in the SOD activity of zooxanthellae compared to the coral tissue, which is accordance with these studies and suggests that the zooxanthellae of the coral *Galaxea fascicularis* are more susceptible to temperature changes than the coral host is. In addition, Takahashi and Asada (1983) reported that $\bullet O_2^-$ exhibits low permeability through the cell membrane. Therefore, it is probable that the $\bullet O_2^-$ formed in the zooxanthellae did not penetrate into the coral tissues.

Effects on CAT activity

Figure 2 shows the effects of H_2O_2 and/or high water temperature on the activity of CAT in the coral tissue and in the isolated zooxanthellae. CAT activity was significantly increased by both H_2O_2 and high water temperature in coral tissue and in isolated zooxanthellae (P<0.05).

CAT activities in the host coral and in zooxanthellae increased rapidly within the first day of exposure and were similar for both increased H_2O_2 concentrations and high seawater temperature conditions. Levy et al. (2006) reported that CAT activities increased within a few minutes to several hours. Moreover, as shown in Fig. 2, CAT activities in the coral tissue increased gradually each day with either H_2O_2 exposure or high temperature conditions. It is possible that the coral tissue acclimatized day by day against increased H_2O_2 or high temperature. Yakovleva et al. (2004) also reported that the CAT activities in the coral tissue of *P. ryukyunensis* and *S. pistillata* exposed to high water temperature were higher after 12 h than after 6 h of exposure. CAT activities in zooxanthellae also increased quickly within the first day of the experiments, but CAT activities in zooxanthellae appeared to level off for the remainder of the incubation period. Yakovleva et al. (2004) also reported that CAT activities of the zooxanthellae were slightly lower after 12 h than after 6 h of exposure, which is different from the response of the host coral.

Increases in CAT activities were a result of increased H_2O_2 concentrations in the host coral and zooxanthellae, which suggests that H_2O_2 in the surrounding seawater reached the cytosol of the coral. Dykens and Shick (1982) reported that enzymatic defenses in the animal host occur in proportion to the

potential for photooxidative damage in symbiotic cnidarians. Our results showed that exposing corals to ten-fold differences in H_2O_2 concentrations did not result in significant differences in CAT activities in zooxanthellae, which suggested that zooxanthellae probably had a limit in increasing CAT activities, unless penetration of H_2O_2 into the zooxanthellae was controlled by the cell membrane. Once inside the coral cytosol, H_2O_2 should be inactivated by protective enzymes such as CAT, or could be converted into OH radicals, which could damage the photosynthesis system and can eventually lead to coral bleaching (Levy et al. 2006). Higuchi et al. (2009) showed the photosynthesis rate of the coral *G. fascicularis* was reduced by the higher concentration of H_2O_2. It was suggested that higher H_2O_2 in the coral cytosol remained unless all the H_2O_2 was neutralized by the enzymes because of the limitation of increased CAT activity.

Relationship between ROS and coral bleaching

Figure 3 shows the changes in the density of zooxanthellae, measured as the number of cells per cm^2 of the surface area of the coral colonies, during the 5-day incubation period. There were no significant differences in the density of zooxanthellae with increased concentrations of H_2O_2 during the exposures, even with an addition of 3 µM H_2O_2 (Fig. 3). Thus, coral bleaching was not observed with the elevated H_2O_2 exposure within the 5-day experiments. Downs et al. (2002) suggested that bleaching is a final defense of corals against oxidative stress. As shown in Fig. 2, corals (both host coral and zooxanthellae) were under oxidative stress with increased H_2O_2, but coral was not bleached. This could be because the oxidative stress was not due to the *in situ* formation of H_2O_2 in the symbiotic algae, and therefore the corals did not expel zooxanthellae. Alternatively, it is possible that the oxidative stress posed by the 3 µM H_2O_2 in seawater was within the tolerance level for the tested corals.

Figure 3 also shows the effects of increased seawater temperature on the density of the zooxanthellae. When seawater temperature was elevated from 27 to 31°C, the density of zooxanthellae decreased rapidly by approximately 50% within 1 day, and leveled off for the rest of the 4-day exposure. Significant differences in zooxanthellae density were observed between the corals kept at 27°C and those kept at 31°C during the 5-day high temperature conditions (P<0.05). The amount of Chl. *a* per surface area was also decreased by high temperature (data not shown).

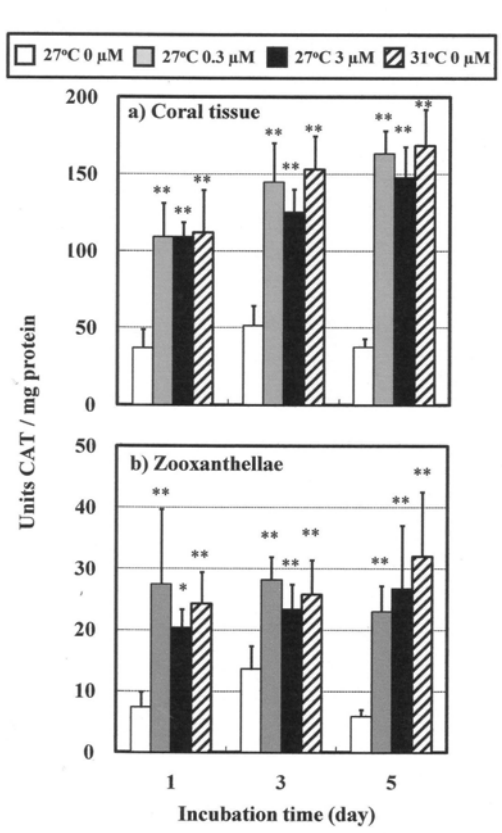

Figure 2: Effects of hydrogen peroxide and/or temperature on the activity of catalase (CAT) in (a) coral tissue and (b) zooxanthellae. Means±S.D. (n=6).
**: P<0.01, *: P<0.05 significantly different with Dunnett's test for comparing treatments to a control.

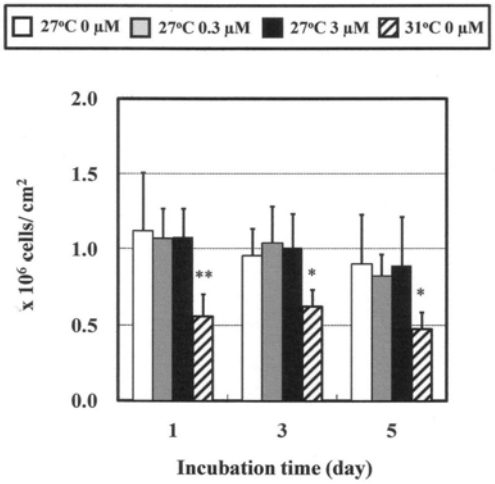

Figure 3: Effects of hydrogen peroxide and/or temperature on the density of zooxanthellae per coral surface area. Means±S.D. (n=6).
**: P<0.01, *: P<0.05 significantly different with Dunnett's test for comparing treatments to a control.

Summary

We studied the effects of increased H_2O_2 in the surrounding seawater and/or high seawater temperature on antioxidant enzyme activities of SOD and CAT in the coral *G. fascicularis*. With increased concentrations of H_2O_2, CAT activities in both coral tissue and zooxanthellae were increased, but SOD activities were relatively unaffected. In contrast, with high seawater temperature, both SOD and CAT activities in coral tissue and zooxanthellae were increased, indicating that both $\bullet O_2^-$ and H_2O_2 were formed within the cell by the increased temperature stress. CAT activities in zooxanthellae were not significantly different even with a ten-fold difference in H_2O_2 concentration. It is possible that the coral tissue and zooxanthellae reached a limit of CAT activity. Coral bleaching was not observed at the levels of H_2O_2 used for our experiments during the 5-day exposure period. Although the long-term effects of H_2O_2 remain unknown, our results suggest that coral bleaching would likely not occur as a result of short-term (5 days) exposure to increased H_2O_2 in the seawater, and that high seawater temperature had a greater impact on the enzymatic activities of the coral.

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References

Beers RF, Sizer IW (1952) Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133–140

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein dye binding. Anal. Biochem. 72: 248 -254

Brown BE (1997) Coral bleaching: causes and consequences. Coral Reefs 16: 129-138

Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM (2002) Oxidative stress and seasonal coral bleaching. Free Radical Biology & Medicine 33(4): 533-543

Dykens JA, Shick JM, Benoit C, Buettner GR, Winston GW (1992). Oxygen radical production in the sea-anemone *Anthopleura elegantissima* and its endosymbiotic algae. J. Exp. Biol. 168: 219-241

Dykens JA, Shick JM (1982) Oxygen production by endosymbiotic algae controls superoxide dismutase activity in their animal host. Nature 297: 579-580

Elstner EF, Heupel A (1976) Inhibition of nitrite formation from hydroxylammonium chloride - simple assay for superoxide dismutase. Anal. Biochem 70: 616 -620

Fitt WK, Brown BE, Warner ME, Dunne RP (2001) Coral bleaching: interpretation of thermal tolerance limits and thermal thresholds in tropical corals. Coral Reefs 20: 51-65

Fridovich I (1986) Biological effects of the superoxide radical. Arch Biochem Biophys 247: 1-11

Fujimura H, Higuchi T, Shiroma K, Arakaki T, Hamdun AM, Nakano Y, Oomori T (2008) Continuous-flow complete-mixing system for assessing the effects of environmental factors on colony-level coral metabolism. Biochem. Biophys. Methods 70: 865-872

Higuchi T, Fujimura H, Arakaki T, Oomori T (2009, in press) The synergistic effects of hydrogen peroxide and elevated seawater temperature on the metabolic activities of the coral,*Galaxea fascicularis*. Marine biology

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, *c*, and c_2 in higher plants, algae and natural phytoplankton. Biochem Physiol Pflanz 167:191-194

Lesser MP, Stochaj WR, Tapley DW, Shick JM (1990) Bleaching in coral reef anthozoans : effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. Coral Reefs 8: 225-232

Levy O, Achituv Y, Yacoci YZ, Stambler N, Dubinsky Z (2006) The impact of spectral composition and light periodicity on the activity of two antioxidant enzymes (SOD and CAT) in the coral *Favia favus*. Journal of Experimental Marine Biology and Ecology 328: 35-46

Miller WL, Kester DR (1988) Hydrogen peroxide measurement in seawater by (p-hydroxyphenyl)acetic acid dimerization. Anal. Chem. 60: 2711-2715

Mydlarz LD, Jacobs RS (2004) Comparison of an inducible oxidative burst in free-living and symbiotic dinoflagellates reveals properties of the pseudopterosins. Phytochemistry 65: 3231-3241

Oyanagui Y (1984) Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. Analyt. Biochem. 142: 290 -296

Ross C, Santiago-V´azquez L, Paul V (2006) Toxin release in response to oxidative stress and programmed cell death in the cyanobacterium *Microcystis aeruginosa*. Aquatic Toxicology 78: 66–73

Stone L, Huppert A, Rajagopalan B, Bhasin H, Loya Y (1999) Mass coral reef bleaching: a recent outcome of increased El Nino activity? Ecol. Lett. 2:325-330

Shick JM, Lesser MP, Dunlap WC, Stochaj WR, Chalker BE, Won JW (1995) Depth dependent responses to solar ultraviolet radiation and oxidative stress in the zooxanthellae coral *Acropora microphthalma*. Mar. Biol. 122: 41-51

Smith DJ, Suggett DJ, Baker NR (2005) Is photoinhibition of zooxanthellae photosynthesis the primary cause of thermal bleaching in corals? Global Change Biology 11: 1–11

Suggett DJ, Warner ME, Smith DJ, Davey P, Hennige S, Baker NR (2008) Photosynthesis and production of hydrogen peroxide by *Symbiodinium* (Pyrrhophyta) phylotypes with different thermal tolerances. Source: Journal of Phycology 44: 948-956

Takahashi M, Asada K (1983) Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. Arch Biochem Biophys 226:558-566

Takahashi S, Whitney S, Itoh S, Maruyama T, Badger M (2008) Heat stress causes inhibition of the *de novo* synthesis of antenna proteins and photobleaching in cultured *Symbiodinium*. Proceedings of the National Academy of Sciences of the United States of America 105: 4203-4208

Yakovleva I, Bhagooli R, Takemura A, Hidaka M (2004) Differential susceptibility to oxidative stress of two scleractinian corals: antioxidant functioning of mycosporine-glicine. Comparative Biochemistry and Physiology, Part B 139: 721-730