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Temperature-Induced Stress on Growth and Expression of Hsp in Freshwater alga *Scenedesmus quadricauda*[1]

S. ZARGAR, K. KRISHNAMURTHI, S. SARAVANA DEVI,
T. K. GHOSH, AND T. CHAKRABARTI[*]

Environmental Biotechnology Division, National Environmental Engineering Research Institute (NEERI), Nehru Marg, Nagpur-440 020 (MS), India

Objective To investigate the impact of various levels of sublethal temperature (26℃, 31℃, 33℃, 36℃, and 39℃) on growth and heat shock protein (hsp) expression in freshwater green alga *Scenedesmus quadricauda*. **Methods** Impact of selected levels of temperature on growth rate (based on optical density), population count, chlorophyll-*a* and biomass of the alga was evaluated in artificial growth medium for 19 days. To determine the induction of hsp in the alga, it was exposed to selected temperature levels for 3 h and further kept for 6 h at culturing condition at 26℃. Induction of hsp was confirmed by immuno-detection followed by SDS-polyacrylamide gel electrophoresis. **Results** The selected growth parameters such as growth rate, population count, chlorophyll-a and biomass were reduced significantly ($P<0.001$) at 39℃. However, hsp 70 expression was observed only at 39℃. **Conclusion** Temperature up to 36℃ may be considered as the limit of safe exposure for thermal stress for the alga *Scenedesmus quadricauda*.

Key words: Temperature; *Scenedesmus quadricauda*; Heat shock protein (Hsp)

INTRODUCTION

Temperature is one of the major environmental factors and plays a critical role in growth, reproduction, migration, succession pattern and metabolism of organisms and communities[1]. In general, elevated water temperature causes changes in species composition, species dominance, standing crop and productivity of biota including phytoplankton communities in any aquatic ecosystem. Thus warm water discharges from power plants into receiving water bodies may adversely affect aquatic ecology. The productivity of ecology depends on the quality and quantity of the plankton biomass production. Phytoplankton, being placed in the bottom rung of the food chain in aquatic biotope, fluctuates in density and the biomass directly affects the entire biotic structure of ecosystem. Every organism has a range of temperature that it can tolerate, which is known as tolerance levels. As temperatures get too far above or below this preferred range, the number of individuals of the species decreases until finally there are few, or none. The range of temperature tolerated by the life form is completely wide but each species shows characteristic-limited temperature preference and tolerance[2-3]. It is known that plants and animals are able to thrive best in certain temperature ranges and changes in the temperature of a body of water will influence the types and number of organisms in aquatic ecosystems.

Living organisms respond at the cellular level to unfavorable conditions such as temperature-shock, chemical and other stressful situations of many different origins, by the rapid, vigorous, and transient acceleration in the synthesis of a class of proteins known as heat shock proteins (hsps) or stress proteins[4]. In many of the model organisms studied, a family of four major heat-shock proteins (hsps) of 90, 70, 60, and 12-16 KDa are the most prominent, and these proteins have been frequently referred to as hsp 90, hsp 70, hsp 60, and low-molecular weight (LMW) hsps respectively[5]. Heat shock proteins are important for regulation of gene expression, inducing

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[*]Correspondence should be addressed to T. CHAKRABARTI, Director-Grade Scientist, Environmental Biotechnology Division, National Environmental Engineering Research Institute (NEERI), Nehru Marg, Nagpur-440 020 (MS), India. Tel: 01-712-2249757. Fax: 01-712-2249961. E-mail: twmneeri_ngp@sancharnet.in, kmurthi_saravana@hotmail.com, santoshzargar@yahoo.com

Biographical note of the first author: Santosh ZARGAR, born in 1974, research fellow, major research work emphasizes on the effects of various nutritional conditions on fish physiology.

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thermotolerence and protecting cells from undergoing apoptosis. Hsps are highly conserved and found in all organisms, from archaebacteria to eubacteria, yeasts, plants and vertebrates, including humans[6-7]. Some hsps are constitutively expressed and play a critical role in normal physiology, growth and development of cells[8]. Plants respond to stress as individual cells and whole organisms. Evidence from literature and experimental studies suggests that small chloroplast hsps are involved in plant thermotolerance but their site of action is unknown[9]. Green microalgae are of great value, both as organisms for fundamental biological industry and as a resource for biotechnological industry. Sizova *et al.* [10] studied the expression of aph VIII gene in *Chlamydomonas reihardtii* (a green alga) in combination with different regulatory elements of the nuclear genes rbc S2, hsp 70A, cop (chlamyopsin) and the first intron of the rbcs2 gene. These experiments led to the development of the first stable enzymatic selection marker for the nuclear genome in the alga. Hsps are strong candidates for biomarkers of environmental pollution since they are activated very early in the cascade of cellular events that follow toxic exposure and at concentrations below the lethal dose. Still, little is known about the induction of Hsp under different environmental conditions. Bierkens *et al.*[11] conducted a study to detect the synthesis of hsp 70 in *Raphiodocelis subcapitata* (a green alga) in response to changes in pH, temperature, humic acids, nitrates, and phosphorus, and found that only temperature and pH are able to induce acquired tolerance. Investigations, carried out by Wolfe *et al.*[12], using *Isochrysis galbana*, a golden brown alga and primary producer in marine food chains, found that the organism can efficiently induce hsp 60 in response to low concentrations of petroleum hydrocarbons modeled after an oil spill and dispersant clean-up. Golldack *et al.*[13] saw different protein induction with cultures of unicellular alga, *Dunaliella parva*, acclimated to different salinities, suggesting that differences in stress tolerance in the same species under different physiological conditions are related to differences in hsp expression[14].

The focus of this study was to investigate the impact of various sublethal levels of temperature on survival, growth, and heat shock protein formation of the green alga *Scenedesmus quadricauda*.

MATERIALS AND METHODS

Cell Culture and Exposure

The alga *Scenedesmus quadricauda* was selected for the present study because it is easy to culture in laboratory, cosmopolitan in habitat and stands as a vital component among aquatic food chain organisms. The alga was isolated from Kadra reservoir, Kaiga, Karnataka of India (where cooling water from Kaiga Nuclear Power Plant was discharged) and its pure culture was maintained in Beckers' nutrient solution for Chlorella[15]. All cultures were kept in 1-*l* glass flasks containing 500 mL of test culture solution at the density of 4×10^4 cell mL^{-1} for 19 days at 26℃, 31℃, 33℃, 36℃, and 39℃. Experiments were run in triplicate.

Parameters Selected

Impact of various levels of temperature on growth rate (based on optical density), population count, chlorophyll-*a* and biomass of the alga was evaluated. Optical density of the culture was measured at 560 nm in Perkin Elmer, Spectrometer Lambda 900. For measurement of growth through biomass, 15 mL of samples was filtered through pre-weighed millipore filter paper (GF/C; size 4.7 cm), oven dried at 100℃ and finally weighed in balance up to the 4th decimal place. For chlorophyll-*a* estimation, 15 mL of samples was centrifuged with $MgCO_3$. The intensity of colour due to pigment concentration was read spectrophotometrically at 630 nm, 647 nm, and 664 nm. To evaluate population density of the alga on different days, Lackey Drop method[16] was used. Maximum specific growth rate (μmax) was calculated for assessing the maximum growth rate of the alga during the period of experiment in each concentration. The cultures were kept at a light intensity of 35 μE (12:12 light/dark regimen). Details of the methodology have been referred in Standard methods[17-18].

Heat Shock Protein Expression

In order to determine the induction/expression of heat shock protein in the alga, 1-*l* glass flasks containing 500 mL of test culture solution exposed to selected temperature levels viz. 31℃, 33℃, 36℃, and 39℃ for 3 h in thermostatic water baths in triplicate. Each flask contained 10×10^4 cell mL^{-1} of the alga. After thermal exposure, the algal cultures were maintained at culturing conditions at 26℃ for 6 h. During this period equal volume (100 mL) of the culture was taken out at 0 (just after thermal exposure), 1, 3, and 6 h. Appropriate control (algal culture at 26℃) was maintained during the experiment. The cultures were kept at culturing conditions after thermal exposure because during the thermal exposure hsps could not be induced.

Protein Quantification

In order to extract proteins from each sample, the cultures were centrifuged at 1000 ×g for 10 min at 4℃. The pellets were then lysed with SDS gel loading buffer {50 mmol/L Tris. HCl (pH 6.8), 2% SDS, 0.1% Bromophenol blue and 10% Glycerol} and sonicated for 15 s (20 KHz, 75 W) thrice in ice. The homogenate was again centrifuged at 5000×g for 30 min at 4℃. Total protein concentration in supernatant was determined spectrophotometrically using Lowry's method[19].

SDS-PAGE/Electroblotting/Immunodetection

The sample containing 50 µg of protein was loaded in 10% SDS polyacrylamide gel[20], which was then transferred to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia, UK) using electroblotting apparatus. The electroblotting (western blotting) was carried out according to the laboratory manual of molecular cloning. Further, immunodetection was carried out according to the instruction manual (Hybond ECL, Amersham Pharmacia, UK).

Statistical Analysis

All the experiments were run in triplicate, and the results were presented as means and levels of significance, following Student's *t* test using a statistical software Statistica version 5.0.

RESULTS

Tolerance of Alga to Elevated Temperature

In the present investigation, the alga was exposed to selected temperatures, viz 26℃, 31℃, 33℃, 36℃, and 39℃ for 19 days in laboratory. During the experiment selected growth parameters, viz, density, chlorophyll-*a*, biomass and growth rate (based on optical density values) of the alga were reduced drastically at 39℃ (Figs. 1-4). The maximum growth rate of *Scenedesmus quadricauda* was observed at 26℃ compared to other selected temperatures during experiment. The growth rate was decreased at 39℃ after 6 days of exposure. Density, chlorophyll-*a* and biomass were increased even after 17 days of exposure at 31℃ and 33℃, and decreased at 36℃ after exposed for 15, 17, and 15 days respectively. Student's *t* test revealed that the selected growth parameters were significantly higher at 26 ℃ (P<0.001) than at 31℃, 33℃, 36℃, and 39℃. Specific growth rate (µ) based on OD, was calculated for all the temperature levels and maximum specific growth rates (μ_{max}) were 0.377, 0.486, 0.510, 0.360, and 0.182 at 26℃, 31℃, 33℃, 36℃, and 39℃ respectively (Fig. 5). While μ_{max} was achieved on the 3rd day at 26℃. and on the 11th day at 31℃. Temperature above 36℃ appeared to be unfavourable for proper growth of the alga. Temperature up to 36℃ may be considered as the limit of safe exposure for thermal stress of the alga.

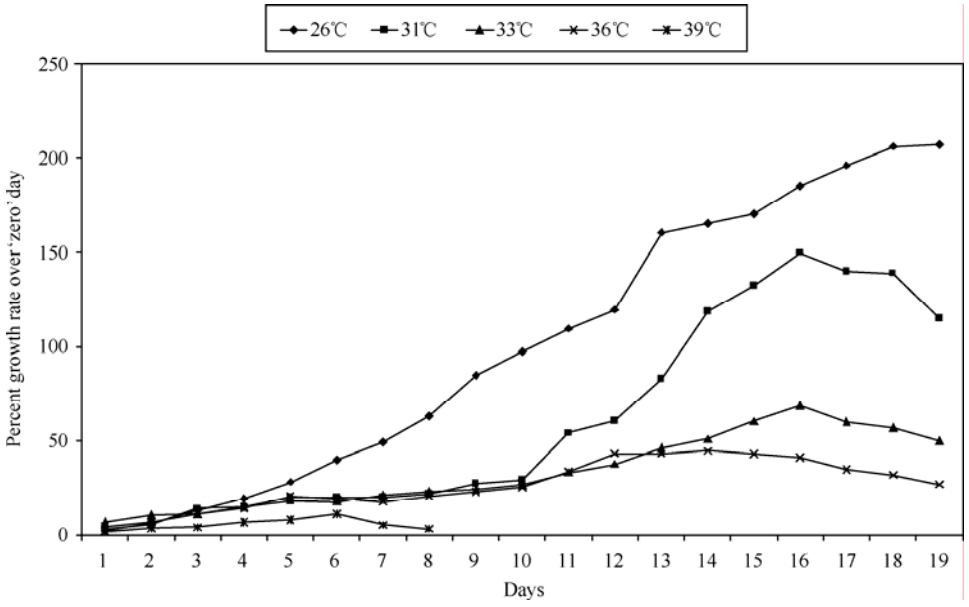

FIG. 1. Effect of different temperature levels on percent growth rate of the alga *Scenedesmus quadricauda.*

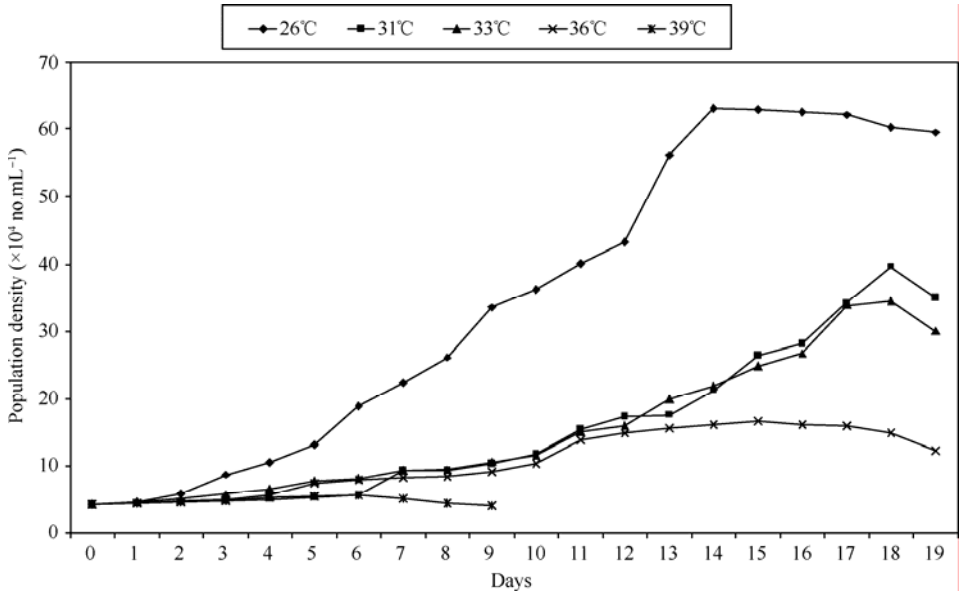

FIG. 2. Effect of different temperature levels on population density of the alga *Scenedesmus quadricauda*.

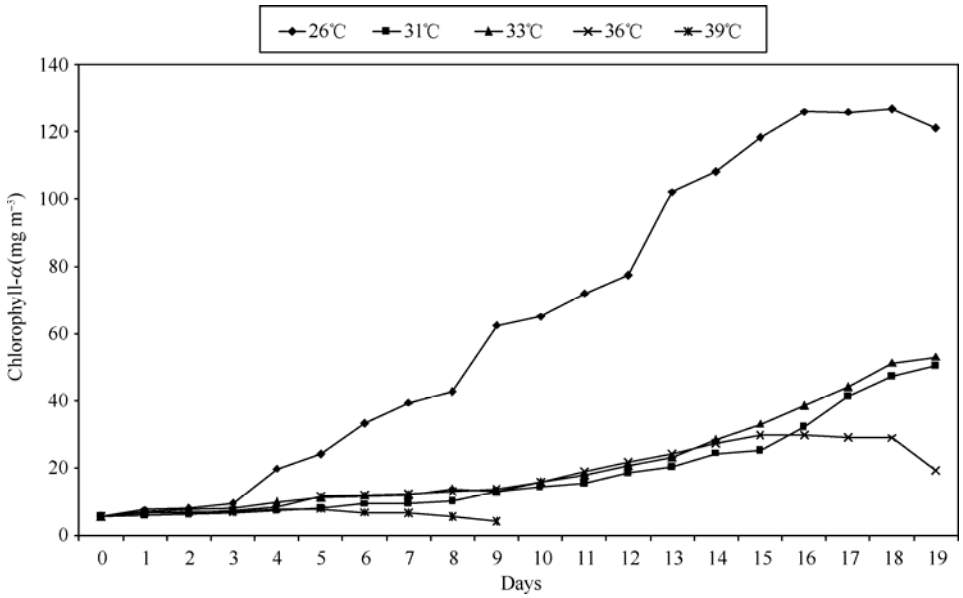

FIG. 3. Effect of different temperature levels on chlorophyll-*a* of the alga *Scenedesmus quadricauda*.

Heat Shock Protein Expression

In another experiment, when the alga was exposed to elevated temperatures (31℃, 33℃, 36℃, and 39℃) for 3 h and further incubated at culturing conditions at 26℃ for 6 h, hsp 70 was expressed only at 39 ℃ in the alga. The *Scenedesmus quadricauda*, incubated for 3 and 6 h showed a higher level of expression of the hsp as than that incubated for 0 and 1 h (Fig. 6). The expression level of control sample was the lowest.

DISCUSSION

Tolerance of alga to Elevated Temperature

Scenedesmus, with about 100 species is widely distributed in almost all pools of standing water. The number of cells in a coenobium is usually 4-8 cells. The cells are ellipsoid to fusiform in shape. Each cell is uninucleate and contains a single longitudinal laminate chloroplast with one pyrenoid. Each cell in a colony is capable of producing a daughter colony by

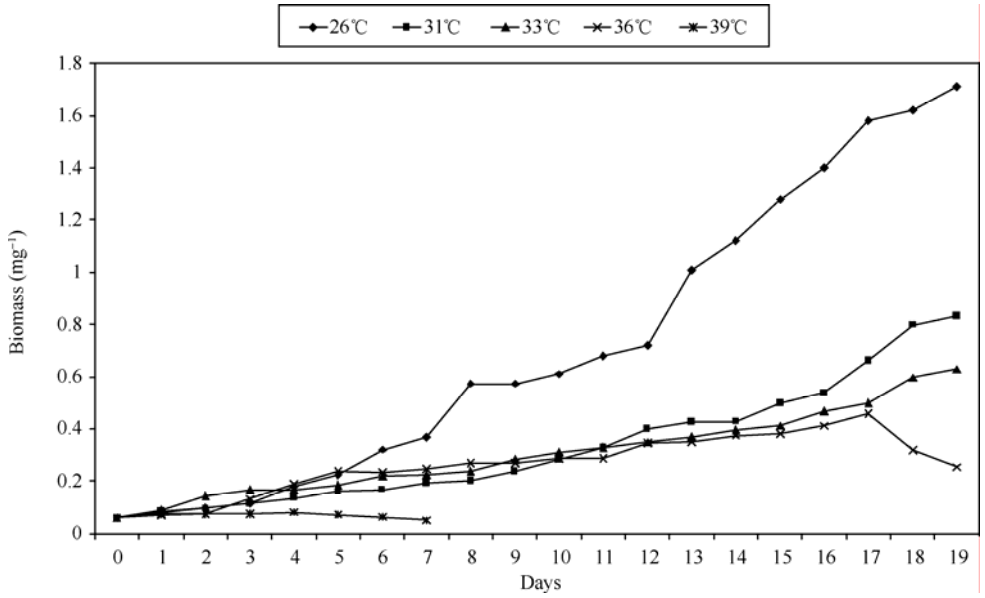

FIG. 4. Effect of different temperature levels on biomass of the alga *Scenedesmus quadricauda.*

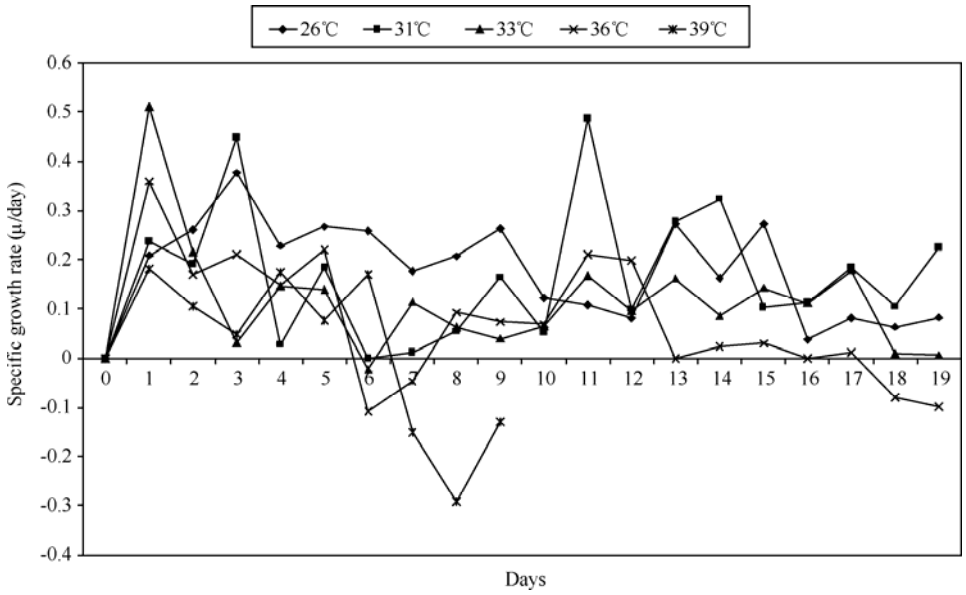

FIG. 5. Effect of different temperature levels on specific growth rate of the alga *Scenedesmus quadricauda.*

division. In a dividing cell, which is about to form a daughter colony, the protoplast divides transversely followed by vertical division. A single cell may produce as many as 2, 4, 8, 16, and 32 autospores.

During the study period all the selected parameters showed a considerable growth of the alga *Scenedesmus quadricauda* at 36℃ Similar results are observed in an another green alga *Chlorella vulgaris*[21]. A definite correlation between chlorophyll-*a* and biomass, containing 51%-58% protein[22], at certain levels of temperature was not observed in *Scenedesmus quadricauda*. It can be mentioned that the degradation product of chlorophyll-*a* is phaeophytin-*a*, which is formed when magnesium is lost from the porphyrin ring, and the product has an absorption spectrum similar to that of chlorophyll-*a*[23]. Based on studies with *Scnedesmus obtusiuscula*, Felfoldy[24] found that 35℃ is the optimum temperature for photosynthesis and growth of the alga. Reynold[25] grew another green alga *Selenastrum capriocornutum* successfully within 15℃ and 37℃, and found that the growth is arrested at

38℃. Margalef[26] found that cultures of *Scenedesmus oblicus* grown at 23℃ have smaller cells than those grown at 13.5℃. He, however, did not get any correlation between dry weight biomass and size of cells.

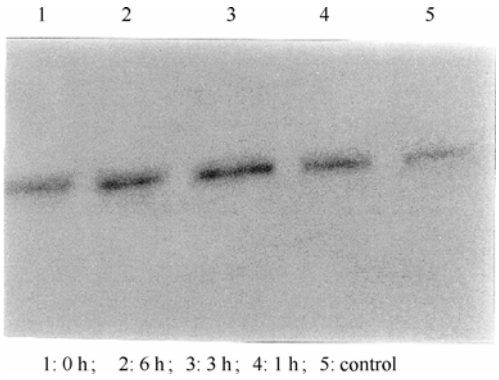

FIG. 6. Western blots (hsp 70) of freshwater alga *Scenedesmus quadricauda* at different hrs of exposure at 39℃.

Literature reveals that green alga is dominant 30℃ and 35℃[27]. *Mougeotia sp.* (green alga) becomes conspicuous when temperature is below 35℃. In view of physiological differences, various species under different trophic levels exhibit different temperature tolerances (Table 1). In general, bacteria, cyanophyceae and protozoa can tolerate higher levels of temperature. It appears that cold-water fishes are most sensitive to elevated temperatures followed by zooplankton and phytoplankton. Maximum temperature tolerance limits of most aquatic organisms in India are not available in literature. Recently, Zargar and Ghosh[28] and Das *et al.*[29] have estimated the preferred temperatures of selected zooplankton (26℃-31℃) and Indian major carps (31℃-36℃) respectively. Tolerance levels, often reported for groups, are sometimes misleading. For example, while maximum tolerance level for the diatom is referred as 30℃[30] (Table 1), the same for a species (*Nitzchia filiformis*) of the same group is reported as 31℃-35℃ by Patric *et al.*[27] (Table 1). Data on this aspect need to be generated in India at variable environmental conditions. These temperature requirements are important in determining the distribution and maximum allowable temperature limit (MATL) of organisms in aquatic ecosystems.

Heat Shock Protein Expression

In the present investigation hsp 70 was induced in the alga. The induction of hsp 70 has been confirmed in a wide range of organisms, i.e. microorganisms to humans[31]. Misfolded proteins formed during stress, bind to hsp 70. Hsp 70 is associated with the ribosomal subunits. Hsp70 gene is a sensitive biomarker for different classes of environmental pollutants in green algae[11]. A blue green alga *Synechocystis sp*. PCC6803 has been used as a model organism for investigating hsp's function and when a particular hsp is withdrawn, the alga becomes thermo sensitive[32]. Hsp's function is seen mainly in blue green algae, especially in genera *Oscillatoria*, *Synechocystis*, *Anabaena*, and *Synechococcus* in various countries[5, 33-35]. At both cellular and organism levels, induction of synthesis of stress protein correlates with acquired tolerance, a phenomenon which increases the ability of the exposed organisms to survive a subsequent more severe stress that would have otherwise be lethal[31]. Whether the kinetics of hsp 70 induction is similar after exposed to selected temperature levels for days remains a topic for further investigations. The

TABLE 1

Temperature Limits for Different Fresh Water Organisms

Groups/organisms	Maximum Temperature Tolerance Limits
Bacteria[36]	88℃
Chlorophyceae[28]	25℃-35℃
Ankistrodesmus sp.[36]	20℃-25℃
Cocconeis schlettum[36]	26℃-41.5℃
Mastogloia smithi[36]	20℃-25℃
Scenedesmus oblicus[30]	38℃
Cyanophyceae[36]	80℃
Oscillatoria chalybae[27]	47℃
Phormidium ambiguum[37]	47℃
Diatoms[30]	30℃
Nitzchia filiformis[27]	31℃-35℃
Protozoa[36]	54℃
Brachionus angularis[30]	25℃
Filinia hafmanni[30]	15℃
Cladocera[27]	35.5℃
Daphnia laevis[36]	27℃-29℃
Daphnia pulex[30]	25℃
Copepoda[27]	34℃-42℃
Nauplius[37]	25℃
Diptera[27]	40℃
Benthos[36]	30℃
Barbus capensis[38]	21℃
Brook trout[38]	22℃
Brown trout[38]	29℃
Salmo salar[39]	10℃

differences in the experimental set-up for the determination of hsp 70 synthesis on the one hand and growth inhibition in *Scenedesmus quadricauda* on the other hand, do not allow strict comparison of the sensitivity of both assay systems. It should be noted that preliminary experiments showed that increasing the growth parameters such as OD, population count, chlorophyll-*a* and biomass of the alga in growth inhibition assay could not yield a higher sensitivity. It seems that hsp 70 induction occurs at a much lower temperature level. In the present study, since the synthesis of stress-70 in the alga following exposure to 39℃ was apparently transient, this response to stress was likely to have any role in the physiological adaptation of the organisms, thus not supporting the suggestion that synthesis of stress protein correlates with acquired tolerance. The induction of response in the alga may be due to conformational changes in protein caused by the exposed temperature.

Temperature up to 36℃ may be considered as the limit of safe exposure for thermal stress of the alga, however the adverse effect on growth depletion and hsp induction was observed at 39℃. Since application of stress protein to environmental monitoring is still limited, the present study provides validation for the development of hsp as a biomarker of exposure and effect. The generated data on the general biotic features of aquatic ecosystem would be useful in determining threshold levels of temperature and in diagnosing the tolerance levels of different plankton. The database can serve as a reference source for initiation of review and reaffirm the thermal water quality standards.

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