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Evaluation of heat shock protein 70 as a biomarker of environmental stress in *Fucus serratus* and *Lemna minor*

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Heat shock proteins (Hsps) are known to be induced in response to short-term stress. The present study aimed to evaluate the potential of Hsp70 as a biomarker of stress produced by increased temperature, osmotic pressure, and exposure to cadmium and sodium chloride in marine macroalgae and fresh water plant species. An indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) was developed with a working range of 0.025–10 μg ml^{-1} using a monoclonal antibody raised against purified Hsp70 of *Phaseolus aureus* (mung bean). *Fucus serratus* (toothed wrack), *Chondrus crispus* (Stackhouse or Carrageen moss), *Ulva lactuca* (sea lettuce) and *Lemna minor* (common duckweed) sample extracts were stressed for up to 24 h and then tested in the IC-ELISA. The presence of Hsp70 and cross-reactivity of the monoclonal antibody was confirmed by Western blot. The heat shock response was confirmed in each species using a 2-h 42°C treatment. Following heat shock, Hsp70 concentrations increased to a peak at 2 h (*F. serratus*) or 4 h (*L. minor*), after which concentrations decreased. Osmotic and cadmium stresses also resulted in elevated Hsp70 concentrations in samples of *F. serratus* and *L. minor* when compared with unstressed controls. In both, osmotic and metal stress, the production of Hsp70 increased to a maximum and subsequently decreased as the stressor levels increased. Results suggest that Hsp70 IC-ELISA could potentially be applied to the detection of stress in these aquatic species, although it would probably be most effective when used in conjunction with other measurements to provide a stressor-specific biomarker profile or fingerprint.

Keywords: biomarker, heat shock proteins, Hsp70, enzyme-linked immunosorbent assay (ELISA), stress, *Lemna minor*, *Fucus serratus*.

Introduction

Marine macroalgae and fresh-water plant species have been suggested as potential biomarkers of water quality. However, experimental procedures conducted with such species to determine whether they are subject to stress have predominantly involved the detection of growth impairment. Such studies, however, take several days to complete (Park 1948, Stromgren 1994, Munda and Veber 1996). Responses at the molecular and biochemical level occur more rapidly and are therefore of potential use as biomarkers of environmental quality (Lindquist and Craig 1988, Vierling 1991, Lee 1992, Ernst and Peterson 1994,

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Lewis *et al.* 1999, Bierkens 2000). Heat shock proteins (Hsps) have been proposed as biomarkers in a number of species (Lewis *et al.* 1999, Nadeau *et al.* 2001).

The synthesis of Hsps is one of the biochemical responses to environmental stress (Hightower 1980, Morimoto *et al.* 1994). Hsps are a group of highly conserved proteins that are expressed in response to stresses such as increased/decreased temperature, heavy metal exposure and oxygen depletion (Wu *et al.* 1993, Morimoto *et al.* 1994, Boston *et al.* 1996, Sanita di Toppi and Gabbrielli 1999, Bierkens 2000). They are generally grouped into families: Hsp100, Hsp90, Hsp70, Hsp60 and low molecular weight Hsps (De Pomerai 1996, Latchman 1999). The Hsp70 family is one of the most abundant of these proteins. Different organisms produce variable numbers of isoforms of Hsp70 (Boorstein *et al.* 1994). At least one isoform, heat shock cognate (Hsc) 70, is constitutively expressed and assists in the correct folding of nascent polypeptides (Hartl 1996). The stress-inducible form (Hsp70) is considered to have a variety of roles including augmentation of Hsc70, refolding partially unfolded proteins and facilitating the removal of denatured proteins (Morimoto *et al.* 1994, Hartl 1996). Because Hsp70 concentrations are increased in response to environmental stress, it has potential for use as a biomarker (De Pomerai 1996, Lewis *et al.* 1999, Bierkens 2000, Nadeau *et al.* 2001). A Western blotting method for Hsp70 has recently been proposed as a biomarker for pollutants in the earthworm (*Lumricus terrestris*) (Nadeau *et al.* 2001).

The species chosen for the present study represent the marine (*Fucus serratus*, *Chondrus crispus*, *Ulva lactuca*) and fresh water (*Lemna minor*) environments. The absence of a vascular system in the seaweeds and the direct uptake of material from the surrounding water suggest that biochemical changes within these species should reflect the quality of the seawater. *F. serratus* (toothed wrack) is a brown seaweed found at mid-tide level. *C. crispus* (Stackhouse or Carrageen moss) is a red seaweed also found at mid-tide level and in tidal pools. *U. lactuca* (sea lettuce) is a small green alga found at all intertidal levels. These species were selected partly because of their distribution in marine ecosystems and partly because of their potential for use as indicator species (Dickinson 1963, Gutierrez and Fernandez 1992, Vasquez and Guerra 1996, Lewis *et al.* 2001). *L. minor* (common duckweed) is a widely distributed freshwater species that is free floating. This would suggest that any biochemical changes would directly reflect water quality (Wang 1986, Taraldsen and Norberg-King 1990, Teisseire and Vernet 2000).

The type of assay used was the enzyme-linked immunosorbent assay (ELISA). ELISAs can provide a cheap, rapid and specific means of quantitating diverse target analytes, including pesticides or biomolecules, in complex matrices such as environmental samples (Bonwick *et al.* 1996, Lewis *et al.* 1999, Bierkens 2000, Nadeau *et al.* 2001). ELISA development requires the use of antibodies specific for the target analyte. A monoclonal antibody previously raised by our group (Wilkinson *et al.* 1990) against purified *Phaseolus aureus* (mung bean) Hsp70 was used. This antibody has been shown to recognize Hsp70 from a variety of species including cotton, wheat and barley (Wilkinson *et al.* 1990, Adham *et al.* 1991, Wu *et al.* 1993, Marmiroli *et al.* 1997). Given the highly conserved nature of Hsp70, it was considered likely that the anti-*P. aureus* Hsp70 antibodies would cross-react

with Hsp70 from the species used in the current work. The specificity of the anti-Hsp70 antibody in complex mixtures would enable development of an IC-ELISA whilst avoiding time-consuming and technically demanding procedures such as the purification of Hsp70 from these species and the production of additional monoclonal antibodies. Furthermore, an assay based on the use of these antibodies could be used to examine the potential of Hsp70 as a biomarker in additional aquatic species.

The main aims of the study were as follows: (1) to determine the specificity of a monoclonal anti-*P. aureus* Hsp70 antibody to *F. serratus* and *L. minor* plant extracts using Western blotting; (2) to develop a simple and rapid ELISA for the quantification of Hsp70; and (3) to assess whether Hsp70 could be used as a biomarker by determining the ELISA response to exposure of the different species to a variety of stressors.

Materials and methods

Materials

All chemicals were obtained from Sigma Chemical Co. (Poole, UK) unless stated otherwise.

Sample collection and culture

F. serratus, *C. crispus* and *U. lactuca* were collected from the intertidal zone as soon as it was uncovered by the ebb tide at Penmon Point, Anglesey, UK (Grid Ref. SH6481). *L. minor* was collected from a local pond (Middlewich, UK).

Seawater and *L. minor* culture medium was filtered (0.2 μm filter) before use. Samples of seaweed were kept in seawater sampled from source for 24 h with timed artificial illumination (220 $\mu mol\ m^{-2}\ s^{-1}$, 12 h photoperiod, 16°C) before treatments to equilibrate.

A sterile culture of *L. minor* was prepared by immersing the samples for 30 s in 1% sodium hypochlorite followed by washing with sterile dH_2O. Samples were then cultivated for 28 days in glass tanks of aerated culture medium (2% w/v Phostrogen [Fisons Ltd, Loughborough, Leics, UK] in dH_2O) with timed artificial illumination (220 $\mu mol\ m^{-2}\ s^{-1}$, 12 h photoperiod, 18°C) after which subcultures were taken and the process repeated.

Heat stress

A temperature of 42°C was chosen to demonstrate the heat shock responses. This temperature has been shown to illicit strong Hsp70 responses in plant material (Wilkinson *et al.* 1990).

Seaweed. Subsamples (5 mm discs) of *F. serratus* were transferred to 42°C seawater for 0, 2, 4, 6, 8, 12 or 24 h, after which they were dried with filter paper, snap frozen in liquid nitrogen and stored at −70°C until required. Samples of *C. crispus and U. lactuca* were treated in the same manner except that tests were carried out at 0 and 4 h after exposure to 42°C seawater.

Duckweed. Subsamples of *L. minor* were transferred to 42°C medium for 0, 2, 4, 6 or 8 h, dried with filter paper, snap frozen in liquid nitrogen and stored at −70°C until required.

Osmotic stress

Seaweed. Filtered seawater was diluted with dH_2O to give a series of solutions with decreasing osmolarity. Subsamples of seaweed were incubated in these solutions for 4 h before being dried with filter paper, snap frozen in liquid nitrogen and stored at −70°C until required.

Duckweed. Subsamples of *L. minor* were placed in filtered culture medium containing 0, 31.25, 62.5, 125, 250, 500 and 1000 mM sodium chloride for 4 h before being dried with filter paper, snap frozen in liquid nitrogen and stored at −70°C until required.

Cadmium stress

Subsamples were placed in filtered seawater (*F. serratus*) or filtered culture medium (*L. minor*) containing 0, 5, 10, 25, 50 and 100 mM cadmium chloride (Cd^{++}) for 4 h before being dried with filter paper, snap frozen in liquid nitrogen and stored at −70°C until required.

Protein extraction and quantification

Frozen subsamples of seaweed and duckweed were homogenized (Waring blender) at 1:5 (w/v) with 10% poly(vinylpolypyrrolidone) and 90% extraction buffer — 25 mM Tris-HCl, pH 7.4, 10 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM -mercaptoethanol (TEB), 2 mM phenylmethanesulphonyl fluoride (PMSF), 2.5 mM 6-amino-*n*-hexanoic acid, 1 mM benzamidine. Sample extracts were centrifuged (13 500*g*, 20 min, 4°C) and the supernatants decanted before snap freezing in liquid nitrogen and storage at −20°C until required for analysis.

Salt was removed from sample extracts using a pressurized stirred cell with a 10-kDa membrane filter (XM10) in accordance with the manufacturer's instructions (Amicon Corp., Stonehouse, UK).

The protein content of extracts was determined using a Bio-Rad Protein Assay (Hemel Hempstead, UK) in accordance with manufacturer's instructions for microassay.

Hsp70 purification

Hsp70 was purified using a modification of a protocol previously used by the present authors (Wilkinson *et al.* 1990). Briefly, 350 g mung beans were transferred to 42°C for 2 h after which proteins were extracted. A 500-ml sample was loaded onto a DEAE Diethyl amino ethyl sepharose column previously equilibrated with 25 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM β-mercaptoethanol (TEB). The column was then washed with approximately 2 vols TEB. The Hsp70-containing fraction was eluted with 0.25 M NaCl in TEB. $MgCl_2$ was added to the eluted fraction to give a final concentration of 3 mM $MgCl_2$. The sample was then loaded onto an adenosine triphosphate (ATP) agarose column previously equilibrated with TEB 3 mM $MgCl_2$ (TEBM). The column was washed in 0.5 column vol. 0.5 M NaCl in TEBM. Pure Hsp70 was eluted with 3 mM ATP in TEBM.

Western blotting

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 4% stacking gel and 10% resolving gel in a Mini Protean II electrophoresis system and by electrophorectic transfer onto 0.45-μm nitrocellulose membrane (Bio-Rad). The nitrocellulose was blocked for 1 h with Tris-buffered saline (TBS) (25 mM Tris-HCl, pH 7.4, 1 M NaCl) with 1% (w/v) bovine serum albumin. The nitrocellulose was then probed with a monoclonal anti-*P. aureus* Hsp70 antibody diluted 1:1000 in TTBS (TBS with 0.05% v/v Tween 20 [polyoxyethylenesorbitan monolaurate]) with 1% (w/v) bovine serum albumin and incubated overnight at 4°C. The membrane was washed (thrice, 15 min per wash) with TTBS and then incubated with anti-mouse immunoglobin IgG (whole molecule) peroxidase conjugate diluted 1:1000 in TTBS (25°C, 1 h). After washing (thrice, 15 min per wash) with TTBS, bound antibody was visualized with the addition of a metal enhanced 3,3′-diaminobenzidine tetrahydrochloride substrate in accordance with the manufacturer's instructions (Perbio Science UK Ltd, Tattenhall, UK). Western blots were washed (thrice, dH_2O, 5 min) then dried in filter paper (Whatman No. 3) and stored in the dark.

Indirect competitive (IC)-ELISA

Optimization of IC-ELISA. IC-ELISA was optimized using a series of checkerboard titrations in which the concentrations of the 96-well plate (Immulon 4HBX, Thermo Life Sciences, Basingstoke, UK) coating antigen and the dilutions of the first and second antibodies were sequentially varied. The Hsp70 was heat treated (100°C, 5 min) before use as this enhanced binding by the anti-Hsp70 antibody (data not shown). Hsp70 in phosphate-buffered saline (PBS, pH 7.2) was added to the wells of 96-well plates (100 μl/well) and incubated overnight at 4°C. The wells were then emptied, blotted dry and blocked with 3% (w/v) dried non-fat milk powder in PBS (250 μl/well, 37°C, 1 h). After washing three times with wash buffer (PBS with 0.05% v/v Tween 20), a monoclonal anti-*P. aureus* Hsp70 antibody diluted in ELISA buffer (PBS with 0.001% v/v Tween 20) was added (50 μl/well) and the plates incubated (37°C, 1 h) then washed (thrice, wash buffer) and blotted dry. Goat anti-mouse IgG (whole molecule) peroxidase conjugate (diluted in 3% w/v dried non-fat milk powder in ELISA buffer) was added (50 μl/well) to the plate and incubated (25°C, 1 h), washed (thrice, wash buffer) and blotted dry. The bound antibody was visualized with the addition (100 μl/well) of 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) substrate solution (35 mM ABTS, 120 mM citric acid, pH 4.0, 0.05% v/v H_2O_2) incubated at 25°C for 30 min, then the absorbance was measured at 405 nm (MRX II Microplate Photometer, Thermo Life Sciences).

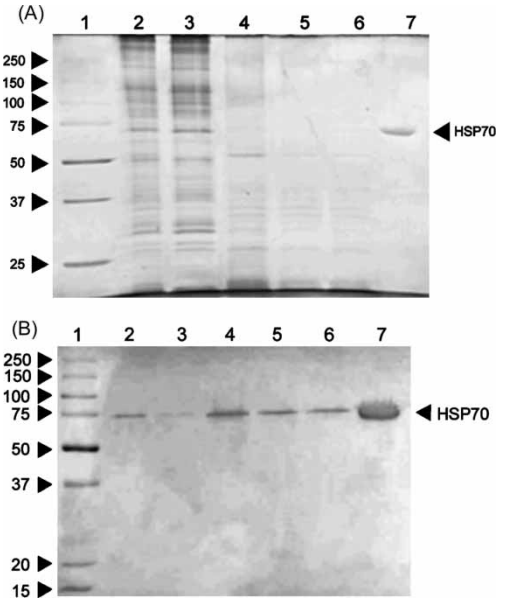

Figure 1. Samples during a purification protocol for Hsp70 from mung beans heat shocked at 42°C for 2 h. (A) sodium dodecylsulphate-polyacrylamide gel electrophoresis (10%), (B) Western blot. Lanes 1, molecular weight markers; 2, crude mung bean extract; 3, DEAE sepharose non-bound; 4, DEAE separose eluant; 5, adenosine triphosphate (ATP) column non-bound; 6, 0.5 M NaCl eluant; 7, 3 mM ATP eluant — pure Hsp70.

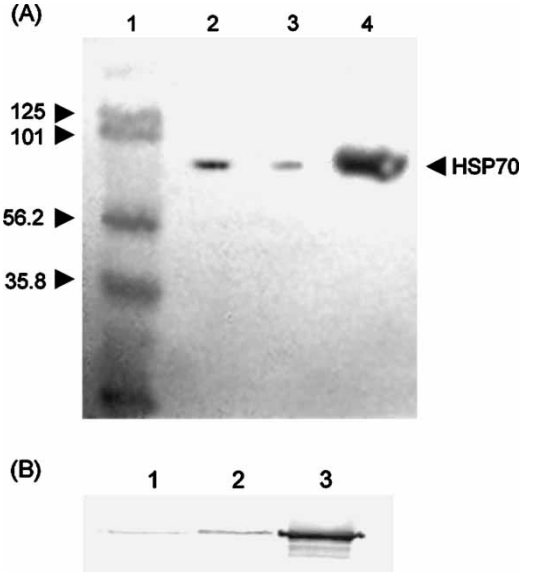

Figure 2. Western blot analysis of Hsp70 in sample extracts of *Lemna minor* and *Fucus serratus*. (A) 1, Prestained molecular weight markers; 2, *L. minor* heat shocked at 42°C for 4 h; 3, *L. minor* control (18°C); 4, purified Hsp70 extract from *Phaseolus aureus*. A total of 30 μg total protein were loaded onto each well. (B) 1, *F. serratus* control (17°C); 2, *F. serratus* heat shocked at 42°C for 4 h; 3, purified Hsp70 extract from *P. aureus*. A total of 30 μg total protein were loaded onto each well.

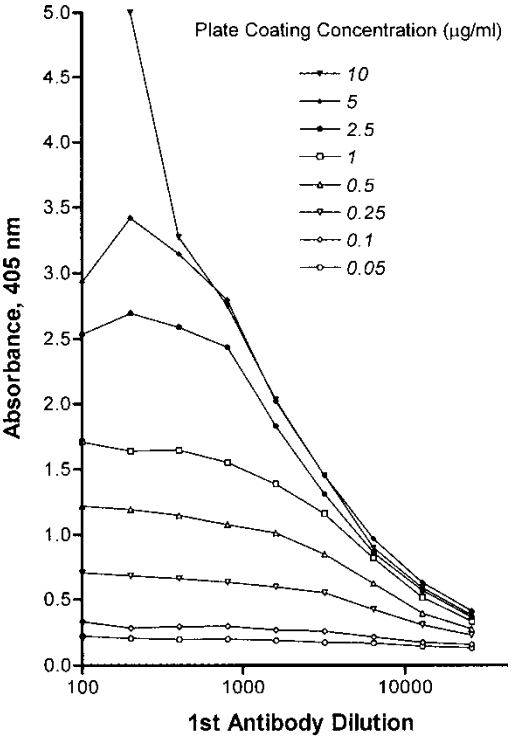

Figure 3. Optimization of an indirect competitive enzyme-linked immunosorbent assay plate coating and primary antibody dilution. Plates were coated with variable concentrations of purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, variable; second antibody 1/1000.

Optimized ELISA. Plates were coated with 5 $\mu g\,ml^{-1}$ in PBS (100 μl/well) heat treated Hsp70 (100°C, 5 min) and incubated overnight at 4°C. The wells were then emptied, blotted dry and blocked with 3% (w/v) dried non-fat milk powder in PBS (250 μl/well, 37°C, 1 h). After washing three times with wash buffer, plates were blotted dry. Sample extracts, standards (purified *P. aureus* Hsp70) and controls (25 μl, standards diluted in PBS) were mixed with an equal volume of anti-Hsp70 antibody solution (diluted 1:500 in ELISA buffer) and pipetted into wells on the plate (50 μl/well), incubated (37°C, 1 h) then washed (thrice, wash buffer) and blotted dry. Goat anti-mouse IgG (whole molecule) peroxidase conjugate (diluted 1:1000 in 3% (w/v) dried non-fat milk powder in ELISA buffer) was added to the plate (50 μl/well) then incubated (25°C, 1 h), washed (thrice, wash buffer) and blotted dry. Bound antibody was visualized with the addition of ABTS substrate (100 μl/well) incubated at 25°C for 30 min then the absorbance read at 405 nm.

Data obtained were corrected for non-specific binding by the inclusion of first and second antibody controls.

Statistical analysis

All data are means ± standard error unless otherwise noted. The difference between sample means was evaluated by using Dunnett's multiple comparison test, *post hoc*.

Results

Hsp70 purification

The SDS-PAGE gel and Western blot of samples during the purification protocol confirmed the efficiency of the protocol in rapidly producing significant

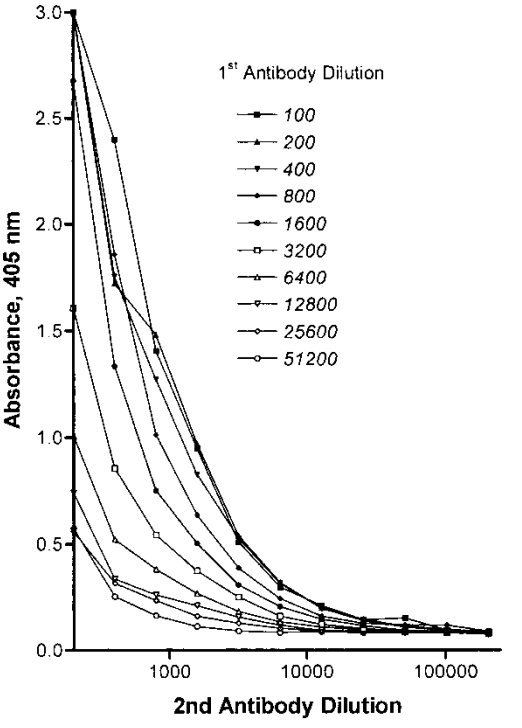

Figure 4. Optimization of indirect competitive enzyme-linked immunosorbent assay primary and secondary antibody dilution. Plate coating, 5 μg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, variable; second antibody, variable.

quantities of pure Hsp70. In the purified sample, a single band was visible in both the silver stained gel and the Western blot (figure 1).

Western blotting

The anti-*P. aureus* Hsp70 monoclonal antibody was shown to cross-react with bands from *L. minor* and *F. serratus*, having an approximate molecular weight of 70 kDa. The mobility of these bands was similar to the pure *P. aureus* Hsp70 control (figure 2). This was taken as confirmation that these bands represent the Hsp70 molecule in both *L. minor* and *F. serratus*. Although staining intensity cannot be used to determine accurately the Hsp70 content of the extracts, the extracts from the heat shocked (42°C, 4 h) samples produced a band that was more intense than sample controls (figure 2). Although the anti-*P. aureus* Hsp70 antibody was shown to cross-react with heat-shocked *F. serratus* sample extract, a multiple banding pattern was observed for the *P. aureus* control sample (figure 2B). Although the antibody is known to recognize both constitutive and inducible Hsp70, the lower bands were probably due to the formation of Hsp70 breakdown products. The anti-*P. aureus* Hsp70 antibody has been shown to have the ability to recognize breakdown products (data not shown).

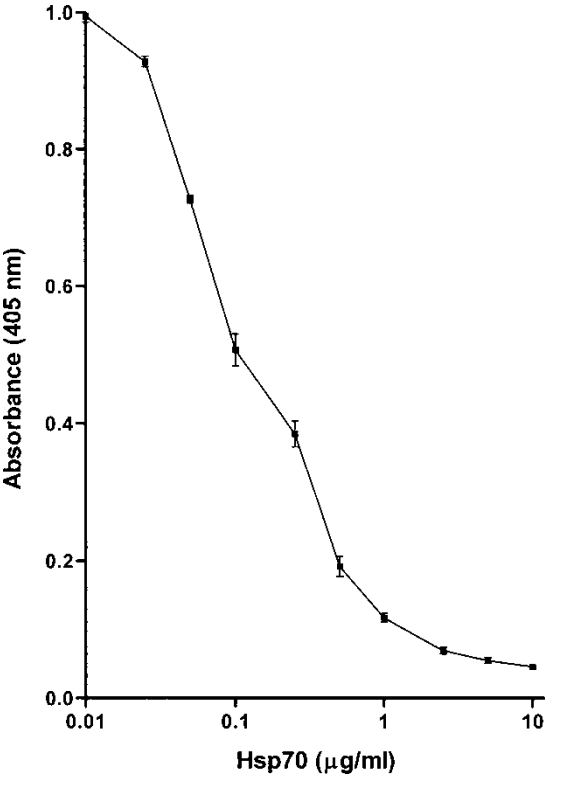

Figure 5. Hsp70 standard curve using optimized indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 μg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. The mean absorbance for 0 μg ml^{-1} Hsp70 was 1.017 ± 0.003. Data points are the mean ± standard error of the mean, $n = 3$.

IC-ELISA development

A checkerboard titration of the Hsp70 coating concentration and first antibody dilution shows a typical set of curves (figure 3). At 10 μg ml^{-1} coating concentration, absorbance decreased with dilution of first antibody (figure 3). As the coating concentration decreased, there was no effect on the curve until 5 μg ml^{-1}, after which there is a decrease in the response (figure 3). The data suggest that the coating concentration of 5 μg ml^{-1} and first antibody dilution of 1/1000 would be suitable for the ELISA.

A checkerboard titration of the first and second antibodies also shows a typical set of curves (figure 4). At high concentrations of second antibody, the absorbance decreased as the first antibody was diluted. As the second antibody was diluted, the slope of the line decreased. The target absorbance for the ELISA was an optical density of 1.0. This absorbance was achieved at antibody dilutions of 1/800 for both antibodies (figure 4). For convenience, dilutions of 1/1000 were used for both antibodies in the optimized ELISA.

Following optimization, an IC-ELISA using purified *P. aureus* Hsp70 as the inhibitor molecule was successfully developed that had a working range of

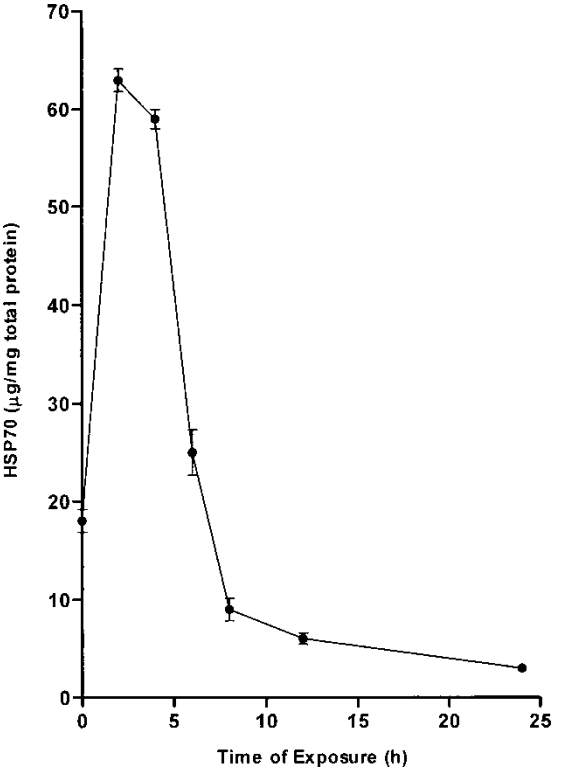

Figure 6. Hsp70 in *Fucus serratus* following a heat shock treatment of 42°C for up to 24 h. Samples were analysed by indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 µg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. Data points are the mean ± standard error of the mean, $n = 3$.

0–10 µg ml^{-1} and a detection limit of 0.025 µg ml^{-1} ($p < 0.01$ compared with 0 µg ml^{-1}) (figure 5).

Seaweed treatments

Increasing the temperature of *F. serratus* to 42°C resulted in a significant increase in Hsp70 concentration, from 18 to 63 µg Hsp70 mg^{-1} protein ($p < 0.01$) within 2 h. Between 4 and 6 h, the Hsp70 concentration returned to control values. At 8 h and longer, the Hsp70 concentrations were significantly lower than control values ($p < 0.01$) (figure 6).

Samples of *C. crispus* and *U. lactuca* were exposed to 42°C treatment for 4 h. There was a significant increase in Hsp70 concentration in *C. crispus* (21–36 µg Hsp70 mg^{-1} protein, $p < 0.01$), however the increase was not significant in *U. lactuca* (figure 7).

Samples of *F. serratus* were exposed to osmotic stress for 4 h by dilution of the seawater culture. A 50% decrease in osmolarity resulted in a sevenfold increase in Hsp70 concentration (from 12 to 83 µg Hsp70 mg^{-1} protein, $p < 0.001$). Hsp70 concentration continued to increase as osmolarity was decreased to a peak at 50 mOsmol kg^{-1} of 137 µg Hsp70 mg^{-1} protein (figure 8).

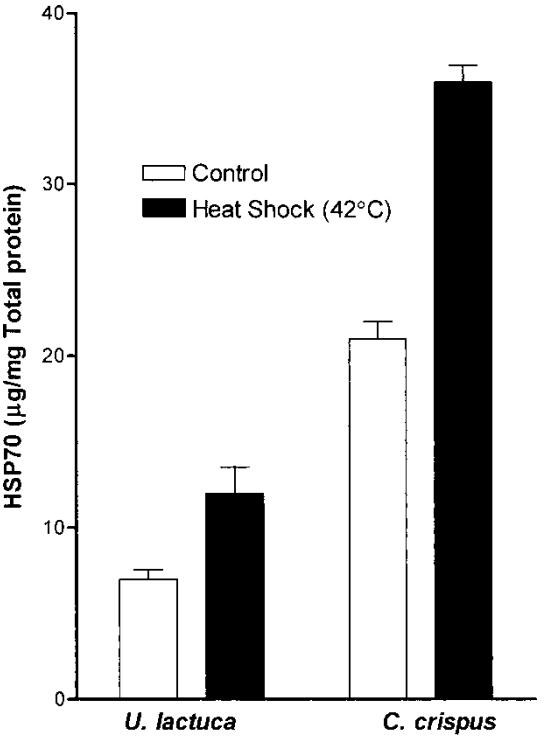

Figure 7. Hsp70 in *Ulva lactuca* and *Chondrus crispus* after heat shock treatment 42°C for 4 h. Samples were analysed by indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 µg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. Data points are the mean ± standard error of the mean, $n = 3$.

Exposure of samples of *F. serratus* to 0–25 mM Cd^{++} for 4 h resulted in a dose-dependent increase in Hsp70 concentration. At 25 mM, the increase in Hsp70 was more than tenfold above control values ($p < 0.01$). Between 25 and 50 mM Cd^{++}, the Hsp70 concentration decreased, and by 100 mM, Cd^{++} had decreased to below control values ($p < 0.05$) (figure 9). It is possible that these doses were resulting in some cell death although there was no visible indication of necrosis.

Duckweed treatments

Increasing the temperature of *L. minor* to 42°C resulted in a significant increase in Hsp70 concentration compared with controls at all times up to 8 h after treatment ($p < 0.01$). Hsp70 concentrations increased up to 4 h (18–74 µg Hsp70 mg^{-1} protein) when values were more than threefold greater than control plants. Concentrations decreased at 6 and 8 h, although they remained significantly higher than control values (figure 10).

L. minor samples were exposed to osmotic stress by the addition of NaCl. Hsp70 concentrations increased in a dose-dependent manner up to 250 mOsmol kg^{-1}, by which concentrations were over fivefold those of controls ($p < 0.01$). Further increases in osmolarity to 500 mOsmol kg^{-1} and above resulted in a decrease in Hsp70 concentration to values not significantly different

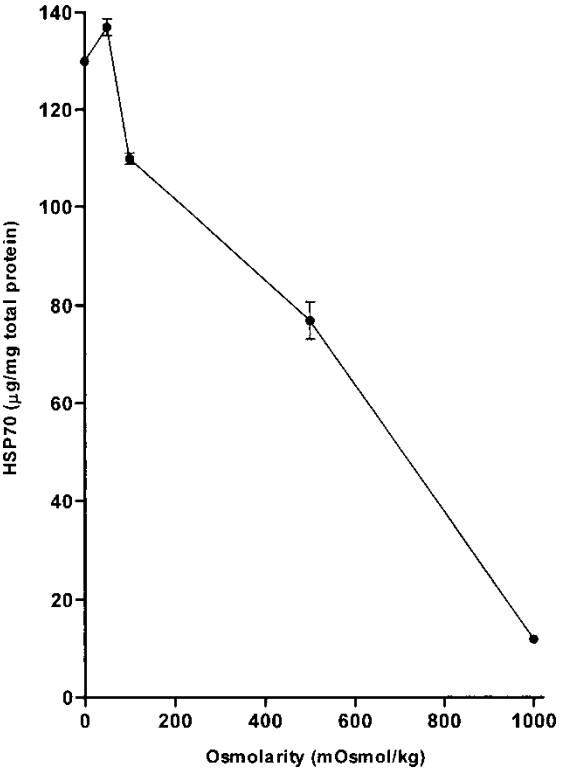

Figure 8. Effect of osmotic stress for 4 h on Hsp70 concentration in *Fucus serratus*. Samples were analysed by indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 µg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. Data points are the mean ± standard error of the mean, $n = 3$.

from controls (figure 11). It is possible that these doses were resulting in some cell death, although there was no visible indication of necrosis.

Exposure of samples of *L. minor* to Cd^{++} for 4 h resulted in a dose-dependent increase in Hsp70 concentration up to 20 mM Cd^{++} ($p < 0.01$). At 20 mM Cd^{++}, the increase in Hsp70 concentration was more than fivefold the control values. Hsp70 concentrations then decreased to control values by 60 mM Cd^{++} (figure 12). Treatments of Cd^{++} higher than 20 mM were not significantly different from control values and may be inducing cellular damage.

Discussion

The overall aim of the present paper is to evaluate Hsp70 as a biomarker in marine macroalgae and freshwater plant species. The paper first confirmed that the *P. aureus* anti-Hsp70 antibody cross-reacts with a protein from *L. minor* and *F. serratus* with a similar molecular weight to Hsp70. It has now been confirmed that this antibody cross-reacts with Hsp70 from species ranging from macroalgae to higher plants (Wilkinson *et al.* 1990, Adham *et al.* 1991, Wu *et al.* 1993, Marmiroli *et al.* 1997). There is a high degree of sequence homology between Hsp70 from

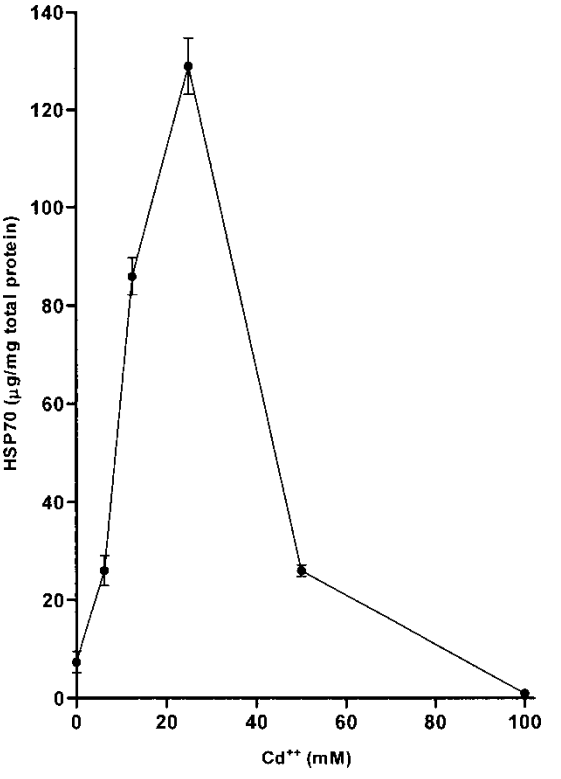

Figure 9. Effect of Cd^{++} exposure for 4 h on Hsp70 concentration in *Fucus serratus*. Samples were analysed by indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 µg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. Data points are the mean ± standard error of the mean, $n = 3$.

different species (Lindquist and Craig 1988, Boorstein *et al.* 1994, Morimoto *et al.* 1994, Latchman 1999). The wide cross-reactivity suggests that this antibody probably recognizes a highly conserved epitope such as the ATP binding domain of Hsp70.

Using this antibody, an IC-ELISA has been developed and optimized that has a wide working range (0.025–10 µg ml^{-1}). The assay is robust with coefficient of variations typically below 5% within the working range. The optimized ELISA requires small amounts of first and second antibodies, therefore reducing the cost. The format used here requires the plates to be coated with purified *P. aureus* Hsp70. The published purification method (Welch and Feramisco 1985, Wilkinson *et al.* 1990) was modified to allow the production of pure Hsp70 within 6 h. Typical yields from 350 g mung bean are 3 mg Hsp70, which are enough for 50 ELISA plates. The assay could be modified to increase sensitivity, for example through inclusion of biotin–avidin-linked labelling; however, the Hsp70 concentrations of the extracts in this study were well within the detection limits of the assay.

The antibody used probably recognizes both Hsc70 and Hsp70 isoforms. The control data would seem to confirm this because in the absence of an applied stress, Hsp70 was present at 0.5–2.0% total protein. As expected, all species tested

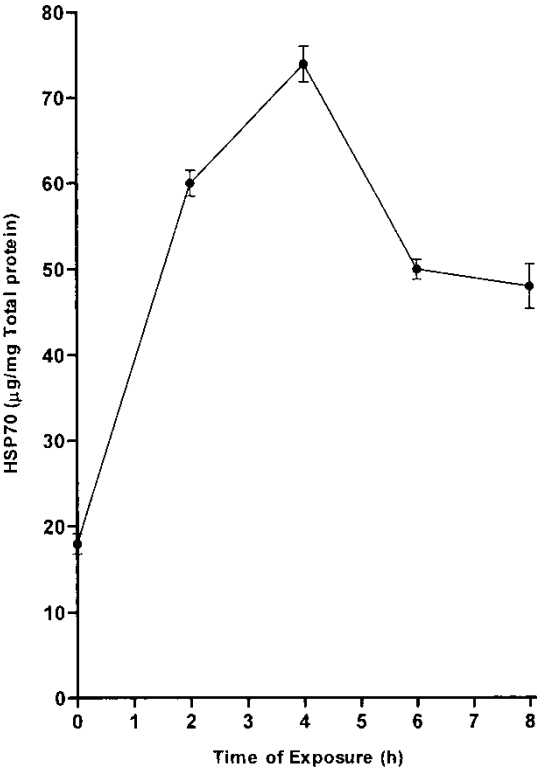

Figure 10. Hsp70 in *Lemna minor* following a heat shock treatment of 42°C for up to 8 h. Samples were analysed by indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 µg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. Data points are the mean ± standard error of the mean, $n = 3$.

responded to a heat shock by producing more Hsp70. Both *F. serratus* and *L. minor* responded to the 42°C treatment with a 200% increase in Hsp70 concentration, whereas in *C. crispus* and *U. lactuca*, the increase was only 50%. The Hsp70 concentrations in *U. lactuca* were significantly lower than the other species at all times. *U. lactuca* is known to be very fibrous compared with the other species (Dickinson 1963, Howson and Picton 1997), which could have affected the extraction process.

All further experiments on macroalgae were restricted to *F. serratus* due to the higher Hsp70 concentrations found in this species. The time courses for the temperature treatments were very similar in *F. serratus* and *L. minor*. As in other species (Wu *et al.* 1993, Sanita di Toppi and Gabbrielli 1999), there was an initial increase in Hsp70 concentration followed by a decrease. The temperature (42°C) used for the heat shock is not outside the range that may be experienced by either species; however, it is a significant shock and despite the fact that there was no physical sign of necrosis, the decrease may be due to protein degradation or cell death. Future experiments should include tests of cell viability.

F. serratus normally lives in an aqueous environment with a high osmolarity, whereas *L. minor* grows in very low osmolarity solutions. *F. serratus* is intertidal and

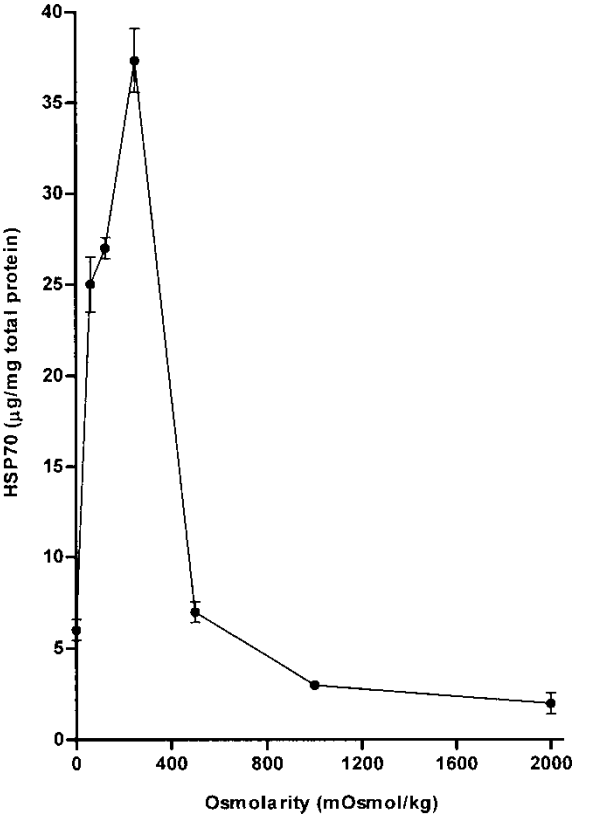

Figure 11. Effect of osmotic stress for 4 h on Hsp70 concentration in *Lemna minor*. Samples were analysed by indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 $\mu g\,ml^{-1}$ purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. Data points are the mean $\pm$ standard error, $n = 3$.

so is exposed to a range of salt concentrations and can compensate for changes in salinity by adjusting its internal ion concentrations (Stromgren 1994). Despite this ability, a decrease in osmolarity is a significant stress to *F. serratus*, as shown by the increase in Hsp70 as osmolarity is decreased. An increase in osmolarity would be expected to stress *L. minor*. It is a relevant stressor because in fresh water plant species might be subjected to osmotic stress through effluent discharges and chemical spillage. *L. minor* is typical of freshwater vascular plants that have low tolerance to increased levels of salinity. The reduction in Hsp70 levels observed when exposed to high osmolarities (>500 mOsmol kg^{-1}) is probably the result of cell death due to osmotic shock.

The temperature and osmolarity treatments might be considered as environmental stressors and do not represent responses to pollutants. A large number of compounds pollute fresh and marine waters. The heavy metal Cd^{++} was selected as a representative non-essential toxic heavy metal. *F. serratus* is known to have low sensitivity to heavy metal contamination (Stromgren 1980) and *L. minor* has demonstrated the ability to bioaccumulate heavy metals (Rahmani and Sternberg

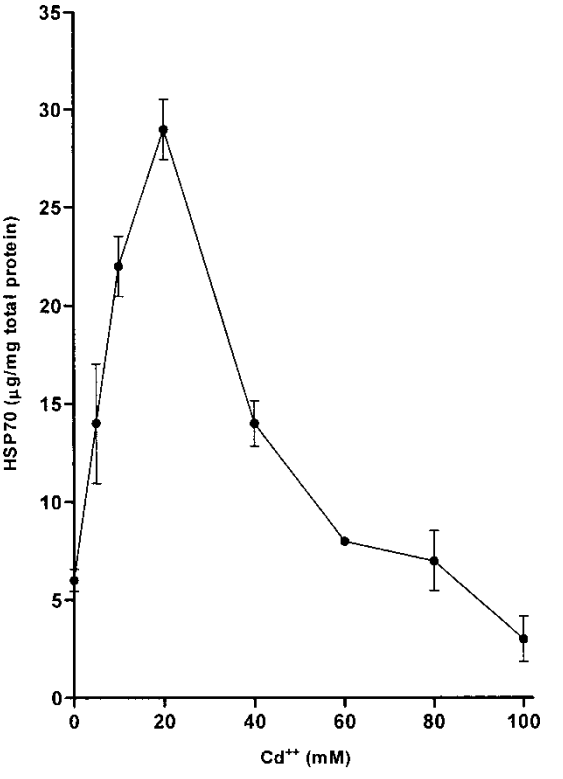

Figure 12. Effect of Cd^{++} exposure for 4 h on Hsp70 concentration in *Lemna minor*. Samples were analysed by indirect competitive enzyme-linked immunosorbent assay. Plate coating, 5 µg ml^{-1} purified Hsp70 from *Phaseolus aureus*. Dilutions of antibodies were anti-*P. aureus* Hsp70 antibody, 1/1000; second antibody, 1/1000. Data points are the mean ± standard error, $n = 3$.

1999). In the present study, Cd^{++} exposure up to 25 mM resulted in induction of Hsp70 in both species. Higher concentrations of Cd^{++} resulted in a reduction in the response, which was probably due to cytotoxicity (Bauman *et al.* 1993, Sanita di Toppi and Gabbrielli 1999). Relatively high Cd^{++} concentrations were chosen for the present study as there is little information available on acute Cd^{++} toxicity (Sanita di Toppi and Gabbrielli 1999).

Their responsiveness to a wide range of stressors has naturally suggested Hsps as potential biomarkers (Ali *et al.* 1996, De Pomerai 1996, Lewis *et al.* 1999, Bierkens 2000, Nadeau *et al.* 2001). Several different Hsps have been used in this context: Hsp60 (Kammenga *et al.* 1998, Wheelock *et al.* 2002), Hsp70 (Pyza *et al.* 1997, Lewis *et al.* 1999, Nadeau *et al.* 2001) and Hsp90 (Ali *et al.* 1996). Although a large number of studies have demonstrated that Hsps do respond to a wide variety of stressors, several problems in their use have been highlighted (Pyza *et al.* 1997, Werner and Hinton 1999, Bierkens 2000). Some of these problems are highlighted in the data presented in this study. There is a clear time dependence to the Hsp70 response, i.e. typically Hsp70 will increase up to a peak and then decline often to lower than control values (Pyza *et al.* 1997, Werner and Hinton 1999). However, it has been suggested that over longer periods (days or weeks) of stress, a secondary

peak for Hsp70 occurs (Kohler *et al.* 1999). The dose–response to a stressor is typically that a peak is reached above which Hsp70 decreases (Pyza *et al.* 1997, Bierkens *et al.* 1998, Kohler *et al.* 1999, Werner and Hinton 1999). Any study of this type on Hsp70 responses needs to consider these factors.

The ELISA developed in the present paper has been shown to give rapid, accurate measurements of Hsp70 concentrations from a number of species. Many previous studies evaluating Hsp70 as a biomarker have used a Western blotting method (Lewis *et al.* 1999, Werner and Hinton 1999, Nadeau *et al.* 2001). The IC-ELISA method developed herein is more rapid and more suitable to quantitative determinations of Hsp70. There are two ways in which its application can be of worth in the future. First, in the assessment of water quality using laboratory-grown plant species. Second, it has potential as one of a number of Type 1 biomarkers used to produce a profile or fingerprint that can indicate the presence of a specific stressor.

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