Induction of heat-shock (stress) protein gene expression by selected natural and anthropogenic disturbances in the octocoral *Dendronephthya klunzingeri*

Matthias Wiens\textsuperscript{a}, Mohammed S.A. Ammar\textsuperscript{b}, Ahmed H. Nawar\textsuperscript{b}, Claudia Kozioł\textsuperscript{c}, Hamdy M.A. Hassanein\textsuperscript{a}, Michael Eisinger\textsuperscript{c}, Isabel M. Müller\textsuperscript{a,}\* Werner E.G. Müller\textsuperscript{a,}\*

\textsuperscript{a}Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Duesbergweg 6, D-55099 Mainz, Germany

\textsuperscript{b}National Institute of Oceanography and Fisheries, Hurghada, Egypt

\textsuperscript{c}Institut für Ökologie, Universität Essen, Universitätsstraße 15, 45117 Essen, Germany

Received 20 July 1999; received in revised form 3 November 1999; accepted 11 November 1999

**Abstract**

Previously it was found that the expression of selected heat-shock proteins is upregulated in corals after exposure to elevated temperature. We published that HSPs are suitable markers in sponges to monitor the degree of environmental stress on these animals. In the present study the heat-shock proteins (HSPs) with a molecular weight of 90 kDa have been selected to prove their potential usefulness as biomarkers under controlled laboratory conditions and in the field. The studies have been performed with the octocoral *Dendronephthya klunzingeri* from which the cDNA coding for HSP90 was cloned first. The expression of the HSP90 gene is upregulated by thermal stress; treatment of the animals for 2 h at 4°C below or above the ambient temperature resulted in a \( > 4.5 \)-fold higher steady-state level of the respective mRNA. Also animals taken from stressed locations in the field showed an increased expression. The amount of HSP90 protein in *D. klunzingeri* was found to be strongly increased under thermal stress, or exposure to polychlorinated biphenyl (congener 118), but not after treatment with cadmium. Field studies revealed that samples taken from a nonstressed area have a low level of HSP90, but those collected from locations at which the corals are under physical stress (sedimentation through landfiling) show a high expression of HSP90. It is concluded that the chaperone HSP90 might become a suitable biomarker to monitor environmental stress on corals. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** *Dendronephthya klunzingeri*; Coral; Heat-shock protein; Biomarker; Cadmium; PCB 118; Landfilling; Environmental stress

*Corresponding author. Tel.: +49-61-3139-5910; fax: +49-61-3139-5243.
E-mail address: wmueller@mail.uni-mainz.de (W.E.G. Müller)

0022-0981/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0022-0981(99)00167-7
1. Introduction

Coral reefs are fragile ecosystems which are highly vulnerable to environmental stress (Johannes, 1975; Loya, 1976; Loya and Rinkevich, 1980). Natural stress, such as storm-generated waves (Stoddart, 1963; Hernandez-Avila et al., 1977) or population explosions of the crown-of-thorn starfish *Acanthaster planci* (Chesher, 1969; Pearson, 1981) and anthropogenic disturbances, e.g. sedimentation, landfilling, construction activities (Sheppard, 1980), sewage and eutrophication (Pastorek and Bilyard, 1985), oil (Loya and Rinkevich, 1980) and thermal stress (Johannes, 1975) affect the growth and ecology of corals in the reefs (reviewed in: Grigg and Dollar, 1998). An effective management of the health state of coral reefs requires regulation of human activities based on a rational monitoring of the extent of stress in a given coral reef and concepts for assessing of coral reef health. One suitable way to assess sublethal effects of stressors on growth and metabolism of corals is to quantify the physiological responses of corals to those influences. Therefore, biomarker(s) for a biological monitoring program are needed. A biomarker is defined as ‘a xenobiotically-induced variation in cellular or biochemical components or processes, structures, or functions that are measurable in a biological system or sample’ (NRC, 1989).

Our earlier findings revealed that sponges [*Porifera*] react to environmental stress with an increased expression of the heat-shock protein(s) (HSPs) (Koziol et al., 1996, 1997a; and reviewed in: Koziol et al., 1997b). The response to stressors occurred in sponges not only under controlled conditions in the aquarium, but also in animals collected from the field (Müller et al., 1998). In the present study we investigated if corals also react to anthropogenic stress with an overexpression of the gene coding for a selected HSP. Five families of stress proteins are found in eukaryotes; four of them are grouped according to their molecular weights as HSP90, HSP70, HSP58-60 and HSP20-30 whereas the fifth HSP is termed ubiquitin (Schlesinger et al., 1982). The HSPs are known to be essential cell components, most of them being involved in the formation of transient protein complexes (Lindquist and Craig, 1988; Nover, 1991). They may also play key roles during cell cycle and development. As an example, members of the HSP90 family, which might possess ATPase activity (Nadeau et al., 1993), are involved in the folding of nascent and denatured proteins or protein complexes (and hence act as molecular chaperones) (Miyata and Yahara, 1992; Wiech et al., 1992). The HSP90s are characterized by their high level of specificity in associating with particular proteins; they form in most cases long-lived complexes and acquire important regulatory features (Pratt et al., 1992; Xu and Lindquist, 1993).

In the present study we determined if in corals the level of expression of HSPs can be used as a marker for environmental stress. From earlier studies with the technique of Western blotting (Sharp et al., 1994; Hayes and King, 1995) or by determination of putative related proteins (Miller et al., 1992; Black et al., 1995), it is known that in corals an upregulation of selected HSPs, especially of HSP70, occurs. Here HSP90 was selected because it was previously found that this chaperone is upregulated by cadmium (Barque et al., 1996), is stress-inducible (Ali et al., 1996) and binds to the estrogen receptor by which it is — in turn — regulated in its activity (Sabbah et al., 1996). The latter property could qualify the HSP90 polypeptide as a marker for xenoestrogens (Soto et al., 1995).
The experiments have been performed with the octocoral *Dendronephthya klunzingeri* which is abundantly found in coral reefs at the Red Sea (M. Eisinger, unpublished); in addition this species does not contain zooxanthellae (unpublished) like *Dendronephthya hemprichi* (Fabricius et al., 1995). As stressors, thermal stress as well as the heavy metal cadmium (Cd) and the polychlorinated biphenyl (PCB) congener 118 were selected and tested under controlled aquarium conditions on the response of this coral. Furthermore, the effects of stressors like destruction of the biotope by storm or by landfiling were analyzed in the field. The data revealed that the selected chaperone HSP90 might become a biomarker which is useful to assess the extent of stress on the coral *D. klunzingeri*.

2. Materials and methods

2.1. Materials

DIG [digoxigenin] DNA labeling kit, DIG-11-dUTP, anti-DIG AP Fab fragments, CDP [disodium 2-chloro-5-(4-methoxyxypirrol)[1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1.1^3,7^]decan-4-yl]phenyl phosphate] and CSPD [disodium 3-(4-methoxyxypirrol,1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^3,7^]decan-4-yl]phenyl phosphate] were from Boehringer Mannheim (Mannheim, Germany); 2,3′,4,4′,5-penta-chlorobiphenyl (PCB 118) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The monoclonal antibody directed against the mold *Achlya ambisexualis* HSP90, which also cross-reacts with vertebrate and plant HSP90s (H1775), and heat-shock protein 90 from bovine brain (H6774) were from Sigma (Deisenhofen, Germany).

2.2. Coral

Specimens of *Dendronephthya klunzingeri* (Studer) [Coelenterata: Anthozoa: Alcyonaria (Octocorallia)] were collected near Hurghada (Red Sea, Egypt). They were sampled from a depth of 5 m at 25°C from their habitat, the hard bottom, and either used immediately for experimental analysis or were kept at 22°C for 3 weeks after collection to adapt to this temperature.

2.3. Extracts

Extracts to determine the levels of HSP90 were obtained by grinding frozen tissue samples in three times their volume of phosphate-buffered saline, supplemented with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After centrifugation the supernatant was collected and protein content was determined (Lane, 1957); parallel determinations with the Lowry method (Lowry et al., 1951) revealed that the values did not differ more than 10%.

RNA was extracted from liquid-nitrogen pulverized coral tissue with TRIzol Reagent (Gibco BRL) as recommended by the manufacturer. RNA was determined as described (Ausubel et al., 1995).
2.4. Exposure of *D. klunzingeri* to thermal stress, cadmium or PCB

Animals were exposed as follows. (i) Thermal stress: incubation was performed in filtered, oxygenated seawater. Specimens remained either untreated at 22°C for the entire period, or were treated for 2 h at 26°C (heat shock) or for 2 h at 18°C (cold shock) and subsequently for 2 days again at 22°C in seawater in 20-l aquaria. (ii) Cadmium: the animals were treated with 100 ng/ml (corresponding to 163.2 ng/ml of CdCl₂) or 300 ng/ml of Cd for the entire period of 2 days. (iii) Cadmium and thermal stress: the specimens were treated first by heat shock for 2 h at 26°C and subsequently — in addition — with 100 ng/ml or 300 ng/ml of Cd for 2 days. (iv) PCB 118: coral specimens were injected with 1 ml of PCB 118 (0.1 mg/ml in corn oil) per 10 g of tissue. After 2 days the samples were taken.

Field samples were taken from the intact, undisturbed site ‘Big Giftun’ (latitudes (N): 27°11.063’; longitudes (E): 033°57.843’; depth, 19 m, temperature 25.2°C) and sites which are affected by destruction through storm ‘El-Fanadir’ (27°17.708’ — 033°49.867’; depth 12 m, temperature 25.5°C) or by sedimentation ‘Gota Torfa’ (27°13.428’ — 033°57.094’; depth 14 m, temperature 27.3°C) in the vicinity of Hurghada (Red Sea, Egypt) (Ammar, 1998).

The tissue was immediately frozen in liquid nitrogen until use for RNA- or protein isolation.

2.5. Isolation of a cDNA encoding the putative HSP90 protein

The complete coral cDNA, termed *HS9DEKL*, was cloned by polymerase chain reaction (PCR) from the *D. klunzingeri* cDNA library. The data are deposited in the EMBL Nucleotide Sequence Database under the accession number Y17848.

2.6. Northern blot

RNA was extracted from liquid-nitrogen pulverized coral tissue with TRIzol Reagent (GibcoBRL) as recommended by the manufacturer. An amount of 1 μg of total RNA was electrophoresed through formaldehyde/agarose gel and blotted onto Hybond N⁺ membrane following the manufacturer’s instructions (Amersham). Hybridization experiments were performed with the probe *HS9DEKL* (603 bp segment) from *D. klunzingeri*. This probe was labeled with DIG-11-dUTP by the DIG DNA labeling kit. Hybridization was performed with the antisense DIG-labeled probes at 42°C overnight using 50% formamide, containing 5× SSC, 2% blocking reagent (Boehringer), 7% (w/v) SDS and 0.1% (w/v) *N*-lauroylsarcosine, following the instructions of the manufacturer (Boehringer). After washing and blocking DIG-labeled nucleic acid was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase) and visualized by chemiluminescence technique using CDP, the chemiluminescence substrate alkaline phosphatase, according to the instructions of the manufacturer (Boehringer).

For quantitation of the signals of Northern blots the chemiluminescence procedure was applied (Stanley and Kricka, 1990); CDP was used as substrate. The screen was scanned with the GS-525 Molecular Imager (Bio-Rad).
2.7. Gel electrophoresis and Western blotting

Total tissue extracts of coral tissue were subjected to electrophoresis in 7.5% polyacrylamide gels containing 0.1% NaDodSO₄ as described by Laemmli (1970). For Western-blotting experiments the proteins were electro-transferred to PVDF-Immobilon P membranes using a semi-dry blotting apparatus (Wiens et al., 1998). The membranes were incubated with monoclonal antibody against HSP90 (McAb-HSP90; 1:500 dilution) for 1.5 h at room temperature, followed by incubation with peroxidase-conjugated anti-mouse IgG and CSPD; the blots were evaluated using a Model GS 525 Molecular Imager (Bio-Rad) (Stanley and Kricka, 1990). At least five parallel experiments have been performed for each series of investigation. After quantification of the expression pattern the values had no deviation larger than 18%; one representative blot for each experiment is shown.

In one control experiment, 100 μl of the McAb-HSP90 was adsorbed to 50 μg of HSP90 (from bovine brain; 30 min; 4°C) prior to its use.

3. Results

3.1. Expression of HSP90 on transcriptional level

Northern blot experiments, applying 1 μg of RNA per slot, have been performed to determine the level of HS9DEKL gene expression. The level of the 2.7-kb-long transcript in a control animal was arbitrarily set to onefold (Fig. 1; lane b). If animals are treated for 2 h at 4°C below the ambient temperature (lane a) or 4°C above the ambient temperature (lane c) the mRNA level of HS9DEKL reaches a value of 5.6-fold and 4.5-fold, respectively. One sample from the field was collected from a biotope which is disturbed by sedimentation through landfilling; the mRNA level in this animal is 2.7-fold higher than in the control coral (lane d).

Fig. 1. Northern blot analysis to determine the size of the transcripts of the mRNA encoding the coral HSP90. 2.7 kb. RNA was prepared from coral tissue and 1 μg each was subjected to analysis. The following samples were analyzed; control [Ct] (lane b), thermal stressed animals either cold shocked at 4°C below the ambient temperature [−4°C] (lane a) or heat shocked 4°C above the ambient temperature [+4°C] (lane c) and one sample from the field, collected from a biotope, stressed by sedimentation through landfilling [Lf] (lane d).
3.2. Synthesis of HSP90 protein

The level of HSP90 protein in *D. klunzingeri* was determined by Western blotting (Fig. 2). Protein extracts were prepared, size separated and reacted with a McAb-HSP90. The concentrations of protein in the extracts were determined by the methods described by Lane (1957) and Lowry et al. (1951); the values obtained did not differ more than 10% between the two methods. An amount of 40 μg, with respect to protein, was applied per slot. The size of the main band visualized corresponds to an $M_r$ of 86,000 (lane b) which is identical to the one of the deduced amino acid sequence from the HS9DEKL cDNA. Minor bands of a size around 45 kDa are visible which are attributed to similar proteins, as reported before (Kelley and Schlesinger, 1982). For this experiment, a tissue sample was used from a specimen which had been collected from a site affected by landfilling (from ‘Gota Torfa’). In parallel to the Western blot, the gel stained by Coomassie Brilliant Blue is shown (lane a). In one control experiment 100 μl of the McAb-HSP90 were adsorbed to 50 μg of HSP90 prior to its use. This antibody preparation did not react with any coral sample (not shown).

![Fig. 2. Expression of heat shock protein HSP90 in *D. klunzingeri*. In this experiment, an animal from one site which was stressed by landfilling (from ‘Gota Torfa’) was analyzed. Proteins were extracted as described in Section 2, and size-separated by polyacrylamide gel electrophoresis. An amount of 40 μg of protein was applied to each slot. The gel was either stained by Coomassie Brilliant Blue (lane a) or analyzed for the presence of HSP90 by Western blotting technique using McAb-HSP90 (lane b). The position of migration of the HSP90 is indicated (arrow head). The molecular masses of marker proteins, which were run in parallel (lane c), are given in kDa.](image-url)
3.3. **Effect of thermal stress on the level of HSP90**

A shift of the incubation temperature resulted in an increased synthesis of HSP90 protein in the corals. While in the control specimens, which were kept for more than 2 weeks in the aquarium, only occasionally a distinct band, corresponding to a size of 86,000 is seen (Fig. 3A, lane b), a strong increase in the level is measured after incubation of the animals at 4°C or 8°C above the ambient temperature. These animals show a 4.8- to 8.3-fold higher level of HSP90 (Fig. 3A, lanes c and d). Reduction of the ambient temperature by 4°C resulted likewise in an increase of HSP90 (Fig. 3A, lane a).

3.4. **Effect of cadmium and PCB 118**

Cadmium exposure at concentrations of 100 ng/ml or of 300 ng/ml did not cause a change of the steady state level of HSP90 with respect to the controls (Fig. 3B, lanes b and c versus lane a). In contrast, treatment of the animals with PCB 118 as described in Section 2 causes a strong increase of HSP90 (Fig. 3D, lane c versus control at lane a). However, if the animals are treated with cadmium and heat (increase of temperature by 4°C) a strong signal corresponding to HSP90 is seen (Fig. 3B, lane d). Controls were treated with corn oil alone; those animals did not react with an upregulation of HSP90 (Fig. 3D, lane c).

Interesting is the finding that a preincubation of the animals at 4°C above the ambient temperature and a subsequent incubation with Cd resulted in a strong increase of HSP90 expression. This chaperone is synthesized in tissue from animals treated first by an elevated temperature and subsequently with 100 or 300 ng/ml of Cd at a 1.8- and 4.6-fold higher level (Fig. 3C, lanes c and d; as a comparison to the blot shown in Fig. 3C lane c, the expression pattern of HSP90 under identical experimental conditions is given in a second series of experiments (Fig. 3D lane d), if compared with animals treated by a 4°C higher temperature only (Fig. 3C, lane a).

3.5. **Field study**

Preliminary field experiments have been performed to monitor the level of HSP90 in animals from nonstressed and stressed biotopes. As a nonstressed location the ‘Big Giftun’ site has been selected. *D. klonzingeri* specimens from this area, which had been used also for the laboratory experiments (Fig. 3A) show no or only a very low level of HSP90 expression (Fig. 3E, lanes a and b). In contrast, if animals were analyzed which had been collected from sites affected by destruction through storm, ‘El-Fanadir’ (Fig. 3F, lanes a and b), or by landfiling, ‘Gota Torfa’ location (Fig. 3F, lanes d and e), the level of HSP90 is more than twofold higher than that measured in tissue from corals kept at 8°C above the ambient temperature (Fig. 3F, lane c).
Fig. 3. Expression of the HSP90 protein in _D. klunzingeri_ exposed to different stressors; the analysis was performed by Western blotting. (A) Thermal stress: tissue from a control [Ct] animal, or samples from thermal stressed animals either cold shocked at 4°C below the ambient temperature [−4°C] or heat treated 4°C or 8°C above the ambient temperature [+4°C; +8°C]. (B) Cadmium (Cd) exposure: effect of 100 ng/ml or 300 ng/ml of Cd [100 ng/ml or 300 ng/ml], or of 100 ng/ml of Cd after a pretreatment by a temperature shift of +4°C [+4°C/Cd], and the control [Ct]. (C) Thermal stress and cadmium: the specimens were treated first by elevated temperature for 2 h at 26°C and subsequently with 100 ng/ml or 300 ng/ml of Cd for 2 days [+4°C/100 or 300 ng/ml Cd]; in parallel one control [Ct] animal and one animal treated 4°C above the ambient temperature [+4°C] were analyzed. (D) Treatment with PCB 118: the coral specimens were injected with PCB 118 as described in Section 2. After 2 days the samples were taken and analyzed. In controls the animals remained untreated [Ct] or were treated with corn oil alone [c.o.]. (E) Field samples: two specimens from the nonstressed site ‘Big Giftun’ have been analyzed [nonstressed]. (F) Field samples: corals (two samples each) collected from sites affected by destruction through storm ‘El-Fanadir’ [Storm] or by sedimentation ‘Gota Torfa’ [Lf] have been analyzed for HSP90 level. As a reference, a sample from an animal kept under heat-stressed conditions in the aquarium for 2 h at 30°C [+8°C] was run in parallel. Further details are given in Section 2.
4. Discussion

Based on our earlier experience that heat-shock proteins are suitable biomarkers in the lowest metazoan phylum, the Porifera, it was studied if the expression of the HSP species HSP90 can serve as a reliable parameter for stress response in corals. HSP90 is of special interest and importance, because its expression results in a modulation of the activity of the estrogen receptor, the hydrocarbon receptor and it is also involved in signal transduction system(s), mediated by oncogenic tyrosine kinases (see Sabbah et al., 1996). In addition, this protein has been found to be inducible in unicellular- and multicellular organisms and systems (Yonehara et al., 1996). It was also reported that HSP90 is upregulated after treatment of rats with pesticides (Bagchi et al., 1996). Hence, based on these data a systematic survey on the role of HSP90 in one coral species appeared to be promising and was therefore performed.

The cDNA encoding the HSP90 was cloned from the coral *D. klunzingeri* and found to belong to form α of HSP90s; data will be given elsewhere (the EMBL accession number is Y17848).

The expression of HSP90 was determined on the level of RNA and protein. On a transcriptional level it was found that within 2 h the steady-state level of the 2.7-kb-long HSP90 mRNA increases by \(3\)-fold after treatment of the animals by heat or cold shock.

For further elucidation on the functional protein level, and to prove if HSP90 is also regulated in quantity under different stress conditions, studies have been performed both under controlled aquarium conditions and in the field. A monoclonal antibody which recognized *D. klunzingeri*, anti-HSP90, was used to quantify the steady-state level of the HSP90 protein. The experiments revealed that this chaperone is strongly upregulated if the animals are exposed to thermal stress. Even a short exposure time of 2 h and at a temperature shift of 4°C, resulted in a drastic upregulation of HSP90. In contrast, exposure of the coral to Cd at concentrations of 100 or 300 ng/ml displayed no effect on HSP90 expression. In earlier studies it was found that marine sponges undergo in response to these Cd concentrations apoptotic DNA fragmentation (Wagner et al., 1998). This result could indicate that Cd is under such conditions toxic for *D. klunzingeri*. Therefore the result showing that after pretreatment of the animals by thermal stress and a subsequent exposure to Cd the HSP90 protein level is highly increased, suggests that HSP90 protects the corals against Cd if the expression of this chaperone is induced already prior to the influence of this heavy metal. This result is in agreement with an earlier study in which it was demonstrated that the freshwater sponge *Ephydatia fluviatilis* reacts with an increased expression of the stress protein HSP70 if it had been pretreated by a short temperature stress prior to the exposure to environmental xenobiotics (Müller et al., 1995).

It has been suggested that polychlorinated hydrocarbons might affect corals (Chesher, 1969). Polychlorinated biphenyls (PCB) are widely distributed as environmental pollutants in both terrestrial and aquatic ecosystems (Safe, 1990; Clark, 1997). The toxic effects caused by PCBs have been well investigated (Kimbrough, 1985; Silberhorn et al., 1990). In sponges it was recently reported that PCBs cause, besides DNA damage, also an induction of HSP70 (Schröder et al., 1999). In addition, a novel sensitive marker for
PCB load to sponges was found by the demonstration that the chaperone 14-3-3 is highly expressed in response to this class of xenobiotics (Wiens et al., 1998). Here, we tested if one model compound, the nonplanar mono-ortho PCB congener 118, displayed an effect of the expression of HSP90. As reported in sponges also in the *D. klunzingeri* system the expression of heat-shock protein is upregulated after PCB 118 treatment.

In a last series of experiments it was analyzed if *D. klunzingeri* also reacts in the field, at known environmental loads with a change of the intracellular level of HSP90. Animals from one site which appeared to be nondisturbed and comprises a diverse coral community and animals from locations which show a characteristic stressed environment, such as destruction by storm site ‘El-Fanadir’ or by sedimentation site ‘Gota Torfa’, were analyzed. Again, it was found that the chaperone HSP90 is highly upregulated in animals taken from affected areas.

In conclusion, the data reported in this study show that the chaperone HSP90 might become a suitable biomarker for monitoring environmental stress on corals (here *D. klunzingeri*), especially if the animals were taken from areas stressed by natural disturbance and by selected anthropogenic chemicals, here PCB 118. Due to the fact that the species used in the present study, *D. klunzingeri*, does not contain zooxanthellae, possible changes in the HSP90 level cannot be attributed to this symbiont. In previous studies it was shown that zooxanthellae contribute to the upregulation of the heat-shock protein in other corals (Hayes and King, 1995) and are involved in the detoxification process with respect to heavy metals in corals (Harland et al., 1990). In future studies it will be determined, if besides molecular biological and biochemical markers, as have been used here, immunological marker(s) could be chosen to estimate environmental stress also on stony corals, e.g. on the basis of the strength of histoincompatibility reactions as in the hydrocoral *Millepora dichotoma* or the scleractinian coral *Stylophora pistillata* (Müller et al., 1983, 1984). In addition, it must be clarified to what extent the levels of transcripts for HSP90 as well as of the protein vary under nonstressed conditions.

**Acknowledgements**

This work was supported by grants from the Commission of the European Communities [project ‘CORAL REEF RESTORATION’; ERBIC18CT960034] and the International Human Frontier Science Program [RG-333/96-M]. [RW]

**References**


Safe, S., 1990. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). CRC Crit. Rev. Toxicol. 21, 51–88.


