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Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarum*) transplanted in Hong Kong coastal waters

Sharon B. De Luca-Abbott, Bruce J. Richardson *, Katherine E. McClellan, Gene J. Zheng, Michael Martin, Paul K.S. Lam

Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong

Abstract

Green-lipped mussels, *Perna viridis*, and Manila clams, *Ruditapes philippinarum* were sourced from "clean" sites in the Hong Kong region, depurated in a laboratory using uncontaminated filtered seawater for 8 days, and transplanted to a suspected gradient of chemically polluted sites in Hong Kong. After 14- and 28-days of field exposure, several antioxidant parameters including glutathione S transferase (GST), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) were quantified in gill and hepatopancreas tissues. Whole body tissue concentrations of polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons (PHCs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCs) were determined in pooled site samples. Chemical analyses indicated that: (a) clams had higher levels of PAHs, PHCs, DDTs and PCBs, whereas mussels had higher hexachlorocyclohexane (HCHs) and there was no difference between species for dieldrin and remaining OCs; (b) Kat O should not be continued as a "clean" reference site for Hong Kong, because of the levels of contaminants measured and (c) PAH concentrations in the current survey were similar to those previously measured. Toxicological conclusions were: (a) antioxidant responses were different between species; (b) CAT and GST have highest utility in clams for field use in Hong Kong, whereas CAT in both gill and hepatopancreas tissue showed most potential in mussels; (c) significant induction of antioxidant responses over day 0 (excluding GPx in both tissues, and GST in mussel hepatic tissue); (d) groups of contaminants do not consistently induce antioxidant responses and (e) organochlorines and PCBs correlated significantly with CAT and GST in clam hepatopancreas and with CAT in mussel gill and hepatic tissue. Multivariate statistical techniques indicated little relationship between the site patterns for antioxidant responses and the contaminant gradients identified in body burden analysis.

Keywords: Antioxidant responses; Polycyclic aromatic hydrocarbons (PAHs); Polychlorinated biphenyls (PCBs); Organochlorine pesticides (OCs); Mussels (*Perna viridis*); Field transplant

1. Introduction

It is generally recognised that the quality of Hong Kong's coastal waters and sediments has been seriously compromised by significant inputs of contaminants from industry, shipping, population increases, and general urban influences (Connell et al., 1998; Wu, 1998). Common contaminants in marine sediment include polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons (PHCs), polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCs) (Connell et al., 1998). The health and functioning of marine organisms, such as fish and shellfish, in the context of increased and sustained exposure to these contaminants, have raised the question of their toxicological responses

* Corresponding author. Tel.: +852 2788 7042; fax: +852 2788 7406.
E-mail address: bhbrucer@cityu.edu.hk (B.J. Richardson).

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and general condition. The majority of previous studies on marine environmental quality in Hong Kong have focused on determining the concentrations of various contaminants in water and sediment. These studies, however, have often failed to consider the ecological effects of the contaminants on marine organisms (Xu et al., 1999), the health of which may be under serious threat or risk (Richardson et al., 2000). There is a need for the development and implementation of sensitive, inexpensive and informative ecotoxicological monitoring techniques for use with Hong Kong's marine organisms.

Sessile, filter-feeding shellfish that accumulate contaminants have been widely used as indicator organisms for monitoring polluted environments. Predominantly, epifaunal bivalves such as mussels and oysters are used, either sampled directly from natural populations attached to rocks or structures, or from suspended caged populations. These bivalves are exposed to contaminants that are either suspended or dissolved in the seawater. Conversely, little attention has been paid to other bivalves that reside in the sediment, such as clams, which may be exposed to contaminants both in the water and in the sediment. Presumably, there would be a difference in response to the quality of the environment between these two types of bivalves, with infauna potentially exposed to higher concentrations of contaminants via the sediment, whilst epifauna would be dominated by a water column-influenced exposure pathway.

Biochemical alterations in aquatic organisms in response to contaminants are commonly used to indicate the potential for more severe hazards. These biochemical changes, or biomarkers of sublethal stress, have regularly been found to be sensitive to contaminants in the environment (Huggett et al., 1992).

The concept of transplanting shellfish within the marine environment for the purposes of monitoring accumulation rates or subsequent effects of contaminants was initially developed by the US EPA in 1976 (Goldberg et al., 1978), followed by a State of California effort in 1977 (Martin, 1985), and a large scale programme by the United States National Oceanic and Atmospheric Administration (NOAA) programme which began in 1986 (O'Connor, 1992). Sublethal effects of contaminants on marine organisms can also be determined using such approaches, and one advantage of transplanting and caging bivalves for this purpose is that it provides a combination of the experimental control of laboratory bioassays with the environmental realism of field monitoring (Salazar and Salazar, 1997).

In Hong Kong, there are no published studies of soft-sediment bivalves transplanted to contaminated areas for the purposes of identifying sublethal stress induced by contaminants. A study was recently carried out where epifaunal mussels (*Perna viridis*) were transplanted from "unpolluted" to PAH polluted sites in Hong Kong, for the purpose of measuring DNA adduct formation. Although large inter-individual variation in DNA adduct formation was evident within sites, a positive relationship between DNA adduct formation and body burden of total PAHs and benzo[*a*]pyrene was detected (Xu et al., 1999). DNA adduct formation, however, does not directly imply decreased fitness, and therefore it is concluded that biomarkers with clear ecological relevance are likely to provide more relevant ecological information.

Cheung et al. (2001) transplanted green-lipped mussels, *P. viridis*, from a relatively clean site to various polluted sites in Hong Kong. After a 30-day field exposure, different antioxidant parameters including glutathione S transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), NADPH DT-diaphorase (DT-d), glutathione (GSH) and lipid peroxidation were quantified, and tissue concentrations of benzo[*a*]pyrene (B[*a*]P) as well as a total of five polycyclic aromatic hydrocarbons (PAHs) with potential carcinogenicity were determined for individual mussels. Results indicated that: (1) tissue concentrations of B[*a*]P and total PAHs from the same site were highly variable; (2) gill SOD, DT-d and lipid peroxidation showed no response to tissue pollutants; (3) the majority of the antioxidant parameters were induced by increasing tissue pollutant concentrations and (4) amongst the various parameters, oxyradical scavenger GSH best correlated with tissue concentrations of pollutants. Cheung et al. (2002) transplanted mussels to various polluted sites in Hong Kong, measuring PCBs, OCs and antioxidant responses. Only GST and GSH were positively correlated with tissue PCB and OC concentrations. One of the enzymatic antioxidants, GPx, showed significant response to tissue PCB. No correlation was found between tissue concentrations of chlorinated hydrocarbons and other enzymatic antioxidants (SOD, CAT, GR, and DT-d).

The research described herein aims to evaluate differences in sublethal stress responses of bivalve sentinel organisms (as one antioxidant substrate (GSH) and 3 antioxidant enzymes (GST, GPx, and CAT)) when field deployed and exposed to persistent organic contaminants. The evaluation of antioxidant responses considers two different feeding types of bivalve shellfish, a range of coastal sites (4), and 2 exposure durations (14- and 28-day) in Hong Kong waters.

2. Materials and methods

2.1. Field deployment and collection

Mussels (*P. viridis*) and clams (*Ruditapes philippinarum*) were collected from a "clean" reference site, Kat O, and were depurated, with no mortality, for 8 days

in the laboratory using clean filtered, seawater (sourced from Ocean Park, Hong Kong). The duration of the depuration period was based on previous laboratory trials, where significant decreases in contaminants were detected after 1 week (unpublished data). Shellfish were not fed during depuration. Mussels and clams were transplanted on 27 and 28 February 2003 in plastic mesh bags (mesh size approximately 10 mm^2) to four sites, which represented a gradient of contaminants based on previous studies (Richardson and Zheng, 1999): Ma Wan, Kat O, Sai Wan Ho, and Tsim Sha Tsui (Fig. 1). The sampling criteria and sites for mussel transplantation followed those described in Cheung et al. (2001, 2002), with the exception that plastic mesh bags were substituted for metal cages. At each site, 4 replicates of 30 mussels and 40 clams were loosely placed inside separate mesh bags, closed with cable ties, and suspended about 2 m below the water surface. Though it is recognised that the natural habitat of clams is in contact with benthic sediments, in many sampling sites around Hong Kong the bottom sediment comprises extremely fine grain silt, which would smother the clams and greatly reduce survival. Thus, we considered that it was reasonable not to place clams in their natural habitat in order to ensure survival of individuals was sufficient to enable sampling. Significantly, *R. philippinarum* is a filter-feeding bivalve, and we believed (based on previous laboratory and field trials) that the organisms would be able to feed without the presence of sediment, although we recognise that the hydrodynamics involved may be different. It should be noted that during the deployment, no losses of clams were observed. Samples were taken at 0, 14, and 28 days and analysed for total body burdens of PCBs, OCs, PHCs, and lower and higher molecular weight PAHs. There was minimal biofouling of the plastic mesh bags after 28 days.

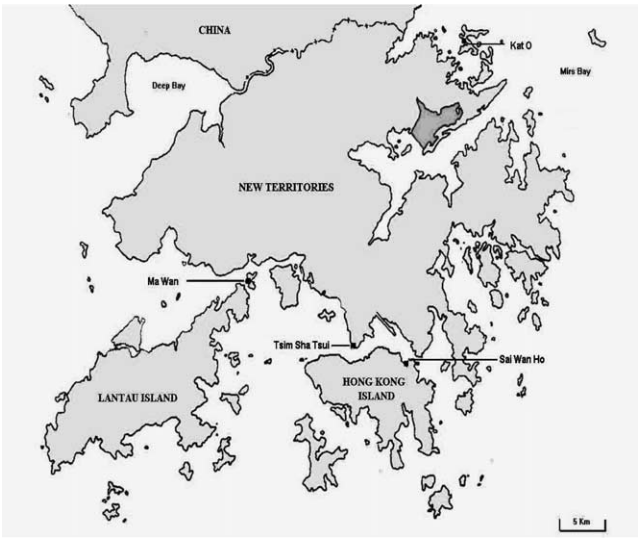

Fig. 1. Site locations in Hong Kong for the mussel and clam field exposure.

Antioxidant responses and methods were developed following recommendations and techniques by Cheung et al. (2001, 2002) and Mak (2003). Eighteen mussels and 23 clams were randomly selected from two bags at each site at each sampling period; 5 individuals of each species were used for condition index analyses, 8 individuals were dissected for biochemical analyses, and the soft tissues of 5 mussels and 10 clams were removed from their shells for composite analysis of contaminants (stored at −20 °C until analysed). The gill and hepatopancreas of each of the 8 individuals selected for biochemical analyses were removed, placed in 1.5 mL microcentrifuge tubes, frozen in liquid nitrogen immediately, and then stored at −80 °C until further biochemical analysis.

2.2. Chemical analyses

Chemical protocols for PCBs followed Cheung et al. (2004) and Mak (2003) for OCs, PHCs and PAHs in tissues. Briefly, the methods for trace organic extraction involved freeze-drying and homogenisation of the clam or mussel tissue into a powder using a tissue grinder. The powder was then extracted in dichloromethane and anhydrous sodium sulfate and the extract centrifuged at 3000 rpm for 5 min. This process was repeated twice, all three supernatants were combined and the sample volume reduced to 5 mL by rotary evaporation under reduced pressure. Sample cleanup was achieved by passing the sample through a silica gel column, with hexane and dichloromethane in hexane elution. The elution fractions were partitioned by hexane (F1), 30 mL 20% dichloromethane in hexane (F2), and 50 mL 20% dichloromethane in hexane. The eluate volumes of each fraction were reduced to approximately 1 mL before analysis of contaminants.

Chlorinated pesticides that were quantitated by gas chromatography with electron capture detection (GC-ECD) included: hexachlorobenzene, four isomers of HCH α, β, γ and δ), heptachlor, heptachlor epoxide, aldrin, endrin, *p*,*p*′-DDD, *o*,*p*-DDD, *p*,*p*′-DDE, *o*,*p*-DDE, *p*,*p*′-DDT, *o*,*p*′-DDT, α-chlordane, dieldrin, endosulfan, and kepone. Identification of PCB isomers and congeners was based on Richardson and Zheng (1999) and Zheng et al. (2000), using Aroclor mixtures (1242, 1248, 1254, and 1260 in a ratio of 1:1:1:1) as reference standards. The detection limit of the analysed organochlorines was 0.005 ng g^{-1} lipid. Total DDTs (∑DDTs) were the sum of all DDT metabolites (6); total PCBs (∑PCBs) were the sum of all identified congeners in comparison with 28 individual standard PCB peaks. The ratio of DDTs:DDEs was determined and used to evaluate the hypothesis that "new" or sequestered sources of technical grade DDTs (Miglioranza et al., 2003) are continuing to influence Hong Kong waters.

PHCs were quantified using gas chromatography with a flame ionisation detector (GC-FID) (Zheng and Richardson, 1999). Total *n*-alkanes were calculated by comparison with C22, and the unresolved complex mixture (UCM) was identified as the peak area under the baseline. Total PHCs were identified as the sum of total *n*-alkanes and UCM.

The 15 PAHs that were quantitated included: naphthalene, acenaphthylene, acenaphthene fluorine, phenanthrene, anthracene, pyrene, chrysene, benzo[*b*]-fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3,*d*]pyrene, dibenzo[1,2,5,6]anthrancene, and benzo(*g*,*h*,*i*)perylene. Gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring (SIM) was used to identify and quantify the concentration of each PAH (Pruell et al., 1986; Zheng and Quinn, 1988). Total PAHs were the sum of the 15 individual PAHs.

The processes responsible for PAH formation cause distinct "fingerprints" in the suite of PAHs, allowing for a general determination of sources. These differences can be identified by separating the molecular weight classes into low molecular weight PAHs (LMW-PAHs), where 2–3 ring compounds (and methylated PAHs) are more prevalent, and these are representative of petroleum, rather than pyrogenically derived materials (Page et al., 1999); the high molecular weight PAHs (HMW-PAHs) are produced through combustion and high temperature of formation. The LMW-PAHs included: naphthalene, acenaphthylene, acenapthene, fluorene, phenanthrene, anthracene; fluoranthene, pyrene, and chrysene; the HMW-PAHs included: benzo(*b*)fluroanthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, indeno(123-*cd*)pyrene, dibenzo(1,2,5,6)perylene and benzo(*ghi*)perylene. All chemical data are expressed as ng g^{-1} lipid. Lipid weights were determined using 1 g freeze dried shellfish powder, extracted with dichloromethane and reduced to a volume of 1 mL. Lipid weight was calculated as the difference between the weight of the extract placed in pre-weighed bottles and the weight of the bottle once the solvent had evaporated.

Standard Reference Materials (SRM 2974, National Institute of Standards and Technology, Washington, DC, USA and SRM 2262, National Institute of Standards and Technology, Gaithersburg, MD20899, USA) were analysed to validate the chemical analytical methods. Recoveries were 84.1% for PAHs, 99.8% for Aroclor 1254 (PCBs), 69.2% for PHC fuel oil, and 92.5% for a spiked OC pesticide test. Reported tissue concentrations have not been corrected for recovery.

2.3. Biochemical analyses and condition index

2.3.1. Condition index

The condition index used was dry soft tissue weight (g) × 100/wet soft tissue weight (Crosby and Gale, 1990).

2.3.2. Protein

Protein concentrations in tissue samples were determined using a commercially-available protein assay kit (Bio-Rad™ Protein Assay Kit). Bovine serum albumin (BSA) was used as the protein standard and quantified by measuring absorbance at 595 nm (Bradford, 1976).

2.3.3. Antioxidant response analysis

Tissue samples were removed from the freezer, thawed on ice and homogenised in buffer (50 mM potassium phosphate buffer, 0.1 M KCl, 0.1 mM EDTA, pH 7.4; with 20% glycerol to protect the enzymes) using an Ultra-Turrax tissue homogeniser. The homogenate was centrifuged at 12,000g for 30 min at 4 °C. The supernatant was retained, divided into several aliquots and stored at −80 °C for subsequent enzyme analyses. All enzyme determinations were carried out in quintiplicate at 25 °C.

GST activity was determined, following the method of Jakoby (1985) using 1-chloro-2,4-dinitrobenzene (CNDB) as the substrate. A unit of GST was defined as the amount of glutathione conjugate formed using 1 mM GSH and CDNB/min per mg protein.

CAT activity was determined with the method of Cohen et al. (1996) using H_2O_2 as a substrate. The consumption of H_2O_2 by the enzyme was monitored colourimetrically using ferrous sulfate and potassium thiocyanate. Unconsumed H_2O_2 oxidises ferrous ions to ferric ions, forming a red coloured ferrithiocyanate with thiocyanate ions. The appearance of the red colour was monitored at 492 nm. The concentration of H_2O_2 was determined 1 and 3 min after initiation of the reaction by the addition of sample. One unit of CAT was defined as $[\ln(A_1/A_2)/(t_1 - t_2)]$/mg protein, where A_1 is the absorbance at time point 1 (t_1), and subsequent time periods.

GPx activity was measured in a coupled enzyme system where oxidised glutathione (GSSG) produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase (Lawrence and Burk, 1976).

GSH was quantified by a method modified from Anderson (1985). GSH in samples is oxidised to GSSG and 5-nitrobenzoic acid (TNB) by 5,5′ dithiobis(2-nitrobenzoic acid) (DTNB). The GSSG in the reaction mixture is then reduced back to GSH, in a reaction catalysed by glutathione reductase at the expense of NADPH. This assay indirectly measures total GSH in samples by estimating the consumption rate of NADPH. The reaction was monitored at 412 nm, colourimetrically. GSH concentration was estimated from a standard curve and reported as nmol GSH/mg protein.

2.3.4. Statistical analyses

Parametric one-way analysis of variance (ANOVA) was used to compare individual contaminant concentrations and antioxidant responses among sites and times,

and Tukey's Studentized Range Test was employed where appropriate (SAS for Windows 6.12). Multivariate analyses were undertaken to test for associations between chemical gradients and antioxidant responses (PRIMER V.5). Biochemical data were transformed using log +1 due to non-normal distribution. Multidimensional scaling (MDS) plots were prepared to compare patterns between species and among sites and times for the contaminant data and the biochemical data separately. The analysis of similarity (ANOSIM) approach was used to test for statistical differences amongst groups. ANOSIM is an analogue of univariate analysis of variance using permutation tests based on ranked data in the form of a similarity matrix. Contaminant data and biochemical data were "matched" using the BIOENV procedure, which calculates the measure of agreement between two similarity matrices (i.e. contaminant and biomarker matrices). Normalised Euclidean distance was used as the similarity measure for MDS and BIOENV techniques. Pearson's correlations were used to test the relationship between individual antioxidant responses and each contaminant group of interest.

3. Results

3.1. Contaminants

3.1.1. Clam and mussel accumulation comparisons

Clams accumulated higher concentrations of $\sum$PAHs, PHCs, $\sum$DDTs and PCBs, while mussels accumulated higher concentrations of $\sum$HCHs. Concentrations of dieldrin and "remaining OCs" (hexachlorobenzene, HCB, heptachlor, heptachlor epoxide, aldrin, endrin, kepone, total chlordanes) were similar between species (Fig. 2). $\sum$PAHs in mussels ranged from 6.895 to 11.890 ng g^{-1} lipid, whereas clams ranged from 17.376 to 23.299 ng g^{-1} lipid. PHCs ranged from 6.635 to 12.967 in mussels and from 16.172 to 23.246 μg g^{-1} lipid in clams. $\sum$HCHs in mussel tissues ranged from 26.88 to 84.53 ng g^{-1} lipid and from 17.23 to 58.54 ng g^{-1} lipid in clam tissues. Dieldrin was detected in mussels in the range 1.58–25.17 ng g^{-1} lipid, whereas clams accumulated 3.64–19.10 ng g^{-1} lipid. $\sum$DDTs ranged from 5.35 to 32.46 in mussels and from 12.05 to 65.53 ng g^{-1} lipid in clams. Total remaining OCs in mussels ranged from 11.49 to 42.02 ng g^{-1} lipid and in clams from 13.68 to 41.55 ng g^{-1} lipid. PCBs in mussel tissues ranged between 162.80 and 437.53 ng g^{-1} lipid and in clam tissue between 321.91 and 734.99 ng g^{-1} lipid.

$\sum$PAHs and PHC concentrations in mussels and clams were significantly higher at Sai Wan Ho and Tsim Sha Tsui compared to Ma Wan and Kat O ($p < 0.001$). $\sum$HCHs, PCBs and remaining OCs were significantly higher at Sai Wan Ho compared to Ma Wan ($p < 0.01$). There was no difference in concentrations of $\sum$DDTs or dieldrin among sites (Fig. 2).

The relative enhancement of each contaminant was somewhat different among days (i.e. day 0, 14 and 28). $\sum$PAHs were significantly higher than day 0 concentration in mussels at Sai Wan Ho on both sampling days, and at Tsim Sha Tsui on day 14 ($p < 0.001$). Depuration of $\sum$PAHs below the day 0 concentration occurred at Ma Wan and Kat O on both sampling days. There was no significant difference in $\sum$PAH concentrations in clam tissues among sampling days. PHCs accumulated above day 0 concentration in mussel tissue at Tsim Sha Tsui and Sai Wan Ho at day 14 only, whereas depuration occurred at Kat O at both sampling times and at Ma Wan on day 14 ($p < 0.001$, Fig. 2). As with $\sum$PAHS, there were no differences in PHC concentrations in clam tissues among sampling times.

$\sum$HCHs significantly accumulated in both mussel and clam tissues above day 0 at Kat O, Sai Wan Ho and Tsim Sha Tsui ($p < 0.001$, $p < 0.05$, respectively). Concentrations of $\sum$HCHs were not significantly different from day 0 at Ma Wan on both sampling days (Fig. 2b). Dieldrin was significantly accumulated above day 0 concentrations in both species at all sites and times, excluding mussels at Ma Wan on day 14 ($p < 0.05$). No significant difference was detected between day 0 and day 28 $\sum$DDT concentrations at Sai Wan Ho and Kat O in mussel tissues, but all other sites accumulated $\sum$DDT above day 0 concentrations ($p < 0.001$). All sites and times had higher concentrations of $\sum$DDT in clam tissues compared to day 0 ($p < 0.05$, Fig. 2).

Of the remaining OCs, significant accumulation over day 0 occurred at Kat O, Sai Wan Ho and Tsim Sha Tsui in clam tissues, whereas accumulation at all sites occurred in mussel tissues ($p < 0.05$). No significant differences among sampling times were detected for either species for PCB concentrations (Fig. 2). Generally, highest concentrations of all of the hydrocarbons (OCs and PAHs) were found at Tsim Sha Tsui and Sai Wan Ho, with lower concentrations at Ma Wan and Kat O.

Continued increase in concentrations of contaminants at day 28 over day 14 was not evident, regardless of species or site. This observation was confirmed by analysis of variance statistics. This indicates that the concentrations appear to be reaching saturation by day 14, although the concentrations of several of the OCs were very low. An ongoing problem with these types of studies in Hong Kong waters is the source of completely depurated experimental organisms. Day 0 organisms (original transplant stocks) of both species, even after laboratory depuration, had relatively high levels of many contaminants of interest, which tends to obscure uptake or depuration patterns and makes results of Hong Kong deployments conservative for accumulation estimates. Depuration of $\sum$DDT only

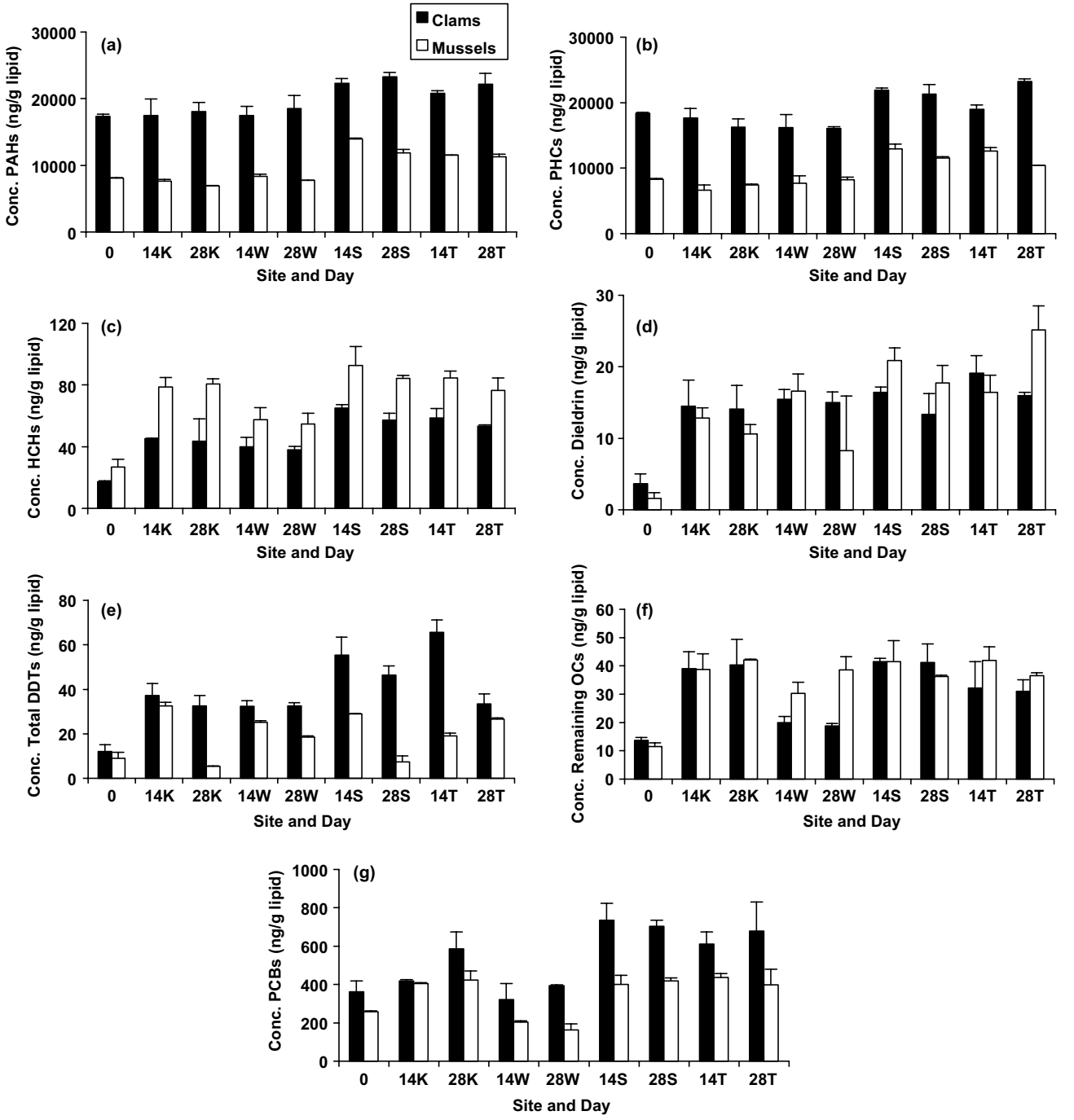

Fig. 2. Concentrations of contaminants (±s.e.) in field-deployed clams and mussels from four Hong Kong sites, determined at 0, 14 and 28 days. Concentrations are in ng g^{-1} lipid weight. Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T).

occurred after 14 days at Kat O, Ma Wan and Sai Wan Ho in mussels, and Tsim Sha Tsui in clams.

Multi-dimensional scaling (MDS) of contaminant concentrations clearly separated clams and mussels into two different groups (ANOSIM, $R = 0.662$, $p = 0.001$). An MDS of contaminants in clam tissue only grouped Tsim Sha Tsui and Sai Wan Ho separately from Kat O and Ma Wan, with a third group of day 0 (Fig. 3). Sites were similarly grouped for contaminants in mussel tissue, with the exception of Kat O day 28 samples which formed another separate group (Fig. 4).

ANOSIM of contaminant concentrations in clam tissue among sites revealed that significant differences existed ($R = 0.732$, $p < 0.001$). Pairwise comparisons indicated that clam day 0 contaminant concentrations were entirely distinct from clam body burden samples determined from transplanted organisms ($R = 1$). Among sites, Ma Wan and Sai Wan Ho were most

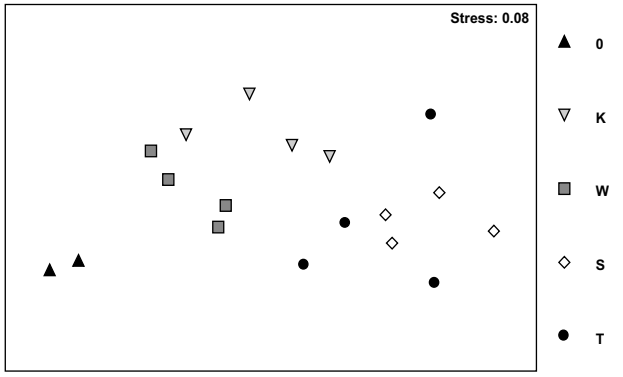

Fig. 3. MDS of contaminant concentrations in clam tissue as determined on day 14 and day 28, plotted against site. Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T). 0 = day zero organisms.

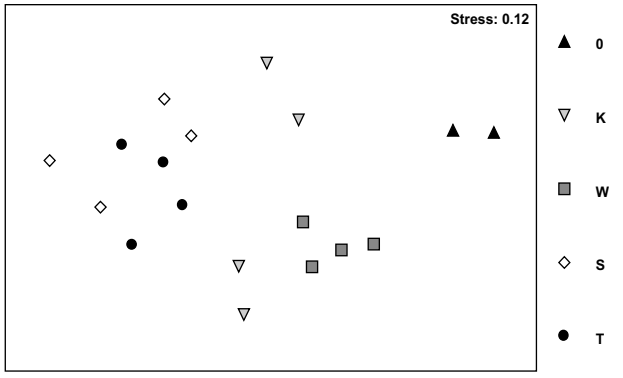

Fig. 4. MDS of contaminant concentrations in mussel tissue as determined on day 14 and day 28, plotted against site. Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T). 0 = day zero organisms.

dissimilar ($R = 1$), followed by Ma Wan and Tsim Sha Tsui ($R = 0.792$) and Kat O and Sai Wan Ho ($R = 0.781$). Kat O and Tsim Sha Tsui, and Kat O and Ma Wan were determined to be less distinct but still clearly different ($R = 0.594$ and $R = 0.531$, respectively).

A virtually identical pattern among sites in mussel contaminant body burden was verified in the ANOSIM test ($R = 0.799$, $p < 0.001$). Pairwise comparisons determined samples collected at day 0 were completely different to those from transplanted mussels ($R = 1$). As with clams, Ma Wan and Sai Wan Ho were the most dissimilar sites ($R = 1$), followed by Ma Wan and Tsim Sha Tsui ($R = 0.958$), Kat O and Sai Wan Ho ($R = 0.917$), Kat O and Tsim Sha Tsui ($R = 0.875$), and Kat O and Ma Wan ($R = 0.646$). For both clam and mussel contaminant body burden there was no difference detected between Sai Wan Ho and Tsim Sha Tsui.

ANOSIM of contaminant concentrations among times for both clams and mussels indicated that significant differences existed ($R = 0.216$, $p < 0.03$; $R = 0.271$, $p < 0.015$). Pairwise comparisons indicated day 0 and day 14 (clams $R = 0.565$, mussels $R = 0.797$), and day 0 and day 28 (clams $R = 0.76$, mussels $R = 0.746$) to be dissimilar, whereas day 14 and day 28 were indistinguishable.

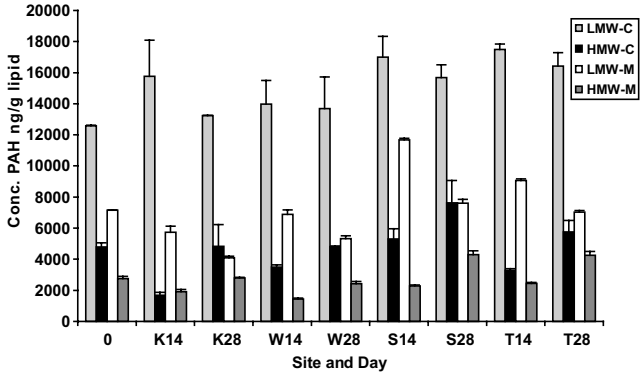

Fig. 5. Concentrations of high and low molecular weight PAHs (±s.e.) in clams (LMW-C, HMW-C) and mussels (LMW-M, HMW-M) tissues from four Hong Kong sites determined at day 0, 14 and 28. Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T).

Comparison of high and low molecular weight PAHs in clam tissue revealed that low molecular weight PAHs were approximately double the concentration of the high molecular weight PAHs (Fig. 5), reflecting likely petroleum hydrocarbon origins. A similar pattern was evident in mussels on day 0 and day 14 at all sites, but there was less disparity between these groups by day 28 (Fig. 5).

The ratio of ∑DDTs to ∑DDEs, representing "new or sequestered" DDT inputs, revealed a significant increase above day 0 at Ma Wan and Sai Wan Ho at day 14 in both species, which decreased by day 28 (Fig. 6). Clams at Tsim Sha Tsui showed a similar trend between sampling days, whereas the ratio in mussels decreased with time at this site. A decrease in DDT:DDE from day 0 was detected in both species at Kat O.

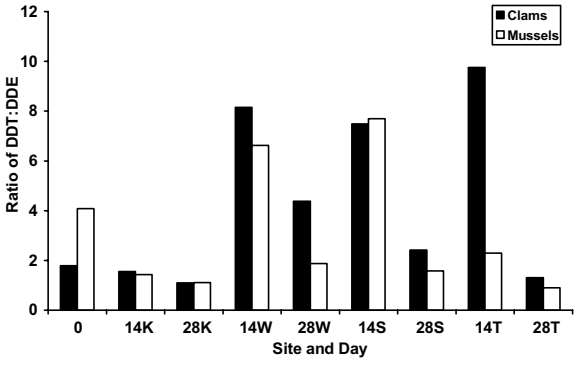

Fig. 6. Comparison of concentration of ∑DDTs to ∑DDEs in tissues from clams and mussels determined at day 0, 14 and 28. Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T).

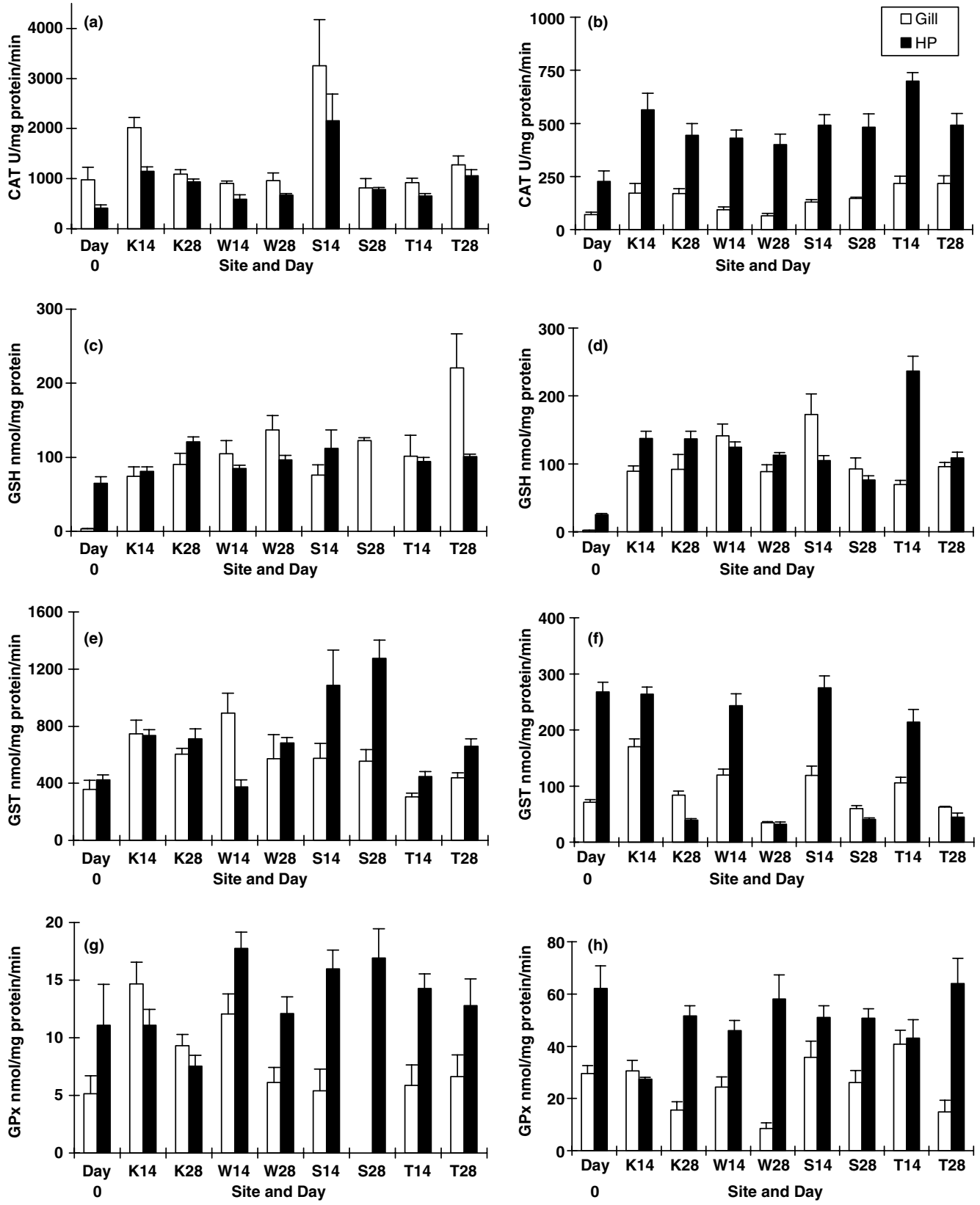

Fig. 7. Antioxidant responses (±s.e.) in gill (white bars) and hepatopancreas (black bars) of clams (a, c, e, g) and mussels (b, d, f, h) at four Hong Kong sites. Sites: Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T). Times: day 0, 14 and 28.

3.1.2. Biochemical analyses

There were no significant differences in condition among sites or times for mussel tissue, whereas clams at Kat O had significantly higher condition index than clams at Tsim Sha Tsui and Ma Wan ($p < 0.0001$).

3.1.3. Antioxidant biomarker responses

In general, mussels had less CAT activity than clams, irrespective of tissue type (Fig. 7a and b). An ANOVA of CAT activity in clam tissue indicated significant differences among sites, days and tissue types ($p < 0.0001$, $p < 0.0002$, $p < 0.0017$, respectively). Tukey's multiple comparisons showed that Sai Wan Ho had higher CAT activity than day 0, Ma Wan and Tsim Sha Tsui, and Kat O had higher CAT activity than Ma Wan. CAT induction at days 0 and day 28 was lower than that at day 14. Gill tissue was found to have higher CAT activity compared to hepatopancreas. A significant interaction term was detected between sites and days, which was driven by the higher induction of CAT in gill tissue at Kat O on day 14, and in both tissue types at Sai Wan Ho at day 14 (Fig. 7a).

Significant differences in CAT activity among sites and times and between tissue types were detected in an ANOVA of mussel tissue ($p < 0.0001$, $p < 0.0357$, $p < 0.0001$, respectively; Fig. 7b). A Tukey's multiple comparison test revealed day 0 CAT activity to be significantly lower than all other sites. Furthermore, CAT was induced to higher levels at Tsim Sha Tsui compared to Sai Wan Ho and Ma Wan. Amongst times, day 0 CAT activity was significantly lower than that at days 14 and 28, which were not significantly different from each other. In contrast to clams, hepatopancreas had higher CAT activity than gills. There was a significant interaction between days and tissues, however, which can be explained by CAT being induced to higher levels on day 14 compared to day 28 in hepatopancreas tissue at Kat O and Tsim Sha Tsui, whereas in gill tissue at all sites and at Ma Wan and Sai Wan Ho in hepatopancreas tissue there was virtually no difference between day 14 and day 28 induction of CAT (Fig. 7b).

ANOVA for both clams and mussels detected significant differences in GSH between factors, but interpretation was compromised by significant interaction terms. It is clear from Fig. 7c and d that there was induction of GSH in gill tissue at all sites and times above day 0 in both species. A similar pattern is evident in mussel hepatopancreas tissue, with the exception of the higher value at Tsim Sha Tsui on day 14. In clam hepatopancreas, however, there was little difference between day 0 GSH level and all other sites and times.

In general, GST was detected at higher levels in clam tissues compared to mussel tissues (Fig. 7e and f). GST induction in clams was significantly different among sites ($p < 0.0001$) and between tissue types (Fig. 7e). Multiple comparisons showed Sai Wan Ho, Kat O and Ma Wan had higher GST than day 0, whereas no difference between Tsim Sha Tsui and day 0 was detected. GST at Sai Wan Ho was also significantly higher than that at Tsim Sha Tsui and Ma Wan. Kat O had higher induction of GST than Tsim Sha Tsui. Clam hepatopancreas tissue had significantly higher GST than gill tissue. A significant interaction between tissues and sites was detected in the ANOVA, the cause of which is evident in Fig. 7e as higher GST in hepatopancreas at Sai Wan Ho and Tsim Sha Tsui, but little difference was observed between tissue types at Kat O, Ma Wan and in day 0 samples.

ANOVA of GST in mussel tissues indicated significant differences among sites, days and between tissue types, but interpretation was compromised by significant interactions among all variables. However, there are clear trends which can be mentioned (Fig. 7f). The pattern of GST response was different between tissue types. In gill tissue, GST increased from day 0 to day 14 at all sites, and by day 28 had returned to the initial day 0 value. In hepatopancreas tissue, however, GST at day 0 was not different to day 14 at all sites, but by day 28 GST was 4 times lower than day 0 at all sites.

It is clearly evident from Fig. 7g and h that GPx induction was generally higher in mussel tissue compared to clam tissue. ANOVA of GPx in both clams and mussels revealed significantly higher induction in hepatopancreas tissue ($p < 0.0001$, $p < 0.0001$, respectively; Fig. 7g). No differences were detected among sites or times for either species.

Although each of the stations and times of deployment had unique patterns of contaminant bioaccumulation and different antioxidant responses, the general pattern of responses followed the order: CAT > GST > GSH > GPx. Hepatopancreas generally showed the highest antioxidant responses for both mussels and clams. Only the gill of clams had a higher response than hepatopancreas for CAT (Fig. 7a and b). The

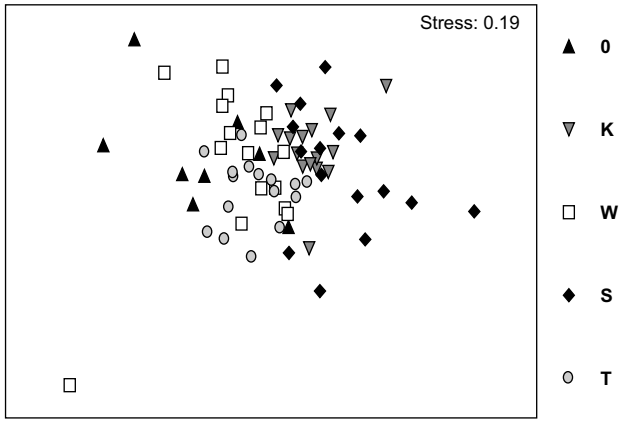

Fig. 8. MDS of antioxidant response in clam tissue as determined on day 0, 14 and 28, plotted against site. Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T). 0 = day zero organisms.

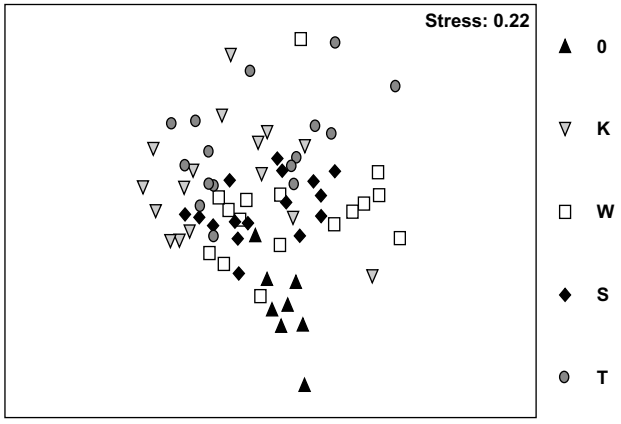

Fig. 9. MDS of antioxidant response in mussel tissue as determined on day 0, 14 and 28, plotted against site. Kat O (K), Ma Wan (W), Sai Wan Ho (S) and Tsim Sha Tsui (T). 0 = day zero organisms.

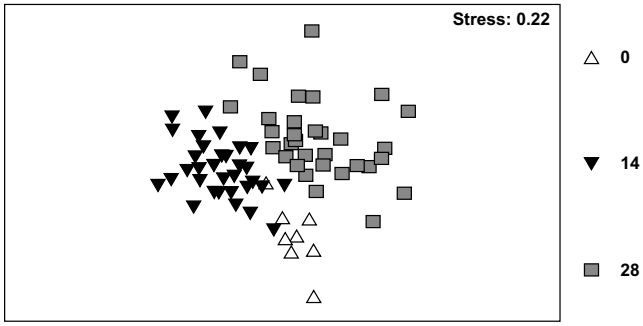

Fig. 10. MDS of antioxidant response in mussel tissue as determined on day 0, 14 and 28.

complexity of substrate and enzyme responses cannot be evaluated on general terms or overall patterns, but rather tissues, species, and individual site data evaluations are necessary to interpret the antioxidant responses.

An analysis of similarity (ANOSIM) test detected significant differences in antioxidant response between mussels and clams ($R = 0.791$, $p < 0.001$). An MDS plot of antioxidant responses in clam tissue revealed no obviously distinct site differences (Fig. 8). An ANOSIM based on the same data, however, indicated differences between sites existed ($R = 0.311$, $p < 0.0001$). Of the pairwise comparisons only day 0 and Kat O were distinctly different ($R = 0.628$). From the remaining comparisons, day 0 and Sai Wan Ho, day 0 and Tsim Sha Tsui, and Kat O and Tsim Sha Tsui were identified as different groups but overlap to various degrees ($R = 0.474$, $R = 0.463$, $R = 0.443$). A one-way ANOSIM comparing antioxidant response among times revealed small differences ($R = 0.216$, $p < 0.001$), with day 0 and day 28 identified as different, but probably overlapping, groups ($R = 0.494$).

As with clams, the MDS plot of antioxidant responses in mussel tissues did not clearly separate sites (Fig. 9). The ANOSIM detected small differences between some pairwise comparisons ($R = 0.238$, $p < 0.001$); day 0 and Kat O, day 0 and Tsim Sha Tsui, and day 0 and Sai Wan Ho were identified as distinct groups but some data points overlap ($R = 0.540$, $R = 0.535$, $R = 0.491$, respectively). An MDS of antioxidant response in mussels highlighting different sampling times (Fig. 10) shows that day 0, 14 and 28 separate into different groups. A one-way ANOSIM confirmed differences existed ($R = 0.524$, $p < 0.001$). Day 0 and day 14 ($R = 0.634$), day 14 and day 28 ($R = 0.515$) and day 0 and day 28 ($R = 0.442$) were found to be clearly different groups, with a small degree of overlapping data points.

BIO-ENV tests were performed on the data for each species, to ascertain the similarity between the contaminant body burden similarity matrix and the corresponding antioxidant response matrix. For clams, GSH and GST in hepatopancreas and GST in gill tissues taken together maximise the matching coefficient (Spearman's correlation 0.352). Five variables give the highest correlation in mussel tissues; condition, protein in gills, CAT in gills and hepatopancreas, and GST in hepatopancreas (Spearman's correlation 0.407).

Pearsons's correlations were undertaken to investigate relationships between individual antioxidant responses and contaminant body burden in each species (Table 1). Data were pooled across sites and times for these analyses. Significant positive correlations for clams existed between some contaminants and CAT in hepatic tissue, GST in hepatic tissue and GPx in gill tissue

Table 1
Significant positive Pearson's correlation coefficients between antioxidant responses and contaminants of interest (data pooled across sites and times)

Antioxidant response	∑PAHs	Dieldrin	∑DDTs	∑HCHs	∑OCs	∑PCBs
Clams						
CAT HP				0.547	0.581	0.504
GST HP	0.530			0.494	0.566	0.605
GPx gill						0.469
Mussels						
CAT HP				0.706	0.694	0.570
CAT gill		0.536		0.647		0.767
GST gill			0.568			

(Table 1). In mussels, significant positive correlations were identified between some contaminants and CAT in both tissue types and GST in gills (Table 1). The majority of the correlations, in both species, occurred in CAT in both tissue types and GST in hepatic tissue. Among these, the common contaminants were ∑HCHs and ∑PCBs, although ∑OCs were also associated with antioxidant responses in hepatic tissue.

4. Discussion

A number of environmental pollutants can cause oxidative damage to biological tissues and systems. Chlorinated biphenyls and pesticides may produce superoxide anion radicles by redox cycling (Winston and DiGiulio, 1991), whilst PAHs have been shown to affect several enzyme parameters including the P-450 content, NADPH cytochrome *c* reductase activity, as well as specific PAH-oxidase activities (Michel et al., 1993). Thus, mussels and clams living in environments contaminated with these hydrocarbons may be exposed to oxidative stressors caused by a variety of oxy-radicals. In physiological responses to these chemical insults, organisms have developed defense mechanisms such as the generation of antioxidants (e.g., GSH) and antioxidant enzymes (GST, CAT, and GPx). Indeed, previous studies in Hong Kong have demonstrated that these antioxidative responses can be induced by oxidative stress, and there appears to be an association (statistically significant positive correlations) with certain trace organic pollutant concentrations (Cheung et al., 2001, 2002).

Other authors have stressed the need to couple chemical determinations of environmental contaminants with the use of biomarkers of exposure and/or their effects to provide evidence of a cause-effect relationship, and to thus provide better environmental risk management actions (Sole, 2000; Porte et al., 2001). This approach has been effective in providing superior metrics for the evaluation of the impacts of pollution in the marine environment, and their application in the field setting with different receptor species has been successfully applied in a number of international environmental situations (e.g., Sole et al., 1998; Livingstone et al., 2000). It is worth mentioning that the accumulation of pollutants in bivalves is determined by a dynamic balance which results in uptake or depuration, and which, in turn, are influenced by a dynamic equilibrium amongst pollutants in sediment, water, air, food particles, and the organisms themselves (Fossato and Canzonier, 1976; Livingstone, 1991; Vernier and Canova, 1996).

Previous Hong Kong field transplant studies with mussels have measured concentrations of 5 higher molecular weight PAHs (Cheung et al., 2001), as well as polychlorinated biphenyls and chlorinated pesticides (Cheung et al., 2002), along with antioxidant response biomarkers. A laboratory based dosing experiment with mussels and benzo[*a*]pyrene and Aroclor 1254 was also completed to evaluate field-based responses; the aim of the study was to examine the exposure–response relationship between the suite of antioxidative biomarkers and two model contaminants (Cheung et al., 2004). Direct comparisons of the current chemicals (and antioxidant responses) with previous work are complicated by measurement of different chemical constituents (only HMW PAHs, Cheung et al., 2001); use of different transplant locations (all previous studies), different seasons (winter, Cheung et al., 2001, 2002) and the measurement of a different suite of antioxidant responses (GSH, GST, CAT, and GPx were selected for evaluation in the current study, based upon all previous work). Cheung et al. (2004), however, determined in a laboratory experiment that CAT and GSH in gill tissue were positively correlated with Aroclor 1254, and GPx in gill and hepatopancreas, and hepatic GST were positively correlated with B[*a*]P concentration.

In order to compare our results with Cheung et al. (2001, 2002), we have converted our contaminant data (lipid weight) to a dry weight basis for this section of the discussion. Cheung et al. (2001) reported concentrations in *P. viridis* of HMW-PAHs from 3 sites (Kat O, Sai Wan Ho, and Tsim Sha Tsui). Tissue concentrations ranged for ∑PAHs from 8 to 307 ng g^{-1} (dry), although the specific sites were not differentiated nor site specific data reported. The lowest concentration of HMW-PAHs in our deployment was 446 ng $^{-1}$ (dry) at Kat O (28 day), whilst the highest reported was 1195 ng g^{-1} (dry) at Sai Wan Ho. PAHs in our deployment were substantively higher than previously reported. We can only speculate on possible reasons for this change, but seasonality of oceanographic and land conditions (more terrigenous runoff and increased circulation in the Pearl River Delta) would be highly probable. Influences of decreased salinity might also change the physiological profiles of the uptake of PAHs, coupled with possible decreases in the mussels' ability to rapidly detoxify or eliminate PAHs. Clams showed little difference in concentrations of HMW-PAHs to those of mussels on a dry weight basis, ranging in concentrations from 800 at day 0 to 1233 ng g^{-1} (dry) at Tsim Sha Tsui (day 14). As all of these analyses have been performed in a single laboratory, with consistent QA-QC and analysts, these differences reflect true differences in the sampling data sets. This is an indication of how variable the environmental conditions are in the Pearl River Delta, and further work needs to be performed to provide a more complete evaluation of the seasonality and spatial distributions of the PAHs in Hong Kong waters.

PCBs and OCs have previously been reported for Hong Kong mussel transplant samples (Cheung et al., 2002). This transplant occurred at the same time of year

as the current experiments, and the duration of the exposure (transplant deployment) was 30 days. The experiments had 3 common sites (Kat O, Sai Wan Ho, and Tsim Sha Tsui) (Cheung et al., 2002). The mean concentrations of PCBs ranged from 149 ng g^{-1} (Kat O) to 515 ng g^{-1} (Tsim Sha Tsui). In comparison, the values for $\sum$PCBs reported herein are dramatically lower, ranging from 18 ng g^{-1} (dry) at Ma Wan (28 day) to 48 ng g^{-1} (dry) at Sai Wan Ho. Total OCs, reported by Cheung et al. (2002) ranged from 33 ng g^{-1} (dry) at Kat O to 172 ng g^{-1} (dry) at Tsim Sha Tsui. In the current investigation, $\sum$OCs ranged from 14 ng g^{-1} (dry) at Ma Wan to 26 ng g^{-1} (dry) at Tsim Sha Tsui. These organochlorine concentrations are lower than previously reported. These lower PCBs and OCs concentrations could be a seasonal cyclic phenomenon, which may be related to dilution, altered physiological state of the mussels, circulation patterns (although PAHs showed a reverse trend), or lowered general inputs from diffuse sources. There have been no previous transplant experiments with clams in Hong Kong.

Laboratory experiments provide an opportunity to simplify and control the experimental environment; however, often observations in such experiments do not hold true in the real environment. Biomarkers that have shown great promise in laboratory trials often show different or at least more variable results in field validation trials. This is, of course, due to the highly complex nature of the environment, which often creates more ''noise'' in the data. Hong Kong's marine environment is an example of such a complex environment, with a broad range of contaminants often at high concentrations in sediments, seawater and organisms. In this study, we encountered inconsistent antioxidant responses which did not tend to agree with overall contaminant patterns. Nonetheless, our field results were not too dissimilar to laboratory trial results of Cheung et al. (2004) in mussels, where CAT and GST were found to be associated with common contaminants.

Low molecular weight PAHs dominated total PAHs in both clam and mussel samples at all sites and sampling times. In general, LMW-PAHs were double the concentration of high weight PAHs, which indicates petroleum sources, as opposed to pyrogenic deposition. The high volume of maritime traffic in Hong Kong supports this finding. The high DDT:DDE ratio at Ma Wan in both species is likely to be influenced by discharges of contaminants from the adjacent Pearl River Delta. Similarly high ratios at the Victoria Harbour transplant sites (Sai Wan Ho and Tsim Sha Tsui) on day 14 are not unexpected, though the marked decrease at day 28 requires further investigation.

Clearly, clams produced larger CAT responses to mussels in this deployment. Gill tissues in both species, however, showed the same trends in CAT response (excluding the spike in CAT response in clams at Sai Wan Ho at day 14), though the response detected in clams was approximately an order of magnitude larger than that in mussels. If we exclude Sai Wan Ho day 14 in clams, then the patterns among sites and times are also similar in both species for hepatopancreas tissue. The large CAT response in clams at day 14 at Sai Wan Ho may have occurred in response to a point source contaminant discharge or spill. A linear regression of CAT in gill and hepatopancreas tissue in clams confirmed high agreement in response ($R = 0.734$), whereas no relationship between tissue types was detected in mussels. The higher response in mussel hepatopancreas compared to gill tissue is in agreement with previous studies (Cheung et al., 2004).

Glutathione (GSH) appears to have quite a range of variation within the sites and tissues (Fig. 7c and d). For all sites and all tissues, the substrate GSH was markedly induced (25–237 nmol/NADPH/mg protein) at day 14, showing a consistent, but differential pattern amongst the sites and exposure durations. Clam gill tissue showed an even more dramatic relative response at day 28 (3–221 nmol/NADPH/mg protein). We have not determined the reason for high GSH at Tsim Sha Tsui at day 28 in clam gill tissue and day 14 in mussel hepatopancreas tissue. Perhaps these data indicate the large variability in response of this substrate.

GST response between species was clearly quite different. A pattern of higher GST at day 14 compared to day 28 was evident at all sites in both tissue types for mussels. Perhaps this indicates some degree of recovery from a chemical insult. No difference was detected among sites though, which tends to negate the hypothesis of a contaminant measured in this study affecting the activity of this antioxidant enzyme. In addition, the very high day 0 value in mussel hepatopancreas tissue complicates the results and suggests that measuring this enzyme in gill tissue might be a more suitable contaminant biomarker in mussels. As with CAT, GST response was greater in clam tissue compared to mussel tissue. With respect to clam tissue, the only striking difference between tissue types is the higher response at Sai Wan Ho in hepatopancreas tissue at both day 14 and day 28. If GST response is affected by a certain contaminant or group of contaminants, it is not obvious from our survey, as we would have expected a similar antioxdant response at Tsim Sha Tsui which is also located within Victoria Harbour and has very similar contaminant profiles. This notwithstanding, it appears that GST in hepatopancreas tissue shows more utility for further investigation than gill tissue in clams.

GPx showed little use as a biomarker of contaminants in either species, regardless of tissue type, due to high day 0 values and an inconsistent response among sites and times. We recommend that in future trials of this nature, GPx be excluded from the suite used.

There is currently some debate in the literature about the usefulness of many biomarkers, including antioxidant responses, to indicate contaminant-mediated stress in molluscs. Nasci et al. (1998), in their study of antioxidant responses in *Mytilus galloprovincialis* in the Venice Lagoon, Italy, did not find significant positive correlations between antioxdant enzyme activities and pollutant body burden. Niyogi et al. (2001) detected significant correlation between antioxidant responses and PAHs in the oyster, *Saccostrea cucullata*, in the Hooghly Estuary, India, though they warn other researchers to be aware of seasonal variation in the activity of these enzymes. Nonidentified pollutants and differences in bioavailability of contaminants were postulated as possible explanations for the lack of correlation between antioxdant enzyme activity and contaminants in the freshwater bivalve, *Unio tumidus* (Cossu et al., 2000). Seasonal variation was also detected in GST and CAT in mussel tissue transplanted to bays near Nice and Cannes in the NW Mediterranean (Romeo et al., 2003). Other researchers, though, have detected significant correlations between various contaminants and antioxdant responses in molluscs (Lionetto et al., 2003; Chen et al., 2002; Nasci et al., 1999).

Comparing mussels and clams as bioaccumulators, clams had higher concentrations for 4 of the 7 contaminant groups used in this experiment, whereas the concentration was higher in mussels for just one group ($\sum$HCHs). One might anticipate, therefore, that clams may have greater biomarker responses compared to mussels. This was true for CAT and GST in both gill and hepatic tissue, but GSH was similar between species for both tissue types. Both species, however, failed to clearly separate transplant sites with respect to antioxidant response (see MDS plots). Furthermore, the suite of antioxidants used in the present study were not found to closely match the total contaminant data for either species (see BIOENV procedure). Based on the correlations of individual antioxidant responses with each contaminant group, we conclude that CAT and GST in hepatic clam tissue and CAT in hepatic and gill tissue in mussels show the best promise as biomarkers in Hong Kong's coastal waters.

Recommendations for future work include more research on CAT and GST, especially in clam tissues, as well as studies to evaluate the lack of consistency in antioxidant response. Seasonal variations in antioxidant responses need to be investigated in *P. viridis* and *R. philippinarum* in Hong Kong coastal waters, as literature suggests significant seasonal alterations may exist. Site-specific antioxidant response evaluations are necessary for various locations in southeast Asia, because of the specific nature of contaminant exposures in these areas.

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