Natural England Research Report NERR009

The application of biological-effects tools to inform the condition of European Marine Sites

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Plymouth Marine Science Partnership

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A summary of the findings covered by this report, as well as Natural England's views on this research, can be found within Natural England Research Information Note RIN009 – The application of biological-effects tools to inform the condition of European Marine Sites.

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It should be noted that the opinions expressed in this report are largely those of the authors and do not necessarily reflect the views of Natural England

Summary

A number of marine sites have been designated as Special Areas of Conservation (SAC) in England under the EC Habitats Directive. Natural England has a duty to monitor, report on the condition, and advise on risks to the features for which each site has been designated. Currently, methodologies for recording condition are based on ecological community-level census techniques, coupled with contextual information (which includes chemical and biological monitoring assessments), and expert judgement.

The aim of this project was to assess the feasibility and value of developing a cost-effective and practical methodology based around biological-effects measurements (biomarkers) to assist in condition assessment of marine SACs, which could be deployed on a 6-year cycle.

The Fal & Helford SAC and Plymouth Sound & Estuaries SAC were chosen as test sites for the deployment of such measurements. Within each SAC, sampling sites were chosen to reflect historical chemical contamination identified by a critical assessment of a series of desk-based site characterisation studies of European Marine Sites (EMS) in the south-west region. The sampling stations spanned a wide range of interest features in each of the SACs.

The field surveys employed a suite of biomarkers incorporating molecular, biochemical, cellular and physiological endpoints applied to coastal invertebrate animals endemic to each SAC site (*Mytilus edulis, Carcinus maenas*). It was important that the species selected occurred at each sampling site.

At the same time as taking biological endpoints, water was sampled from the collection sites and tested using the Microtox rapid toxicity bioassay. None of these samples was acutely toxic.

Biomarker results suggested that there were sites within each SAC that experienced multiple contaminant impacts from PAHs, TBT and metals (and possibly, indirectly, from nutrient enrichment). Individual biomarkers that discriminated impact included metallothionein, TOSC and PAH metabolites.

Taking all the individual biomarker evidence in an integrated biomarker assessment (Biomarker Response Index) indicated that, for the Tamar, mussels and crabs were impacted along a general gradient from Neal Point (highest impact) to Torpoint. For the Fal, the health status of crabs in the upper estuary was impacted (possibly due to elevated PAH concentrations) as was the health of mussels from Weir Point (possibly due to historical trace metals). TBT bioaccumulation in mussels from Penryn Creek and the continuing absence of dogwhelks in the Fal also imply that the condition of the site may be compromised in the Falmouth region.

Despite relying mostly on historical chemical data, biomarker responses often related significantly to chemical hotspots and sources (eg reflecting Restronguet Creek as a source of metals in the Fal; signifying reduced water quality in the upper Fal, and lower Tamar). Equally important, there was usually consistency in characterising good water quality/reference conditions (Whitsand and Maenporth).

This study showed therefore, that some individual biological techniques provide useful diagnostic information on the activity of certain classes of contaminants. In order to facilitate the interpretation of complex biomarker data sets to inform Natural England's environmental risk and condition assessment procedures, a Biomarker Response Index (BRI) was developed. The BRI makes use of a range of "expected" reference values for the biomarkers, classifies the responses based on extent of deviation from reference, and weights responses according to the level of biological integration. The main benefit of the BRI is to provide a simple visual method of comparing integrated biomarker responses from field locations against normal reference responses. It also provides an alternative way of viewing the full biomarker dataset, providing an essentially similar picture to the integrated

assessment based on multivariate analysis, and allows a simple way to prioritise further investigative studies.

For the Tamar, mussels from all sites showed major and severe alteration from normal health ranges; and crabs were impacted along a general gradient and in particular showed major alterations in normal baseline responses at Torpoint and Town Quay. For the Fal, the health status of crabs in the upper estuary was severely impacted as was the health of mussels from Weir Point. In addition, both mussels and crabs sampled from near Falmouth docks (Flushing) showed evidence of major alterations to their health.

These results highlight the potential value of biomarkers in the overall scheme of site assessment, and several examples have been provided on where such information can strengthen Natural England's current condition assessments (for the Fal), and thus help focus future action.

Practitioners should, however, recognise some limitations in using biomarker tools to inform risk assessment and condition assessment; some of these can be reduced by tailoring methodology, sampling design, or by undertaking additional further work. These include:

- The individual tools are not designed to be used on their own, but rather to form part of the weight of evidence in an integrated risk assessment.
- Biomarker responses could be subject to subtle biotic and seasonal influences which would have to be factored in to any routine application of biological-effects tools. While the BRI takes such variation into account, by including studies incorporating different biotic and seasonal influences to set the range of BRI values where ever possible, it would seem that if comparison across sites is a management priority, sampling at a fixed time of year will be essential to avoid seasonal variation.
- Lack of chemical data and detailed ecology measured at the same time as the biomarkers, requires a reliance on historical contextual data to link contamination and health status of exposed organisms. Acquisition of appropriate contemporary information on chemical and biological status, at common sites, should be seen as a high priority.
- As this study has indicated, the environmental relevance of biomarker responses can be increased by using biomarkers from invertebrate species which occupy critical trophic positions, and by applying weight of evidence e.g. BRI techniques to interpret risks. However, additional work is required to understand what these biomarker responses mean for health of an individual, and how this can be extrapolated to the population.

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1 Introduction

Background

1.1 Natural England is charged with the responsibility to ensure that England's unique natural environment, including its flora and fauna, land and seascapes, geology and soils, are protected and improved.

1.2 As part of this responsibility, Natural England has a statutory role to assess the condition of sites designated for their wildlife and habitat interests. These include Special Areas of Conservation (SACs) and Special Protection Areas (SPAs), designated under the European Habitats and Birds Directives, and nationally designated Sites of Special Scientific Interest (SSSI). Natural England also provides statutory advice on the risks and impacts to site interest features (habitats and species) posed by anthropogenic activities.

1.3 The current methods used for recording site condition have been developed to a UK Common Standard through the Joint Nature Conservation Committee (JNCC). This monitoring is based on ecological surveys, the mapping of habitats and biotopes, with more detailed population monitoring applied for some species. In addition, an 'Expert Judgement' is applied, a qualitative estimate based on local expertise and knowledge and which may be unique for each site.

1.4 Contextual information, generated by other relevant authorities and by academia, is also used to inform condition. Examples of contextual information would include the 'Site Characterisation' studies conducted for the South West European Marine Sites (Langston et al., 2003a, b), outcomes from the Environment Agency's review of consents project required under the Habitats Regulations (1994), and also any relevant information collected for the purposes of the Water Framework Directive.

Monitoring the effects of chemicals in the environment and developing biological-effects tools

1.5 Toxic chemicals, such as heavy metals, pesticides, endocrine disruptors, dioxins, polyaromatic and halogenated organics, together with 'non-toxics' such as nutrients, are found widely in UK marine and terrestrial habitats (and wildlife) through exposure to anthropogenic activities. Monitoring the spatial and temporal patterns of these contaminants, and their subsequent effects on ecological receptors, is important in assessing environmental impacts. Sediment chemistry and tissue residue analyses are used widely to determine the bioavailability and bioaccumulation of contaminants, and specific and sensitive methods are available for many classes of compounds. These analyses are, however, relatively expensive to apply and must cope with the continuous development and release of new chemicals, formulations and mixtures.

1.6 To monitor the effects of the full mixture of anthropogenically-derived chemicals found in the UK marine and terrestrial environments, it is considered important to measure the biological effect that occurs in the wildlife species that may be exposed. Consequently, the use of biomarkers (functional measures of exposure to stressors expressed at the suborganismal, physiological, or behavioural level) has been advocated as a vital addition to the risk assessment procedure.

1.7 When damage occurs to organisms, whether caused by disease, trauma or environmental stressors, there are disruptions to molecular and cellular processes. These changes in biological markers (biomarkers) can provide evidence of exposure to the toxic effect of chemical pollution

and can give an indication of an organism's health. Biomarkers can be any molecular, cellular, physiological or behavioural changes that any organism (including humans) experiences. A wide body of evidence has accumulated worldwide in recent years to describe and illustrate the development and application of biomarkers of exposure and effect in environmental monitoring (for some recent reviews, see Anderson and Lee, 2006, Galloway et al., 2004a, b, Gray, 1999, Hagger et al., 2006, Sole, 2000). There is a broad and varied programme of DEFRA and EA-funded biomarker work currently taking place in the UK, which includes ECOMAN (Ecosystem Management Bioindicators DEFRA contract ME3103), PREDICT II (Health of coastal environments: Pathological Risk Evaluation using Diagnostic and Innovative molecular and Cellular Tools DEFRA contract AE1136), Ribble Pilot studies, South West Habitats Directive studies, FULLMONTI (Development of a fully integrated monitoring techniques index DEFRA contract AE1043) and ongoing OSPAR/National Marine Monitoring Programme (NMMP) work.

1.8 In 2004, Natural England (then English Nature) commissioned a review of the potential use of biomarkers to inform the conservation agencies on chemically-induced physiological stress in marine and terrestrial wildlife, the results of which are published in English Nature Research Report 593 (EN, 2004). The report highlighted the scope and potential use of biomarkers in an integrated approach to environmental management, incorporating both biological and chemical data. One of the recommendations in this report was that a trial was required to deploy biomarkers along known pollution exposure gradients, to gather information on their sensitivity and potential for informing environmental risk or condition assessments. The Plymouth Marine Science Partnership (PMSP) team was commissioned by English Nature in 2005 to complete the work presented in this report.

Objectives

1.9 The overall objective of this work was to evaluate the feasibility and value of establishing a rapid, cost-effective methodology of biological-effects based sampling and analysis on SAC designated features in England, which will be deployed on a six-yearly cycle. It is envisaged that ultimately these tools will be used alongside ecological and other contextual (eg contaminant) data, particularly that collected by other relevant authorities, to inform Natural England's condition assessment and advice to government.

1.10 To achieve this objective, a series of field surveys has been carried out on two SACs in the South West of England. The field surveys employed a suite of biomarkers of exposure and sublethal effect, incorporating molecular, biochemical, cellular and physiological assays, which have been applied to common, coastal invertebrate animals endemic to each SAC site. A timetable for the work programme and completion of milestones is shown below.

Table 1 Milestone 1: Review of biomarker techniques, rationale for choice of biomarkers, species and study sites

Milestone	Content	Date of completion
1	Review of biomarker techniques, rationale for choice of species and study sites	October 2005
2	Field deployment, assessment and report	December 2005
3-5	Field deployment, assessment and report	March, June, October 2006
Related action	Joint DEFRA, EA, NE Biomarkers Workshop, Peterborough	October 2006
6	Final report	March 2007

Application of biomarkers: The ECOMAN approach

1.11 As discussed in the Introduction, a diverse array of procedures exists for detecting the impacts of pollutants in coastal and estuarine environments. These include ecological survey procedures for identifying changes in the abundance and diversity of species comprising communities; chemical and biomonitoring procedures for determining the concentrations and bioavailability of anthropogenic contaminants; and biochemical, physiological and behavioural biomarkers which signal exposure to, or the adverse effects of pollution. When these procedures are used in combination in well-designed survey programmes, they can provide insight into which pollutants are responsible for environmental degradation.

1.12 However, the choice of techniques to apply is limited by practical considerations: they may be expensive to perform, require highly-trained personnel and technologically-advanced equipment. An added scientific drawback concerning biomarkers is that current assays may have been validated with only a very limited number of species. At some study sites, these species may not be available, or if they are, their responses may not be indicative of the sensitivity of other functional groups within the community.

1.13 In response to these concerns, the PMSP team has been working for several years on the development of biomarker-testing systems that take these practical and resource constraints into consideration (ECOMAN: Ecosystem Management Bioindicators, DEFRA CDEP 84/5/292, ECOMAN Evidence, DEFRA ME3110, EA SC050062). As a result, a suite of biomarkers has been developed designed to be easy to use, cost effective and environmentally valid, to assess the general health of coastal systems, including estuaries.

1.14 The issue of environmental relevance has been approached by recognising that it is impractical to attempt a comprehensive assessment of each individual species within a given habitat. A more realistic approach is to establish the risk to species occupying critical trophic positions to provide insight into the integrity of the ecosystem as a whole. Therefore, we identified a panel of common, coastal organisms that are widely distributed around the UK and Northwest Europe, are easy to identify in the field and are representative of several invertebrate phyla (Table 2). Most importantly, they exhibit a variety of feeding strategies and occupy a diverse range of habitats in estuaries and coastal areas. The ECOMAN approach has been applied to address the objectives of the present Natural England contract.

1.15 The biomarkers chosen for inclusion in the programme (Tables 3 and 4) have been subjected to literature review and laboratory evaluation to determine their fitness for purpose (precision, reproducibility, concentration response, sensitivity to key contaminants), and field tested to characterise the toxic impact of pollutant releases into the environment at a number of estuarine sites around the UK (Galloway et al., 2004b, Hagger et al., 2007a,b) and internationally (Galloway et al., 2002b, Jones et al., 2004).

1.16 The rationale for this choice of biomarkers is based on the review of contextual information for each site, which is presented in Section 2.7-2.10. The main potential pressures are from PAHs, metals, pesticides and from eutrophication. The biomarkers, therefore, included biomarkers of exposure to PAHs (PAH metabolites in urine), to metals (metal-binding proteins), to pesticides (acetylcholinesterase inhibition) and to the endocrine disrupting organo-metal tributyltin. Sublethal toxic effects are designed to characterise the relationship between these pressures, and the presence of toxic damage and adverse health consequences in exposed animals. The biomarkers of effect therefore included those for genotoxicity (micronucleus), immunotoxicity (phagocytic index), oxidative stress (TOSC and FRAP oxidative capacity assays) and cytotoxicity (cell viability assay). The general health of the animals was measured using heart rate and feeding rate.

Table 2 Common coastal organisms used to develop biomarker protocols during the ECOMAN programme (Galloway et al., 2004a, b, 2006)

Phylum	Species	Habitat	Feeding strategy
Porifera	*Halichondria panacea* Breadcrumb sponge	Large encrustations on lower rocky shore	Sessile filter feeder
Annelida (Polychaeta)	*Nereis diversicolor* Ragworm	Estuarine shores Mud and muddy sand, tolerates low salinity	Omnivore, feeds on plant and animal material trapped on a mucous funnel secreted by the worm
	Arenicola marina Lugworm	Mid to lower shore Sand and muddy sand	Deposit feeder, ingests sediment while in its burrow
Mollusca	*Patella vulgata* Common limpet	Full range of rocky intertidal (sheltered to exposed)	Herbivorous grazer
	Littorina littorea Edible periwinkle	Rocky coasts, estuaries, mud flats, upper shore to sublittoral	Grazes on microorganisms, detritus and algae
	Nucella lapillus Dog whelk	Rocky shores experiencing, most degrees of wave exposure, mid-shore to shallow sublittoral	Carnivore, feeds on barnacles and mussels
	Mytilus edulis Common mussel	Below mid-tide level on rocky shores and estuaries	Sessile filter feeder
	Cerastoderma edule Common edible cockle	Middle to lower shore Sand and muddy sand	Filter feeder
Arthropoda (Crustacea)	*Neomysis integer* Mysid shrimp	Euryhaline, upper region of estuaries, high water level to sublittoral	Omnivore, including detritus
	Carcinus maenas Common shore crab	All shore types, high water to sublittoral	Omnivore, feeds on a wide range of invertebrates
Echinodermata	*Asterina gibbosa* Cushion star	Southern rocky shores, rock pools	Omnivorous scavenger
	Asteria rubens Common starfish	Many shore types, mid-shore to sublittoral	Carnivore, including bivalves, worms, echinoderms
Urochordata	*Ciona intestinalis* Sea squirt	Lower shoreline, attached to rocks and stones	Sessile filter feeder

Table 3 Biomarkers of exposure included in the Natural England suite of assays. These recommended techniques form part of the continuing ICES work to review and evaluate marine biological-effects monitoring techniques in relation to programmes in Member Countries and the OSPAR Coordinated Environmental Monitoring Programme (CEMP). Developed with reference to (ICES 2004)

Method	Organism	QA	Issues Addressed	Biological significance	Refs
AChE inhibition	Molluscs and crustaceans	O	Organophosphates and carbamates or similar molecules. Possibly algal toxins	Measures exposure to organophosphate and carbamate pesticides	Radenac et al. 1998; Narbonne et al. 1999; Cajaraville et al., 2000; Bonacci et al. 2004; O'Neill et al. 2004; Rickwood & Galloway 2004
Metallothionein Induction and metal partitioning	*Mytilus spp.*	O	Measures induction of metallothionein protein by Certain metals (e.g., Zn, Cu, Cd, Hg)	Measures exposure and disturbance of copper and zinc metabolism	Leung & Furness 1999; Cajaraville et al. 2000; Porte et al. 2001b; Geffard et al. 2002; Langston et al. 2002; Chevre et al. 2003; Galloway et al. 2004b
Intersex (also a biomarker of effect)	*Littorina* *Littorea*	B	Specific to reproductive effects of organotins	Reproductive interference in coastal (littoral) waters	Bauer et al. 1997; Oehlmann et al. 1998; Davies et al. 1999; De Wolf et al. 2001; Galloway et al. 2004b
PAH urine metabolites	Crustaceans		PAHs	Measures exposure to and metabolism of PAHs	Dissanayake & Galloway 2004; Watson et al. 2004a; Watson et al. 2004b

B; BEQUALMS; Q: QUASIMEME; O: OTHER (EU projects BEEP; COMPREHEND; UNEP; MEDPOL).

Table 4 Biomarkers of effect included in the Natural England suite of assays. These recommended techniques form part of the continuing ICES work to review and evaluate marine biological-effects monitoring techniques in relation to programmes in Member Countries and the OSPAR Coordinated Environmental Monitoring Programme (CEMP). Developed with reference to (ICES 2004)

Method	Organism	QA	Issues Addressed	Biological significance	Refs
On-line monitoring (including heart rate)	Mussels and crabs		Not contaminant-specific; will respond to a wide range of environmental contaminants.	Measures the effects of chemicals on heart rate using a simple and inexpensive remote biosensor. Gives an integrated response	Styrishave & Depledge 1996; Bloxham et al. 1999; Galloway et al., 2002b; Brown et al. 2004; Abessa et al. 2005
Scope for growth (incorporates clearance rate, oxygen consumption etc)	Bivalve molluscs, e.g., *Mytilus* spp. and oysters	Q	Responds to a wide variety of contaminants	Integrative response; a sensitive sub-lethal measure of energy available for growth	Widdows et al. 2002; Toro et al. 2003; Olsson et al. 2004; Halldorsson et al. 2005
Micronuclei	Fish, bivalve molluscs		Not contaminant-specific, although agent would be genotoxic	A measure of DNA damage which may have higher consequences due to loss of DNA material	Heddle et al. 1983; Scarpato et al. 1990; Bahari et al. 1994; Burgeot et al. 1995; Sugg et al. 1996; Bolognesi et al. 1999; Pavlica et al. 2000; Hagger et al. 2005

Table continued…

Method	Organism	QA	Issues Addressed	Biological significance	Refs
Lysosomal stability (including NRR)	Fish, invertebrates including *Mytilus spp.* Oyster	O/B	Not contaminant-specific, but responds to a wide variety of xenobiotic contaminants and metals	Measure cellular damage and is a good predictor of pathology. Provides a link between exposure and pathological endpoints. Possibly, a tool for immunosuppression studies in white blood cells.	Coles et al. 1995; Pipe et al. 1999; Cajaraville et al. 2000; Wedderburn et al. 2000; Brown et al. 2004; Ringwood et al. 2004
Immunocompetence (including phagocytosis	Fish, invertebrates		Not contaminant-specific; will respond to a wide range of environmental contaminants.	Measures factors that influence susceptibility to disease	Coles et al. 1994; Dyrynda et al. 1998; Auffret et al. 2004; Parry & Pipe 2004
Oxidative stress (including TOSC and FRAP)	Fish *Mytilus spp*.		Not contaminant-specific; will respond to a wide range of environmental contaminants.	Measures the presence of free radicals	Livingstone et al. 1992; Moore 1992; Pipe et al. 1993; Camus et al. 2004; Regoli et al. 2004

1.17 It is important to note that biomarkers are not designed to be used on their own, but rather to form part of the weight of evidence in an integrated risk assessment. The integration of available information and methods applicable to the Natural England SAC sites is shown in Figure 1.

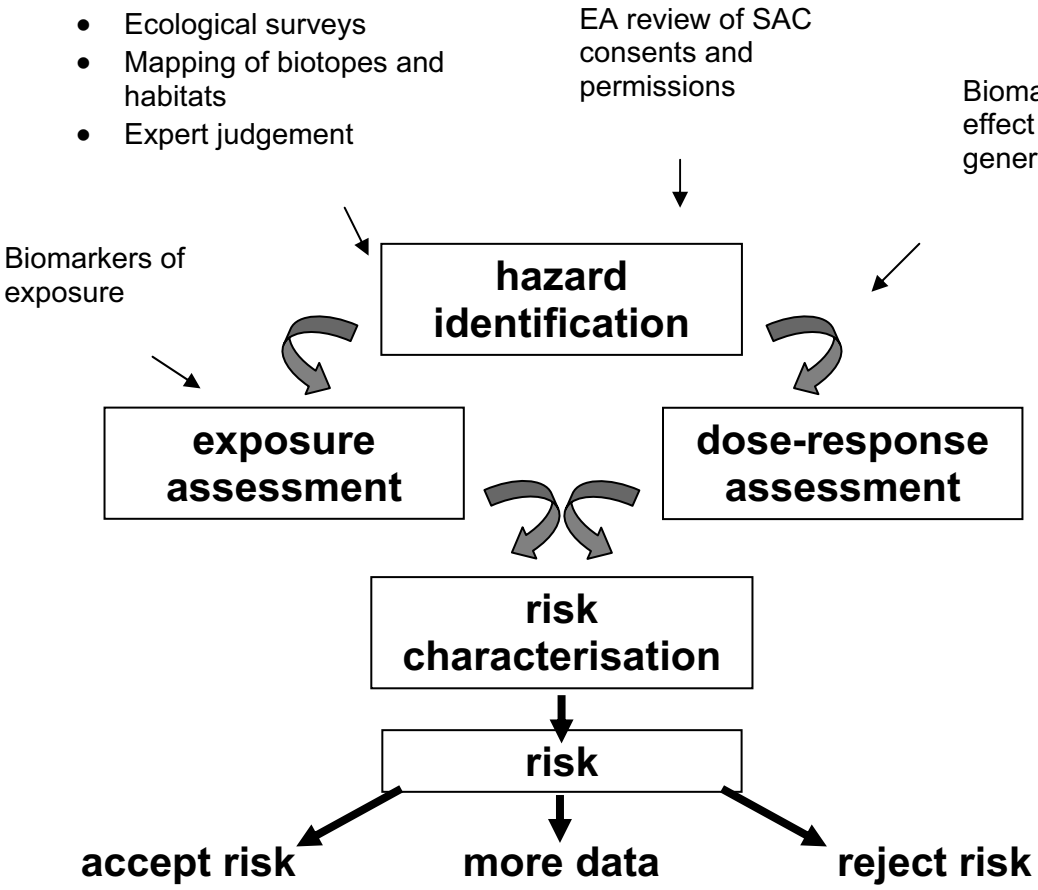


Figure 1 The National Academy of Sciences (NAS) paradigm for risk assessment, as applied to the risk assessment of Natural England SAC sites

2 Study sites

Selection of SACs

2.1 We recommended limiting our sampling to two of the southwest SACs as this allowed:

- a more thorough test of the robustness of the biomarker techniques (including the question of temporal variability);
- the application of a greater range of tests (given the funding constraints);
- a greater emphasis of the budget for science (reducing the costs of travel and subsistence); and
- a more meaningful and cost-effective trialling of the techniques.

2.2 The option of including more SACs was kept 'open' for discussion in the possible extension into the Phase 3 Section of the project (tentatively scheduled for April 2007-March 2008).

2.3 The final selection of the SACs was informed by a critical assessment of a series of in-depth characterisation studies of European Marine Sites (EMS) in the southwest region completed recently by the Marine Biological Association (Langston et al., 2003a, b). The information reviewed in these reports permitted a broad appraisal of the character of each of the nominated sites and identified major threats. However, among the key limitations identified were:

- The biological information (particularly that related to anthropogenic effects) was predominantly qualitative or anecdotal and of restricted spatial or temporal coverage.
- Chemical monitoring was also erratic and has not been designed for the purposes of Natural England's condition assessments, making determination of cause and effect relationships, and links between water quality and biological condition, difficult.

2.4 Given these caveats, it was decided to focus investigations at two of the better studied southwest European Marine Sites (EMS), the Fal & Helford SAC and Plymouth Sound & Estuaries SAC, SPA. Through this report they have sometimes been referred to for brevity as the 'Tamar' and 'Fal' sites respectively.

2.5 These SACs are subjected to a range of potential pressures, sources and gradients of contamination suitable for the examination of biomarker responses. Thus, in the Fal & Helford SAC, there are concerns over impacts from Falmouth dockyard and other boating activity (TBT, metals and hydrocarbons), wastewater treatment works at Truro and Falmouth (and smaller discharges elsewhere including into the Helford), historical inputs of metals, and periodic threats from eutrophication. Plymouth Sound & Estuaries SAC receives diverse inputs including those from dockyards, shipping and marinas, wastewater treatment works (nb those which discharge into the Tamar and Plym), urban run-off, china clay workings (Plym) as well as a historical legacy from metal mining in the Tamar Valley.

2.6 A brief perspective of water quality issues at these sites, based on site characterisation studies, and updates from EA and our own analyses, is summarised in Sections 2.11 to 2.28 of this report.

Rationale for selection of sampling stations within each SAC

2.7 The intention was to give a broad coverage of locations within the SACs and to incorporate biological-effects measurements over as large a range of interest features as possible, given the budgetary constraints.

2.8 Biomarker stations were also chosen to span locations (and gradients) known to be impacted by reasonably well-characterised water/sediment quality issues and known sources, together with sites which are considered as reflecting reference conditions. This selection drew heavily on contextual information from the 'site characterisations', encompassing historical background knowledge on water quality, bioaccumulation, biological impact and other pressures (summarised in Sections 2.11 to 2.28, along with limited additional WQ data gathered during the current project). As indicated, however, appropriate contemporary chemistry was not available for all sites and for all determinants, and the status of some has had to be pieced together from various sources or extrapolated from nearest/adjacent sampling points. This inevitable compromise is far from ideal and a much more rewarding strategy for the future would be to run chemical and biological-effects monitoring simultaneously at common sites (see Appendix I).

2.9 With these limitations in mind, the current deployment of biological-effects techniques was designed to characterise the broader relationships, if any, between anthropogenic contamination, toxic damage and adverse health effects, and to test whether such an approach adds value to Natural England's current Site Assessment protocols.

2.10 Each SAC was visited prior to the start of the fieldwork to confirm practical considerations, such as accessibility for sampling and the availability of test organisms in sustainable numbers. As a result of these visits, the sites shown in Figures 2 and 6 were selected for detailed assessment. [Note these include additional sites at Torpoint (Tamar) and Falmouth Marina which were included at a late stage in the project to test specific hypotheses; not all biomarkers have been measured in these samples on each occasion.]

Summary of existing contextual information on study sites

2.11 Site Characterisations of the Fal & Helford SAC and Plymouth Sound & Estuaries SAC and SPA have been used to summarise 'historical' (pre-2002) information on gradients in water quality and biological effects. Detailed information is contained within individual reports (Langston et al., 2003a, b) but we briefly review here the perceived main water quality impacts, gradients and sources which partly guided the selection of sites.

2.12 Ideally, to help interpret biomarker data from the current project, it would have been useful to assess biodiversity, bioassays and other biological-effects indices, alongside appropriate chemistry, undertaken at the same time and at the same locations. As financial support was not available for this approach, additional contextual information had to be gleaned on an ad hoc basis from other sources, particularly the Environment Agency. Whilst these provided useful summaries of trends, and have been added to the discussions below, it must be recognised that they may be limited in their relevance to the current results, both spatially and temporally.

The Fal & Helford SAC

Table 5 The Fal and Helford SAC

General site character	Percent composition
Marine areas, sea inlets	60
Tidal rivers, estuaries, mud flats, sand flats, lagoons	35
Coastal sand dunes, sand beaches, machair	1
Shingle, sea cliff, islets	1

Why is the Fal & Helford area a designated SAC site?

2.13 The Fal & Helford SAC is a sheltered ria (drowned valley) system with a low tidal range and a wide range of substrata, resulting in rich biological diversity, especially of fully marine invertebrate communities. There are extensive areas of maerl gravel and eelgrass beds, which shelter nationally important sediment communities. Most of the shores of the Fal & Helford and their upper reaches are fringed by sandflats and mudflats. The Annex 1 habitats that are the primary reason for selection of the site include:

- 1110 Sandbanks which are slightly covered by seawater all the time;
- 1140 Mudflats and sandflats not covered by seawater at low tide;
- 1160 Large shallow inlets and bays; and
- 1330 Atlantic salt meadows.

2.14 The Fal & Helford SAC (Figure 2) was chosen as a study site because of potential pressures from a range of sources (notably nutrients, metals and TBT). In addition to diffuse sources, there are a large number of consented discharges into the Fal & Helford system of varying sizes. The locations of some of the more important (by volume) discharge consents at the time of the Site Characterisation study are shown in Figure 3 (from Langston et al., 2003a)

2.15 The location of sampling stations is anticipated to test responses arising from some of the more important of these discharges, on top of the more diffuse gradients exhibited by various categories of contaminants.

Metals

2.16 Parts of the SAC are impacted by metals from past mining activities which continue to influence the area via mine drainage discharges and remobilisation of metals from sediments. This is most evident in the vicinity of Restronguet Creek; some metals have also been transported to other parts of the system, such as Mylor and Pill, and to the upper Fal.

2.17 Concentrations in sediments of Restronguet, Penryn Creek and the upper Fal are above PEL (probable effects levels) for several metals. The latter impact mainly on sites 4-7 in the current project (Table 6).

2.18 At the mouth of Restronguet Creek, Cu concentrations in overlying water may exceed the EQS of 5 µg l^{-1} but beyond that concentrations rarely exceed the standard (EA data) and are unlikely to result in acute toxicity, though chronic effects are possible (hence the selection of site 5, Weir Point). A further source of Cu (and Zn) in the Fal is the outfall at Falmouth Dockyard (average of 11.5 µg Cu l^{-1} during the last 2 years; EA data), and this is reflected by elevated Cu concentrations in waters and sediments of the area (nearest site = 4, Flushing). Occasional high values for dissolved Zn have been recorded in waters of the upper Fal and probably originate in sewage discharges (nearest site = 7, Malpas).

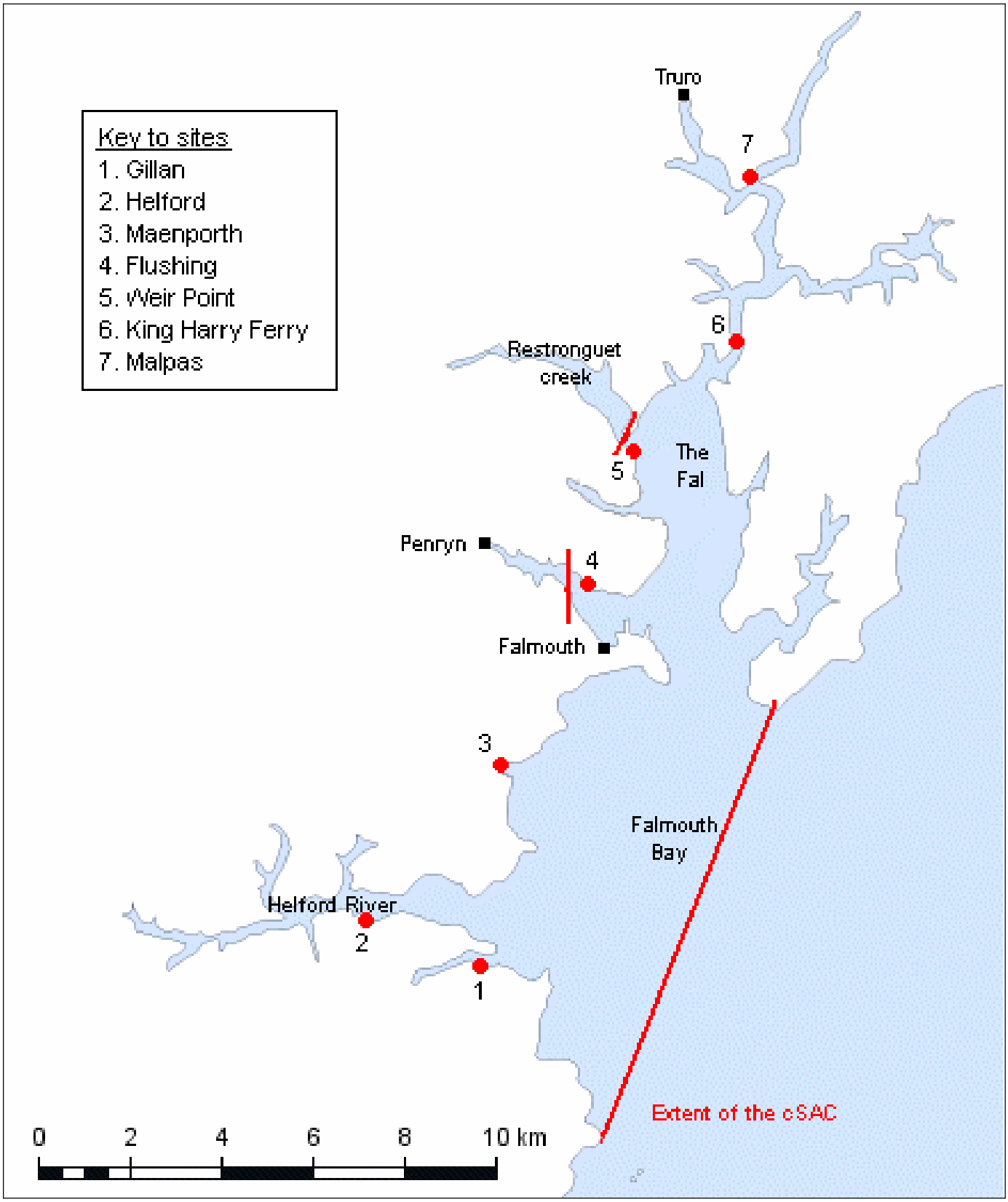


Figure 2 Fal & Helford SAC showing sampling stations

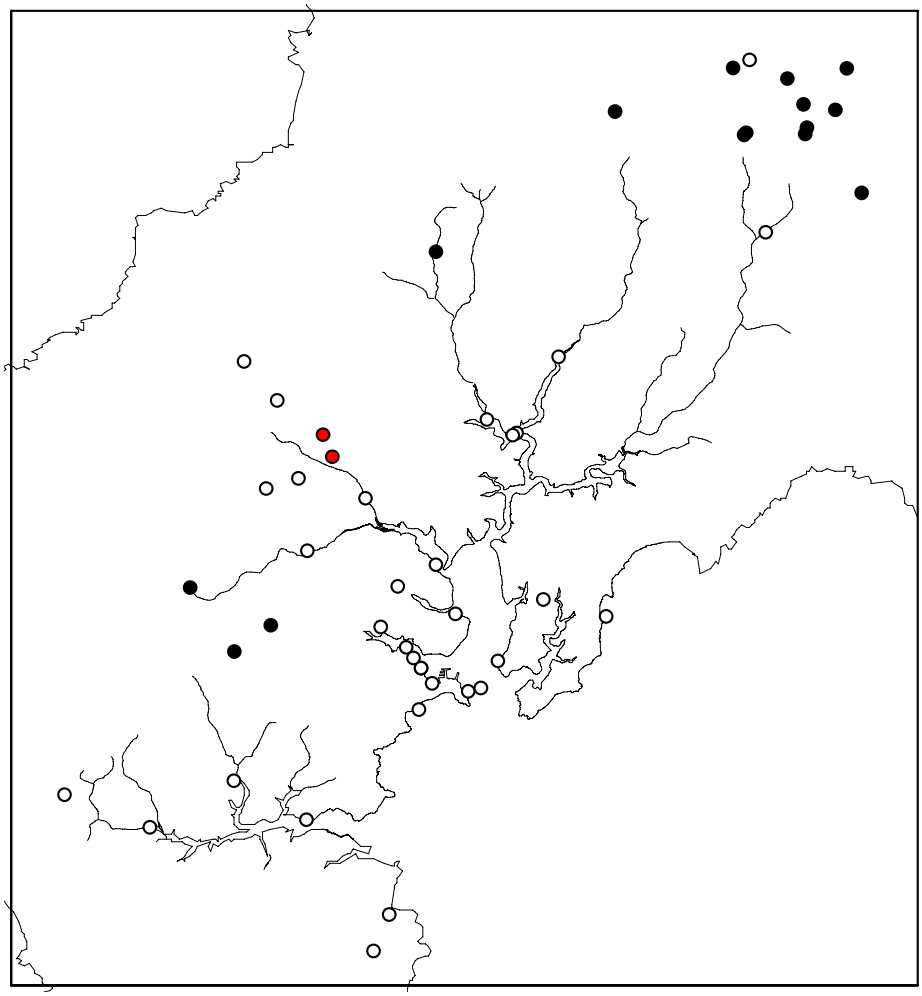


Figure 3 Locations of some of the larger discharge consents to the Fal Estuary system. Consents shown for the discharge of sewage (generally set for SWW) are those >7.3 m³/d MAX (open symbols). Trade consents, and miscellaneous sources of effluents shown (closed symbols) are those> 120 m³/d DWF. NB No distinction has been made between continuous and intermittent discharges. Details of specific discharges should be clarified with the Environment Agency

2.19 Mussel body burdens have been measured in the current project for a suite of metals (see Appendix II). Highest concentrations were generally found near Restronguet (site 5) and Falmouth (sites 4, 4a) confirming environmental 'hotspots' in these areas (see Table 6).

2.20 Evidence of metal-induced impacts on biota, particularly in Restronguet Creek, is reasonably conclusive and has been reviewed previously. The number of studies and amount of information addressing impacts to the fauna and flora of the Helford is rather small in comparison to the Fal.

TBT

2.21 Parts of the Fal, and in particular the Falmouth area (sites 4 and 4a), are affected by organotin contamination. The principal source is Falmouth Dockyard although sediment hotspots have been found at Mylor (between sites 4 and 5) and, recently, at Porth Navas in the Helford (opposite site 2). TBT concentrations are diluted rapidly with distance from the major sources at Falmouth, though levels above the EQS (2ng l^{-1}) may be sustained nearby, as in Penryn Creek (mean 12.8 ng l^{-1}, 2004-2005) and are reflected in body burdens in species such as mussels (see Appendix II). During the current study, concentrations of TBT in mussels from Flushing and Falmouth Marina were above OSPAR upper ecotoxicological assessment criteria (EAC).

2.22 There is clear evidence of impact on sensitive gastropods such as dogwhelk Nucella lapillus populations, which have been eliminated from most of the Fal by TBT. Long-term observations on neogastropods just outside the mouth of the Fal confirm that imposex levels remain high and have not changed since studies began in the 1980s.

Hydrocarbons (PAHs)

2.23 Historical records for hydrocarbons in the Fal and the mouth of the Helford suggest general contamination in the area which originates, possibly, from the dockyard and shipping, but may also be related to run-off and/or aerial deposition. There are scarcely any data for PAHs in the SAC apart from a small number of sediment samples analysed by EA in 2006 which show elevated levels in the vicinity of Penryn Creek (near sites 4 and 4a), and, occasionally, near Malpas (site 7). For some individual PAHs these can exceed the PEL (Table 6).The sources of PAH here are not known for certain.

Nutrient-related pressures

2.24 The upper Fal Estuary in particular may be subject to periodic nutrient enrichment and eutrophication. Although the majority of nutrient inputs in the SAC may be due to diffuse sources such as agricultural run-off, localised enrichment from sewage treatment works (eg Newham STW) is also significant, particularly in the more enclosed reaches of the upper Fal Estuary where chronic contamination and nutrient-associated water quality problems have resulted in toxic algal blooms, DO sags and turbidity. The most recent incidence (in 2002) also affected the Helford Estuary (nb Polwheveral Creek and Porth Navas, just upstream of sites 1 and 2), resulting in invertebrate mortalities.

2.25 The nutrient status in parts of the SAC, notably the upper Fal and parts of the Helford, therefore comprises a potential, if transient, threat to conservation features. In particular, the conditions in the upper Fal have prompted its designation as a Sensitive Area (Eutrophic). Subsequent management actions appear to have reduced nutrient loadings here: dissolved nitrate (and to a lesser extent phosphate) concentrations in the Truro River have decreased substantially over the last five years, presumably in response to improvements to the STW at Newham. Nevertheless, recent EA water quality monitoring in the Fal suggests that related pressures could still be a factor for biota in the Truro River and upper Estuary (eg Malpas, site 7). EA summary data (2004-2006) for nutrient concentrations in the Fal, together with chlorophyll a, DO and salinity are plotted in Figure 4. Nutrient data (and chlorophyll a) still exhibit a substantial gradient downstream as indicated in Figure 4. There are also high (and variable) phosphate levels in Penryn Creek suggesting local sources here.

2.26 There are no recent, equivalent monitoring data for the Helford, though presumably parts of the estuary are still subjected to seasonal nutrient enrichment and associated water quality problems.

2.27 Mean DO and salinity are relatively constant across the range of biomarker stations. However, the variability in DO, expressed as differences between minima and maxima, does appear to differ systematically along the estuary and was greatest at our site 7, Malpas (Figure 5). Although mean DO values appear to meet standard criteria, the tendency to oscillate between high and low extremes could exert oxidative stress on sensitive species in this region.

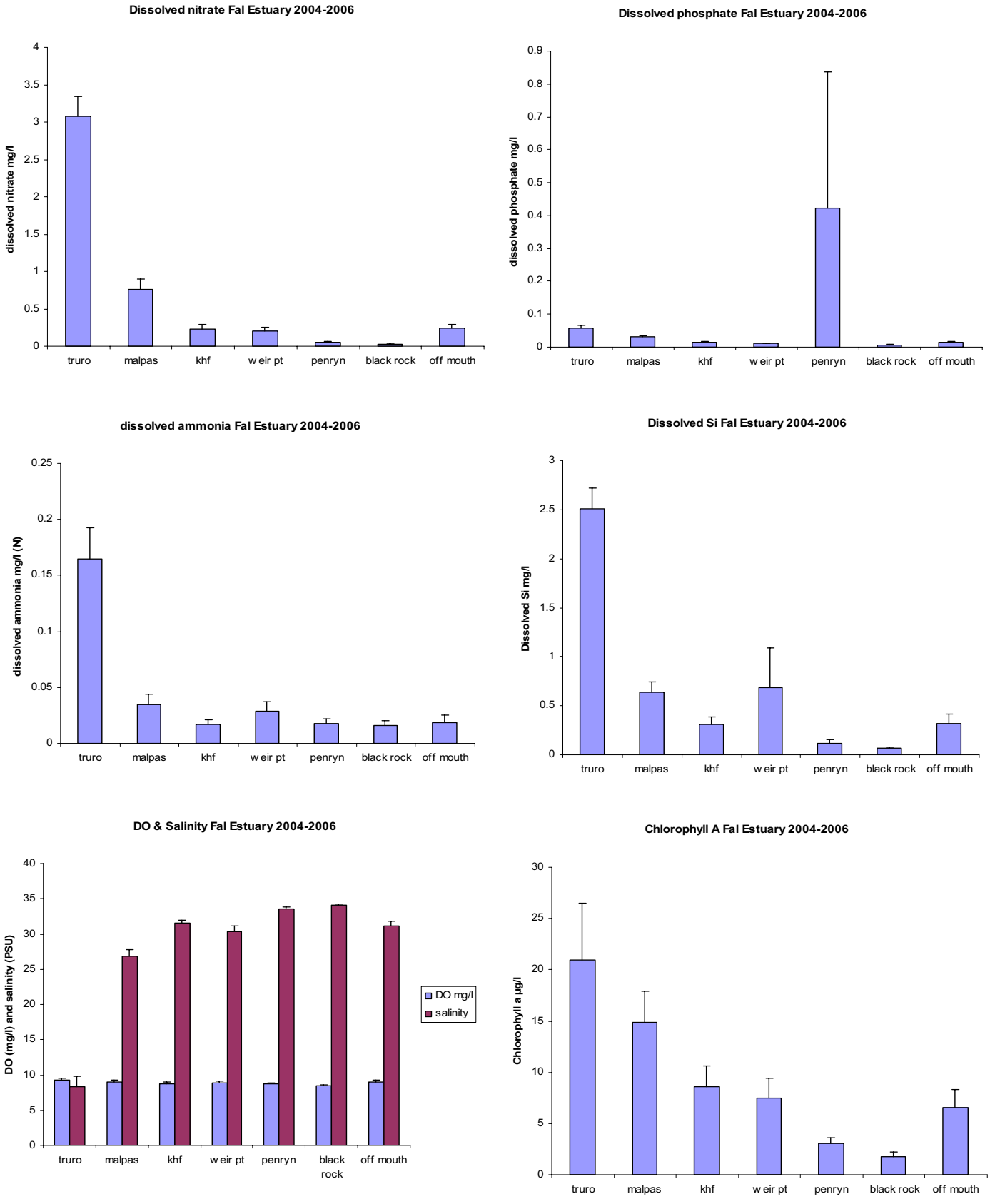


Figure 4 Dissolved nutrients, oxygen, salinity and chlorophyll a Fal Estuary (means + SE, Jan 2004 - Feb 2006. EA data)

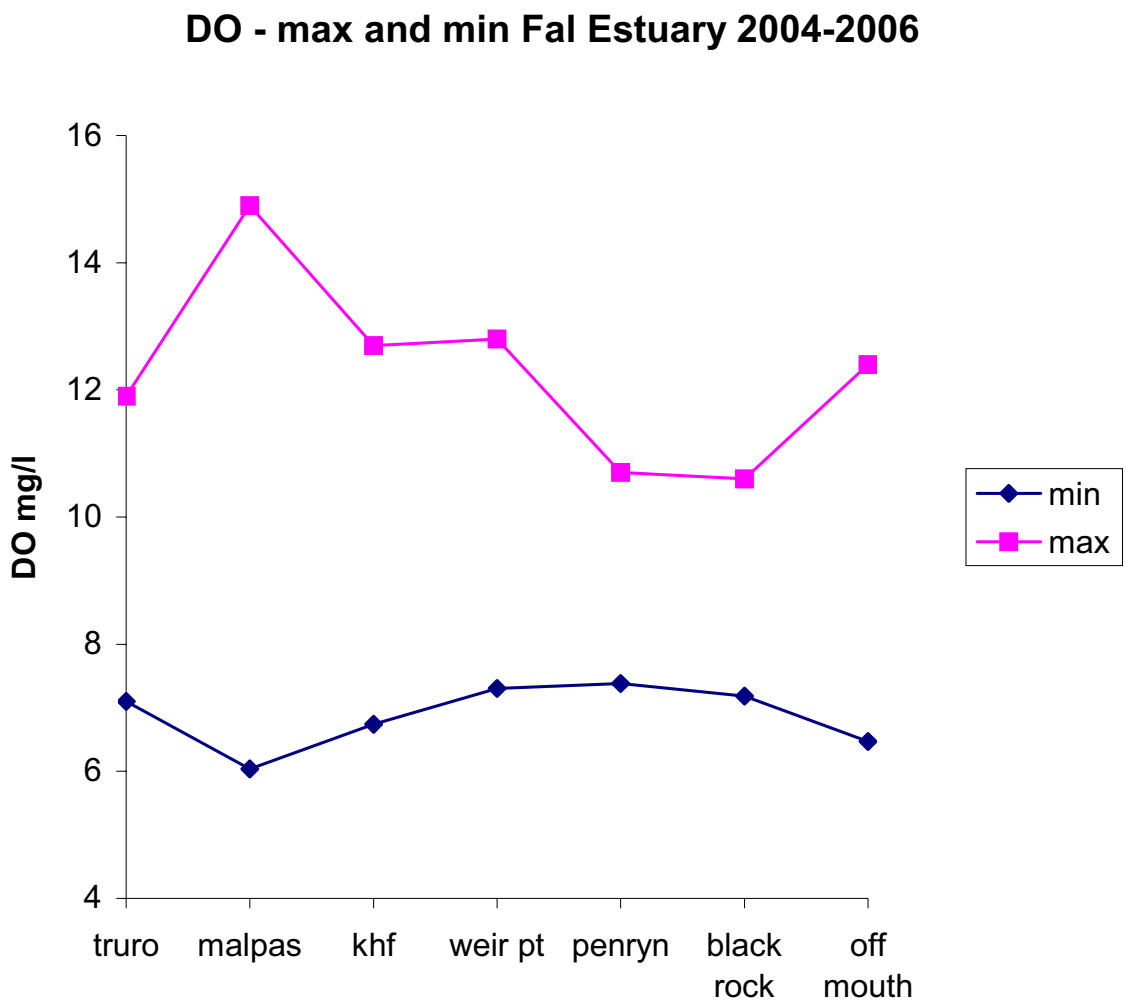


Figure 5 Dissolved oxygen maxima and minima at sites in the Fal Estuary, 2004-2006 (EA data)

2.28 Thus, the status of a number of biological features is considered to be under threat from reduced water quality in parts of the Fal & Helford, albeit based on anecdotal or qualitative assessment. These are discussed in detail in Natural England's Condition Tables and in the Site Characterisation reports. Quantitative evidence from the current project is intended to supplement this somewhat subjective perception. The use of a suite of biological effects protocols is being trialled here to help assess which are the most important threats and the most impacted areas.

2.29 The reference sampling station chosen for the Fal & Helford surveys was Maenporth (site 3) because of its geographical separation from major discharges or known chemical contaminants.

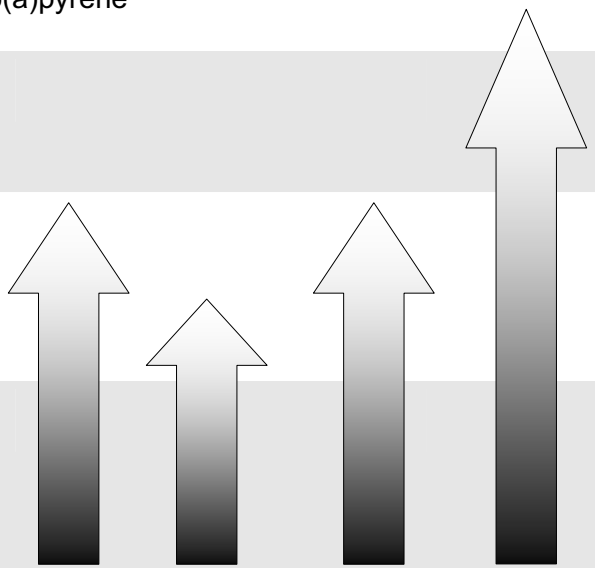
2.30 A summary of sampling stations, interest features and perceived chemical pressures in the Fal & Helford SAC is highlighted in Table 6.

Table 6 Summary of sampling stations, interest features and perceived chemical pressures for the Fal & Helford SAC. The Fal & Helford: known contaminant hotspots and gradients

Sampling station	SAC habitats present/nearby	Body Burdens		sediment			nutrients & eutrophication				
		TBT	metals	TBT	metals	PAHs	nitrate	ammonia	phosphate	chlorophyll	DO
(1) Gillan	Sandflats slightly covered by SW all the time – sublittoral muddy sand. Sandbanks slightly covered by SW all the time – sublittoral muddy sand – mixed sediment communities Mudflats and sandflats not covered by SW at low tide – muddy sand/mud communities nearby. Large shallow inlets and bays – intertidal rocky shore community; sublittoral rock with kelp beds and sublittoral sand & gravel communities nearby.		Cr,Hg				Symptons of eutrophication are observed periodically upstream in the Helford but chemical monitoring has not been undertaken in recent years				
(2) Helford	Mudflats and sandflats not covered by seawater at low tide – mixed substrata shores (muddy sand/gravel). Mudflats and sandflats slightly covered by seawater all the time – sublittoral muddy sand. Maerl bed and eelgrass bed communities nearby. Large shallow inlets and bays – intertidal rocky shore community.		Cr								
(3) Maenporth	Large shallow inlets and bays – intertidal rocky shore community. Sandbanks which are slightly covered by seawater all the time – mixed sublittoral sediments with dead maerl and maerl gravels.										

Table continued...

Sampling station	SAC habitats present/nearby	Body Burdens		sediment		nutrients & eutrophication					
		TBT	metals	TBT	metals	PAHs	nitrate	ammonia	phosphate	chlorophyll	DO
(4) Flushing	Large shallow inlets and bays – sheltered littoral rock with fucoids (seaweeds) – intertidal rocky shore communities – tide-swept sublittoral rock with Laminaria saccharina (kelp). Sandbanks which are slightly covered by seawater all the time – sublittoral muddy gravel	●	Hg, Ni, Pb, Zn	●	As, Cu, Hg, Pb, Zn	Acen apht-hylen-e	● Penryn				
(4a) Falmouth Marina	Outside SAC artificial marina structures. Mixed sediment communities	●	Cu, Pb	●	As, Cu, Hg, Ni, Pb, Zn	Acenapht-hylene Dibenzo(a,h) anthracene Benzo(a)pyrene					
(5) Weir Point	Sandbanks which are slightly covered by seawater all the time – sublittoral muddy gravel – gravel and sand communities		Co, Cu, Hg, Zn		As, Cu, Zn						
(6) King Harry Ferry	Mudflats and sandflats not covered by seawater at low tide – mixed substrata shores – sand and gravel communities. Littoral soft mud. Large shallow inlets and bays – shallow sublittoral rock with kelps and sponges				As, Cu, Zn						
(7) Malpas	Mudflats and sandflats not covered by seawater at low tide – Littoral soft mud – mud communities. Large shallow inlets and bays – sublittoral estuarine mud.				As, Cu, Pb, Zn	Acen apht-hylen-e					Largest fluctuati-on slight DO sag
Data sources; Environment Agency, MBA		>upper EAC	upper quartile	>upper EAC	>PEL	>PEL (ospar)					



(where information is available). (EAC = ecotoxicological assessment criteria as set by OSPAR, PEL = probable effects levels.)

Plymouth Sound & Estuaries SAC

Table 7 Plymouth Sound and Estuaries SAC

General site character	Percent composition
Marine areas, sea inlets	50
Tidal rivers, estuaries, mud flats, sand flats, lagoons	40
Salt marshes, salt pastures, salt steppes	5
Coastal sand dunes, sand beaches, machair	2
Shingle, sea cliffs, islets	3

Why is the Plymouth Sound & Estuaries area an SAC?

2.31 The Plymouth Sound & Estuaries SAC is another ria (drowned estuary) that includes the rias of the rivers Tavy, Tamar, Lynher and Yealm. The upper parts of the Tamar and Lynher include a very well-developed estuarine salinity gradient and the area has a high diversity of habitats and communities characteristic of different salinities, in contrast to the Fal & Helford. Of particular note are the extensive areas of sublittoral sandbanks which include a range of sandy sediments within the inlet and on the open coast. The Yealm has good examples of habitats and communities characteristic of sheltered marine inlets with little freshwater input, including sponge and worm-dominated communities on lower shore mixed sediments. In addition, a wide variety of intertidal and subtidal reef biotopes exists, including limestone reefs along the north shore from West Hoe to Batten Bay.

2.32 Annex 1 habitats that are a primary reason for selection of this site are:

- 1110 Sandbanks which are slightly covered by sea water all the time;
- 1130 Estuaries;
- 1160 Large shallow inlets and bays;
- 1170 Reefs; and
- 1330 Atlantic salt meadows.

2.33 Given the limitations on resources, it was not possible to measure biological responses across all the features of the Plymouth Sound & Estuaries SAC and we have focused on a selection of sites along a gradient in the Tamar Estuary (Figure 6). These span a cross section of the major threats to the system including dockyards, oil storage facilities and shipping (nb sites 2 and 3), waste water treatment works (eg Ernesettle – opposite site 5 - and Camels Head opposite site 3), urban and road run-off (eg Tamar Bridge, near site 4) and historical mine workings in the Tamar Valley (upstream of site 5). The reference site at Freathy, in Whitsand Bay (1), lies outside the SAC and was chosen because of the substantial background information on biomarker responses at this site. Although anticipated to be relatively free of contamination from point sources compared to sites inside the estuary there is some uncertainty as to the influence of offshore dumping (off Rame Head).

2.34 Thus, contaminant inputs in the region of the SAC reflect both diffuse and point sources of which there are a large number discharging into the system of varying sizes. Locations of some of the more important (by volume) discharge consents impacting on the Plymouth Sound and Estuaries system at the time of the Site Characterisations are shown in Figure 7 (from Langston et al., 2003b). Categories of contaminants which may have an effect on the site include the following.

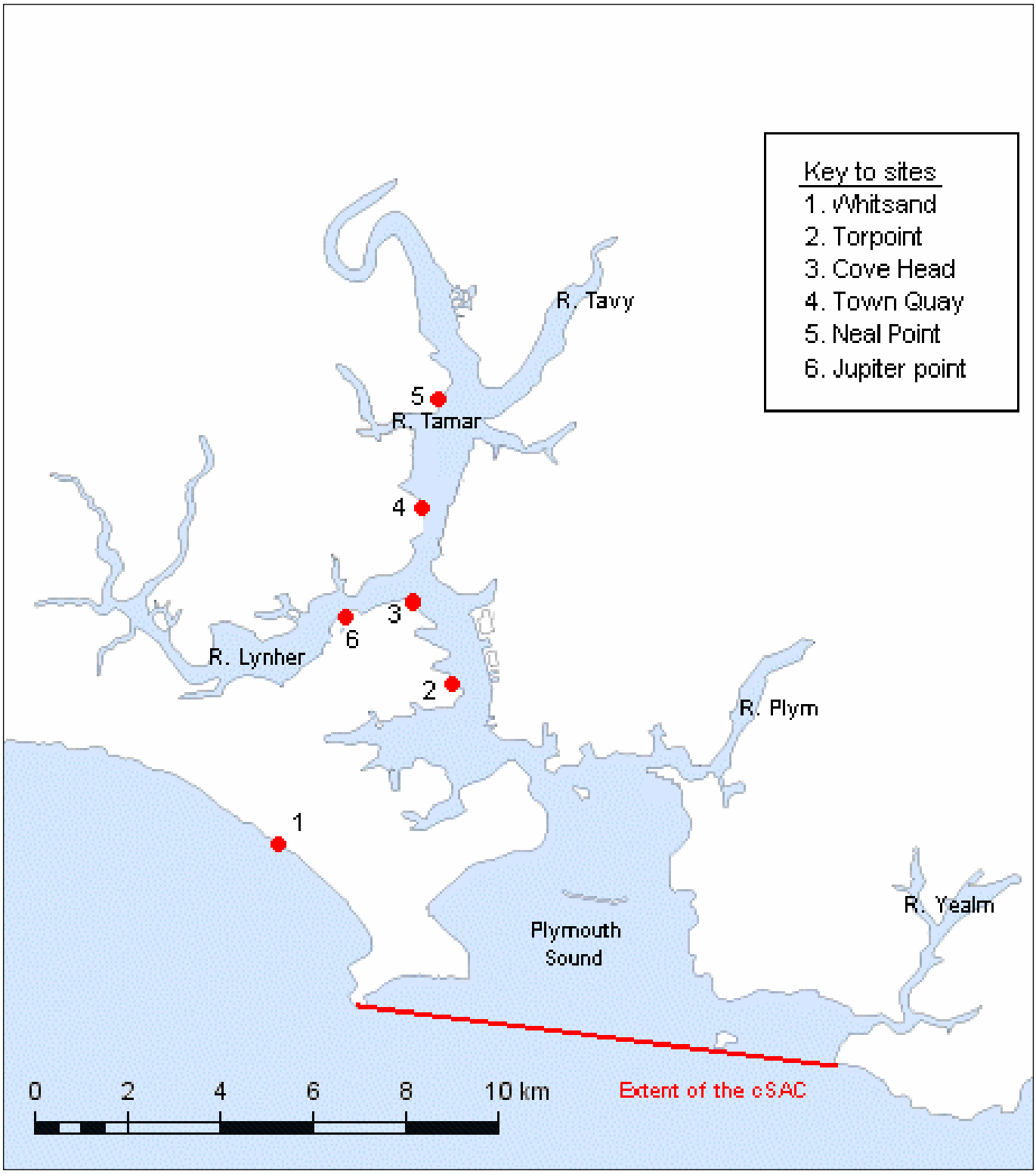


Figure 6 Sampling stations in Plymouth Sound & Estuaries SAC

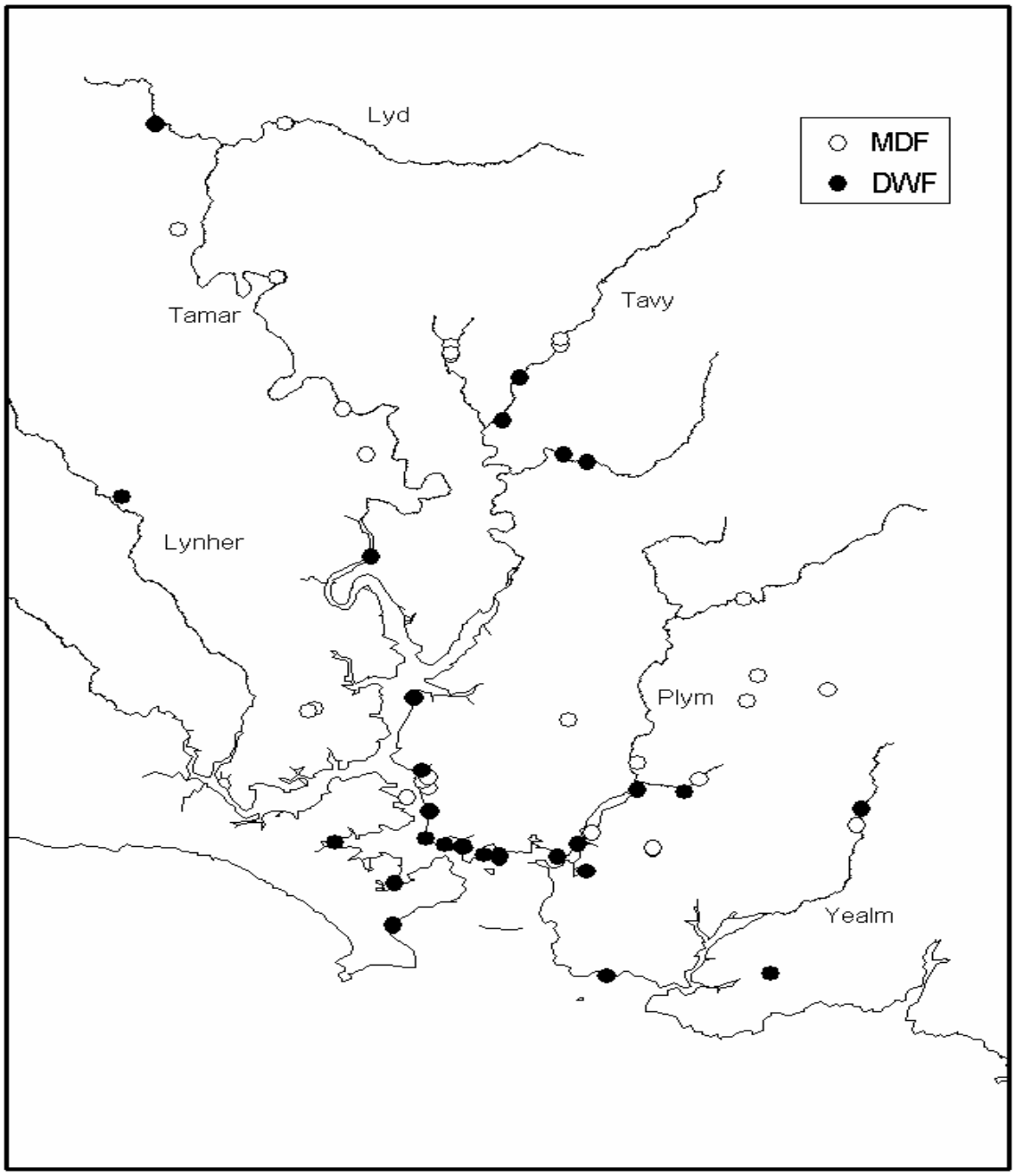


Figure 7 Locations of some of the larger discharge consents to the Plymouth Sound and estuaries system. Consents shown for the discharge of sewage (generally set for SWW) are those >356 m³/d DWF (closed symbols). Trade consents, and miscellaneous sources of effluents shown (open symbols) are those > 500 m³/d MAX (Data supplied by Environment Agency 2002). NB No distinction has been made between continuous and intermittent discharges. Details of specific discharges should be clarified with the Environment Agency

Metals

2.35 The catchment of the upper Tamar (and Tavy) Estuary is influenced by old mines, run-off from spoil heaps and remobilisation of metals from sediments, whilst the lower estuary, near Plymouth, is subjected to substantial urban development and associated metal inputs. Typically, gradients in metals decrease in a downstream direction in the Tamar (Langston et al 2003b; recent EA data eg Figure 8). At the mouth of the Tamar, estuary water is considerably diluted with seawater and, as a result, concentrations in the Sound (and Whitsand Bay) are seldom significantly above background.

2.36 Arsenic, copper and lead concentrations in sediments are above guideline criteria (probable effects level, PEL) for much of the Tamar and Tavy, including the biomarker sites 2-6, and to a

lesser extent, the Plym. Hg and Zn concentrations also indicate some effects are possible (>TEL, but <PEL mainly). Highest Cd levels (>PEL) occur in the upper Plym.

2.37 Generally, bioaccumulation patterns resemble those in sediments with elevated body burdens in the mid-Tamar estuarine complex for most metals, whilst Cd bioavailability is highest in the Plym. Recent body burden data for mussels collected in this project (Appendix II) confirm that bioavailability is significantly higher within the Tamar Estuary than in Whitsand Bay: one exception may be Zn which appears to be bioaccumulated locally at Freathy. The reason for this (and whether it represents a permanent feature), merits further investigation.

TBT

2.38 TBT inputs from ships and leisure vessels situated towards the mouth of the Tamar Estuary were a dominant feature and cause for concern in the late 1980s. Since then, TBT levels in water, particularly in the Hamoaze, have decreased substantially. TBT concentrations in Plymouth Sound water also declined markedly following legislation in 1987, though between 1995 and 1999 values above the EQS (2 ng l^{-1} TBT ≡ 0.4 ng l^{-1} as Sn) were not uncommon. Imposex in littoral dogwhelks was still prevalent and visibly-unaffected females were still a rarity near the Tamar. In contrast, outside the Sound, near Wembury, populations had markedly recovered during this period. More recent TBT data (2004-2006) from EA monitoring suggest compliance with EQS at all monitored sites, though this value appears to represent the detection limit of the methodology.

2.39 Sediment residues in the Plymouth estuaries now probably contribute most to any long-term chronic threat from TBT. We are unaware of any recent comprehensive sediment data but it is unlikely that concentrations have changed substantially in recent years. In the early 1990s, much of the Tamar, including sites 2-6, would be classified as a problem area according to Ospar Ecotoxicological Assessment Criteria (EAC). Arguably, however, these sediment guidelines may be overcautious.

2.40 Body burden data for mussels (Appendix II) indicate a peak in concentrations at Town Quay, Saltash (site 4 - the only site where the EAC for mussels is exceeded).

Hydrocarbons

2.41 PAHs are reported to be relatively high in parts of the Tamar, notably in sediments of the Hamoaze (near site 2) where they may occasionally exceed 'probable effects levels'. PAHs and other hydrocarbon inputs are thought to be principally from urban run-off (nb roads such as the Tamar Bridge), combustion and dockyard activities. Much lower levels occur offshore and are generally below limits of detection in Whitsand Bay.

2.42 Sediment and mussel samples from Neal Point (site 5), Town Quay (site 4), Wilcove (site 3) Cremyll (downstream of site 2) and Whitsand (site 1) analysed in 2006 as part of the PREDICT study (CEFAS and PML) generally confirm the trends observed previously. PAHs levels in sediment were consistently elevated in sediments from the Hamoaze, particularly at Town Quay (near the Tamar Bridges). In this region, concentrations of a number of PAHs exceeded the PEL (probable effects level), though contributions were dominated by 4-6 ring hydrocarbons (predominantly pyrolitic origins). Towards the mouth of the estuary (Cremyll), sediment contained lower overall loadings but with a higher proportion of 3-ring alkylated PAHs (petrogenic), which may represent more bioavailable components: highest total PAH concentrations in mussels were detected here. Despite these gradients, body burdens in all mussel samples were below OSPAR EAC values.

2.43 Unexpectedly, EA sediment data for 2004-2006 suggested that PAH concentrations were higher at Jennycliff, in Plymouth Sound, than at two estuary sites, Warren Pt (≡ Neal Point, biomarker site 5) and the Hamoaze (≡ to Cove Head, biomarker site 3), and imply a localised source, perhaps from the nearby Plym Estuary (Figure 8). PAH concentrations were above TEL but below PEL in all these samples.

Nutrient-related pressures

2.44 There is a lack of up-to-date information regarding the nutrient status of tidal waters: overall, the available published literature does not suggest a major nutrient enrichment problem in the SAC.

However, parts of the site, notably the upper estuaries of the Tamar, Lynher, Plym and Yealm, are subject to some nutrient enrichment and elevated chlorophyll-a. The majority of nutrient inputs in the system appear to be due to diffuse sources such as agricultural run-off, though sewage discharges constitute additional loading and result in localised chronic contamination and possibly nutrient-associated water quality problems in the lower estuary on occasions. In the past, evidence has been presented indicating sewage contamination (coprostanol) in sediment samples taken from adjacent to the Tamar road and rail bridges (Readman et al., 1986), and low DO values in summer have been recorded between here and the mouth of the Tamar (ie roughly between sites 2-4) and in the Lynher (site 6). In addition, depletion of dissolved oxygen has occurred periodically in the upper Tamar and may have been responsible for salmonid deaths in the past (Harris, 1992; Darbyshire, 1996).

Other contaminants

2.45 Certain organic contaminants including pesticides continue to be detected in sediments, indicating both agricultural and STW sources. Recent EA data indicate that PCB levels in sediments increase upstream in the estuary corresponding to trends in organic carbon (Figure 8), but are consistently below PEL. Data from the PREDICT project confirmed that organochlorines (HCB, HCH isomers, dieldrin, DDT, DDE, TDE and PCB) were scarcely detectable in mussels from any of the sites. Screening for various pharmaceutical compounds and some potential endocrine disruptors suggested little likelihood of acute toxicity.

2.46 The radiological significance of current levels of radionuclides discharged into the SAC is considered to be low.

2.47 In summary, PAHs, certain metals and, to a small extent, TBT are the chemicals which are likely contributors to any adverse effects in the Tamar between sites 2 and 5. Periodic reductions in dissolved oxygen content have also been observed in this part of the system in the past and could contribute to stress in benthic organisms. There is a small amount of evidence which tends to support the notion of biological effects in this region of the SAC. Previous biomarker data from the Sound include tentative observations of metallothionein (MT) induction in mussels transplanted near Tamar sources, and close to the (then) offshore sewage sludge disposal grounds (Johnson and Lack, 1985). Reduced cholinesterase activity, a specific biomarker for exposure to neurotoxins (particularly organophosphate and carbamate pesticides), has been observed in flounder from Warren Point in the Tamar - close to site 5 in the current study (Kirby et al., 2000). Bioassays with oyster larvae (up to 1995), conducted as part of the National Marine Monitoring Programme (NMMP), suggested there were areas of poor water quality in the SAC at that time, though no specific compound (or source) has been identified as being responsible.

2.48 Thus, as in the Fal & Helford, the status of a number of biological features could be threatened by reduced water and sediment quality in parts of the Plymouth Sound & Estuaries SAC. A summary of sampling stations, interest features and perceived chemical pressures is highlighted in Table 8. Again it is hoped that the quantitative evidence from the current project will help to assess the extent and severity of these pressures and hence help to inform Natural England's condition assessment.

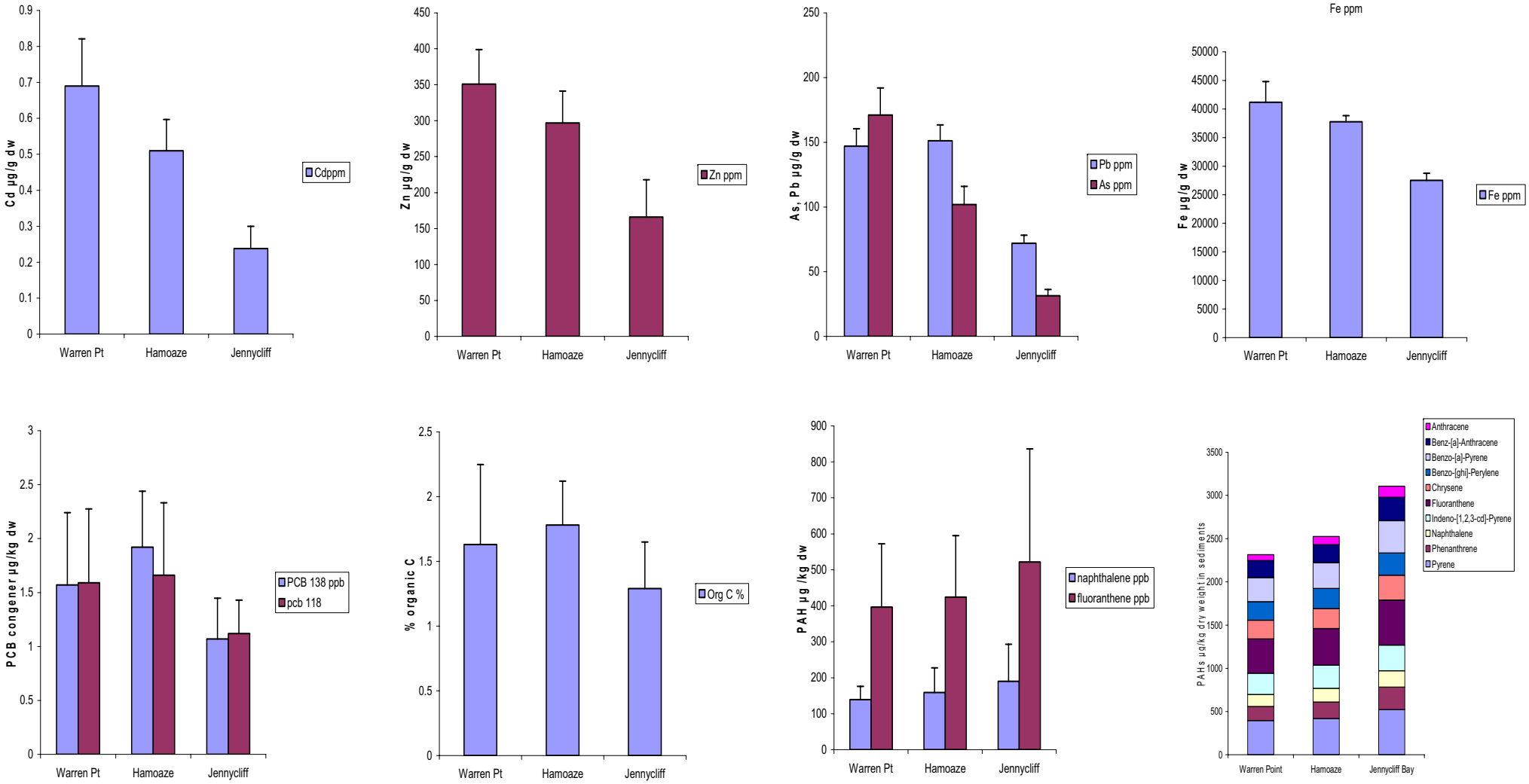
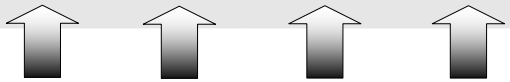


Figure 8 Summary of recent sediment contaminant data in Plymouth Sound (Jennycliff) and the Tamar Estuary at Warren Pt (≡ Neal Point, biomarker site 5) and the Hamoaze (≡ to Cove Head, biomarker site 3), (EA data 2004-2006; n=10)

Table 8 Summary of sampling stations, interest features and perceived chemical pressure for the Plymouth Sound & Estuaries SAC. Plymouth Sound and Estuaries: known contaminant hotspots and gradients

Sampling station	SAC habitats present/nearby	Body Burdens		sediment			nutrients & eutrophication				
		TBT	metals	TBT	metals	PAHs	nitrate	ammonia	phosphate	chlorophyll	DO
(1) Whitsand	Outside SAC rocky shore mixed with sand/gravel. Subtidal sandbank communities		(Zn)								
(2) Torpoint	Large shallow inlets and bays – intertidal rock and boulder shore communities. Estuaries – intertidal mud communities, subtidal mixed muddy sediment communities	Not analysed		●	As, Cu, Hg, Pb, Zn	Often>PEL					Periodic depletion of DO (summer)
(3) Cove Head	Large shallow inlets and bays – intertidal rock and boulder shore communities. Estuaries – intertidal mud communities, subtidal mixed muddy sediment communities		Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb	●	As, Cu, Pb	Often>PEL					Periodic depletion of DO (summer)

Table continued…

Sampling station	SAC habitats present/nearby	Body Burdens		sediment			nutrients & eutrophication				
		TBT	metals	TBT	metals	PAHs	nitrate	ammonia	phosphate	chlorophyll	DO
(4) Town Quay	Estuaries – intertidal mud communities; subtidal mixed muddy sediment communities; estuarine bedrock, boulder and cobble communities	●	Cd, Mn, Ni	●	As, Cu, Hg, Pb, Zn	Most PAHs above PEL		Previously high from STW, now reduced			Periodic depletion of DO (summer)
(5) Neal Point	Estuaries – intertidal mud communities; subtidal communities; saltmarsh communities		Cd, Co, Fe	●	As, Cu, Hg, Pb, Zn	Most often above TEL but below PEL					
(6) Jupiter Point	Estuaries – intertidal mud and mixed muddy sediment communities, subtidal mud communities	Not analysed		●	As, Cu, Pb	Not analysed					Periodic depletion of DO (summer)
											
							Nutrient enrichment, elevated chlorophyll a and periodic depletion of DO predominantly focused on upper estuary but may occasionally apply locally to biomarker sites in lower estuary under certain states of tide and river flow				
Data sources; Environment Agency MBA		>upper EAC	upper quartile	>upper EAC data from 1991	>PEL	>PEL					Data mainly from 1990s

(where information is available). (EAC = ecotoxicological assessment criteria as set by OSPAR, PEL = probable effects levels.)

3 Methods

Species selection

3.1 The choice of study species was determined by their presence at sufficient sites within each SAC to enable biomarker endpoints to be collected from the same species along the gradients of chemical pollution identified from the site contextual summaries (Section 2).

3.2 Preliminary site visits indicated that the following species from Table 2 were present in the SACs and could be collected from the shore by hand, or from baited cages deployed at low water: the shore crab *Carcinus maenas*, the blue mussel *Mytilus edulis*, the lugworm *Arenicola marina*, the common cockle *Cerastoderma edule*, the edible periwinkle *Littorina littorea*, the dogwhelk *Nucella lapillus*, and the peppery furrow shell *Scrobicularia plana*.

3.3 The final selection of *Carcinus maenas* and *Mytilus edulis* was based upon availability throughout the system (Tables 9 and 10). These sentinels encompass different feeding types and represent a wide range of the SAC special interest features and habitats designated by Natural England including large shallow bays and inlets, reefs, mudflats, sandflats and estuaries.

Table 9 Species selection for the Fal & Helford SAC (based on preliminary visits)

Species	Species Present	At all sites	Methodology available	Feeding type, habitat	Selected for study
Halichondria panacea Breadcrumb sponge	No				
Arenicola marina Lugworm	Yes	No			
Nereis diversicolor Ragworm	Yes	No			
Patella vulgata Common Limpet	Yes	No			
Littorina littorea Edible periwinkle	Yes	No			
Mytilus edulis Blue mussel	Yes	Yes	Yes	filter feeder, below mid-tide level on rocky shores and estuaries	Yes

Table continued…

Species	Species Present	At all sites	Methodology available	Feeding type, habitat	Selected for study
Cerastoderma edule Edible cockle	Yes	No			
Noemysis integer Mysid shrimp	No				
Carcinus maenas Shore crab	Yes	Yes	Yes	Omnivore, all shore types, high water to sublittoral	Yes
Asterias rubens Common starfish	Yes	No			

Table 10 Species selection for the Plymouth Sound & Estuaries SAC (based on preliminary visits)

Species	Species Present	At all sites	Methodology available	Feeding type, habitat	Selected for study
Halichondria panacea Breadcrumb sponge	No				
Arenicola marina Lugworm	Yes	No			
Nereis diversicolor Ragworm	Yes				
Patella vulgata Common Limpet	Yes	No			
Littorina littorea Edible periwinkle	Yes	No			
Mytilus edulis Blue mussel	Yes	Yes	Yes	filter feeder, below mid-tide level on rocky shores and estuaries	Yes
Cerastoderma edule Edible cockle	Yes	No			

Table continued…

Species	Species Present	At all sites	Methodology available	Feeding type, habitat	Selected for study
Noemysis integer Mysid shrimp	No				
Carcinus maenas Shore crab	Yes	Yes	Yes	Omnivore, all shore types, high water to sublittoral	Yes
Asterias rubens Common starfish	No				

Sampling programme

3.4 The field programme was planned to include an assessment of temporal (seasonal) variability in biological responses. Two visits each were made to the Fal (spring and autumn) and Tamar (winter and spring).

3.5 For the Tamar, 'winter' samples were collected on 30th-31st January and 13th-14th February 2006, and 'spring' between 10th -11th and 24th -25th April 2006.

3.6 The 'spring' samples for the Fal were collected in March 2006 (13th-14th and 27th-28th March) and the 'autumn' sampling was carried out in October (9th-10th and 23-24th).

3.7 Two sampling trips were incorporated into each season, generally two weeks apart to allow for tide times. On each visit, mussels (n=8 per site) from 3-4 sites per SAC were collected at low tide by hand on the first day of sampling and crab traps (Trappy™), baited with fish, were deployed for collection the following day (n=8 per site). Approximately 10 litres of water was also collected from each site. The animals were transported back to the laboratory in cool boxes to minimise the stress of transportation and were left overnight in water from their respective site in temperature-controlled rooms (15 ± 1°C).

Biomarker selection

3.8 The rationale for biomarker section is presented in the previous section. See Appendix III for the complete methodology for each biomarker as well as appropriate references. We also included a rapid toxicity bioassay to monitor water quality (Microtox™, a laboratory assay based on the inhibition of bacterial bioluminescence).

3.9 The final chosen list includes:

Biomarkers of exposure

- Pesticides - acetylcholinesterase inhibition (Appendix IIII A-22).
- PAHs - PAH metabolites in urine (Appendix IIII A-6).
- Endocrine disruptors (TBT).
- Metals - metallothionein and metal partitioning (Appendix III A-32).

Biomarkers of effect

- General health -Heart rate (Appendix III A-12).

- General health - Feeding rate (Appendix III A-19).
- Genotoxicity - micronucleus (Appendix III A-25).
- Immunotoxicity - phagocytosis (Appendix III A-9).
- Cell viability - Neutral red retention (Appendix III A-6).
- Oxidative stress - TOSC and FRAP oxidative capacity assays (Appendix III A37 and A30 respectively).
- Bioassays.
- Microtox (Instrumental manual and test procedures available for downloading from http://www.azurenv.com/mtox.htm).

Bioassays

- Microtox (Instrumental manual and test procedures available for downloading from http://www.azurenv.com/mtox.htm).

Statistical methods

3.10 Individual biomarkers for each species were analysed using 2 factor analysis of variance (AONVA) using GMAV (General Models of Analysis of Variance). The factor 'site' and 'time' were fixed and orthogonal. Variances were checked using Cochran's test and transformations made where appropriate. To determine significance, post-hoc comparisons were carried out using Student-Neman-Keuls (SNK) test.

3.11 For *C. maenas*, the relationship between urinary metabolites of polyaromatic hydrocarbons and biomarkers (cardiac rate, acetylcholine esterase, ferric reducing antioxidant potential, cellular viability, phagocytosis and protein) was analysed using Spearman Rank Correlation.

3.12 To assess differences in the health of animals between sites within each estuary, data from *M. edulis* and *C. maenas* were analysed separately using one factor Analysis of Similarities (ANOSIM) using the Plymouth Routines in Multivariate Ecological Research (PRIMER) Package. Multidimensional scaling (MDS) and bubble plots were also produced using this package.

3.13 Multidimensional scaling (MDS) is a statistical technique designed to express graphically, in a small-dimensional space, degrees of similarity or dissimilarity between sites. Points representing similar sites tend to cluster together in a specific region of the space; points representing dissimilar sites tend to be far apart. A benefit of MDS is its flexibility in dealing with data sets that have many different kinds of variable, such as may occur in field surveys.

3.14 A bubble plot (for example see Figure 22) is the representation of a univariate measure, in this case a biomarker, in relation to an MDS ordination. The bubbles represent the mean values of the biomarker as a symbol of differing sizes and provide a visual representation of biomarker variation between sites. The sizes of the bubbles provide a useful insight into the role of individual biomarkers in shaping the differences observed per site.

Development of a weighted classification system

3.15 It is evident from the preceding sections, that the application of suites of biomarkers as a component part of the risk assessment process can provide hugely informative, descriptive information with which to characterise the relationship between contamination and the health status of exposed organisms. This is achieved by consideration of individual biomarkers which may diagnose exposure to certain classes of contaminants or by assessing integrated biomarker responses.

3.16 However, one of the difficulties in establishing the environmental quality of an ecosystem in relation to assessments like 'good ecological status' (under the Water Framework Directive) or 'favourable condition' (under the Habitats Directive) is in the identification of a reference value, or knowledge of the state of the system, against which comparisons can be made (eg Maksimov, 1991). The 'reference condition' is a description of the biological quality elements that exist in a situation of low, or very minor, disturbance from anthropogenic impacts. The identification of such an area of high biological status is a crucial element in the development of a classification system suitable for the monitoring of ecological status [with 'ecological status' being an incorporation of biological, physico-chemical and hydromorphological elements (Vincent et al., 2002)].

3.17 Legislation such as the Water Framework Directive identifies four options for deriving reference conditions:

- an existing undisturbed site or a site of only very minor disturbance;
- historical data and information;
- models; and
- expert judgement.

3.18 These criteria for selecting appropriate reference conditions are equally relevant under the Habitats Directive.

3.19 Because reference conditions must incorporate natural variability, in most instances, they will be expressed as ranges. Reference conditions are then derived with a view to distinguishing between minor, slight and moderate disturbance. 'Minor' is generally defined as a change that is just detectable, or more likely to be anthropogenic than not; 'slight' disturbance is then defined as anthropogenic, at a prescribed level of confidence, and so on.

3.20 It follows that the description of the biological reference condition must be detailed enough to permit comparison of monitoring results with the reference condition in a statistically meaningful way. This is generally achieved in the form of an Ecological Quality Ratio (EQR).

3.21 Most of the approaches used for defining an EQR take advantage of multivariate statistical tests such as Principal Component analysis (PCA). Ordination techniques such as PCA aim to replace large, complex data sets (such as are obtained from ecological surveys and chemicals monitoring) with a much smaller set of derived variables that still retain most of the relevant information. These variables are then expressed as two components, or axes, that contain most of the important information in the data. The axes can be viewed as providing a picture of 'statistical distance' between datasets.

3.22 In the case of physico-chemical conditions and ecological survey data, PCA has been used by different authors to provide a measure of the distance (in statistical terms, the Euclidian Metric Distance) between locations designated as 'High Status' and 'Bad Status'. This distance is given a value of 1; as high and bad represent the two extremes, everything in between gets a value ranging from 0 to 1.

3.23 Where:

$EQR_x = CI_x\text{-}CI_{worst})/(CI_{optimal} – Ci_{worst})$

EQR_x = ecological quality ratio of site x

CI is PCA score of site x on first component or axis of the PCA

$CI_{optimal}$ = score of optimal site (reference site) on first component

CI_{worst} = score of worst site on first component

3.24 This then provides a set of boundaries for defining different ecological status, as illustrated below (Romero et al., 2007).

Table 11 Boundaries for defining different ecological status

EQR	Ecological status and colour code	
0.775-1	high	blue
0.550-0.774	good	green
0.325-0.549	moderate	yellow
0.1-0.324	poor	orange
0-0.1	bad	red

3.25 In the case of biological assessments derived from biomarker data, there has been a long and detailed literature exploring the varied ways in which the setting of boundaries for defining different health status may be achieved (eg Galloway et al., 2002, 2004, Hagger, 2006, Beliaeff and Burgeot, 2002, Borja et al., 2004, Aarab et al., 2004, Chevre et al., 2003, Devlin et al., 2006). Again, one of the main problems is in identifying a reference range against which monitoring data can meaningfully be compared.

3.26 In projects such as the European BIOMAR programme, multiple biomarkers were used to assess sites in the Baltic and Mediterranean. Discriminatory factors were then calculated for each biomarker, based on maximum variation range/confidence interval (using ANOVA and the Tukey test), and a response index allocated. For each site, a Global Response Index was calculated as the sum of each of the response indices for each of the biomarkers tested (Narbonne et al., 1999).

3.27 Where:

Discriminatory factor = (RR+CI)/CI

RR = response range

CI = confidence interval (significance level usually $P<0.05$).

3.28 The discriminatory factor then determines the number of significant differences between sites, and a 'level' is applied to indicate the number of places between higher and lower means for each biomarker. This is illustrated in the following table.

Table 12 Index for each biomarker response according to their rank in a scale related to discriminatory factors (from Narbonne et al., 1999)

	Discriminatory factors				
Index of response	1	2	3	4	5
	4	8			
	3	6	10		
	2	4	7	12	
	1	2	4	8	14

3.29 In yet another approach, Beliaeff and Burgeot (2002) outline the formulation of an 'integrated Biomarker Response' value in multiple biomarker studies. In this case, the calculation uses star plots as a simple, graphical way of identifying sites of anthropogenic stress. For each of the

biomarkers tested, the coefficient of variation was calculated and used to derive a 'score' as follows:

S = Z+ [min]

3.30 Where:

S = score

Z = mean estimate – general mean/standard deviation

Min = minimum value (or – maximum value for stimulation) for all stations.

3.31 These brief descriptions illustrate the numerous potential approaches to the construction of a classification system to meet policy needs. Below, we outline the approach taken with the current dataset.

Weighted grading system (Biomarker Response Index)

3.32 Our main concern for this analysis of SAC sites was to provide an adequate reference range against which monitoring results could be compared. We followed the guidance of the Water Framework Directive in identifying reference and impacted ranges, incorporating historical data, and data from sites of minimal impact and expert judgement. Using this information, and incorporating data from the large number of previous studies made by our research group in the South West and UK marine and coastal waters, and in laboratory studies, we compiled ranges for control and exposed values, reference and contaminated sites (Table 13). It has been highlighted in previous studies that because we are looking for human-induced changes, it must be assumed there will be a natural variability in the methodology used as well as in reference conditions (Borja et al., 2004; Muxika et al., 2007). Hence, for most parameters, the defining criteria should be expressed as ranges. An example of the statistical analysis for the determination of the ranges of biological parameters for mussels is presented in Table 13.

Table 13 An example of statistical analysis of laboratory exposures (control and exposed values) and field studies (reference and contaminated sites) for mussels used to develop the graded biomarker responses (for references see Table 14). For each study, the mean biomarker value is a representative of between 8-12 individual responses

Biomarker	Exposure conditions	N° studies	Mean	SD	Min	Max	Range
AChE	Reference/control	13	97.7	36.9	53.8	150	96.2
(µmol AChE/min/mg protein)	Contaminated/exposed	7	44.3	12.8	30	64	34
Feeding Rate	Reference/control	19	2.75	0.93	1.5	4.2	2.7
(Litres/hour)	Contaminated/exposed	8	1.04	0.48	0.14	1.6	1.46
Heart Rate	Reference/control	9	21.4	4.6	15	29	14
(Beats/min)	Contaminated/exposed upregulated	15	34.9	8.3	22	56	34
	Contaminated/exposed downregulated	1	9	0	9	9	0
Micronucleus	Reference/control	12	0.87	0.56	0.25	2	1.75
(number/1000 cells)	Contaminated/exposed	25	4.78	2.31	1.81	9	7.19
Cellular viability	Reference/control	4	4.11	0.69	3.5	4.73	1.23
(OD/mg protein)	Contaminated/exposed	14	2.27	0.85	0.25	3.07	2.82
Phagocytosis	Reference/control	7	30.2	6.6	20.85	38	17.15
(Zymosan particles 107/mg protein)	Contaminated/exposed upregulated	1	48	0	48	48	0
	Contaminated/exposed downregulated	13	18.45	5.8	9	28.11	19.11
Frap	Reference/control	5	15.64	3.2	10.2	19.2	9
(Δ absorbance/mg protein)	Contaminated/exposed upregulated	14	25.53	13.1	15.8	63.4	47.6
	Contaminated/exposed downregulated	8	9.72	2.9	4	14.3	10.3

Table 14 An example of ranking of biomarker responses based on biological responses in mussels in referenced laboratory exposures (control and exposed values) and field studies (reference and contaminated sites) obtained from Table 13 and categorised as per the example in Figure 9

		Alteration in response	None/slight	Moderate	Major	Severe
		Rank assigned	**4**	**3**	**2**	**1**
Exposure	OP	**AChE** (µmol AChE/min/mg protein) Bodin et al., 2004; Brown et al., 2004; Canty et al., 2007; Galloway et al., 2006; Rickwood and Galloway 2004; plus unpublished data	75+	50.0-74.9	25-49.9	0.0-24.5
Effects biomarkers	Physiological	**Feeding/Clearance Rate** (Litres/hour) Camacho et al., 2000; Canty et al., 2007; Marsden and Weatherhead 1999; plus unpublished data	2.0+	1.99-1.5	1.49-1.0	0.99-0
		Heart Rate (Beats /min)	20-29.9	30-39.9	40-49.9	50+
		Astley et al., 1999; Brown et al., 2004; Galloway et al., 2004, 2006; plus unpublished data		15-19.9	10-14.9	0-9.9
	Cellular	**Micronucleus** (number/1000 cells) Anitha et al., 2000; Bahari et al., 1994; Dopp et al., 1996; Galloway et al., 2002; Hagger et al., 2005; Mersch and Beauvais 1997; Mersch et al., 1999; Pavlica et al., 2000; Wrisberg et al., 1992; plus unpublished data	0-1.9	2.0-3.9	4.0-5.9	6.0+
		Cellular viability (OD/mg protein) Hagger et al., 2005; plus unpublished data	4+	3.0-3.9	2.0-2.9	0-1.9
		Phagocytosis (Zymosan particles 107/mg protein) Canty et al., 2007; Galloway et al., 2006; Hagger et al., 2005: plus unpublished data	30-39.9	20-29.9 40-44.9	10-19.9 45-49.9	0-9.9 50+
	Molecular	**Frap** (Δ absorbance/mg protein)	15-19.9	10-14.9	5-9.9	0-4.9
		Galloway et al., 2006; plus unpublished data		20-24.9	25-29.9	30+

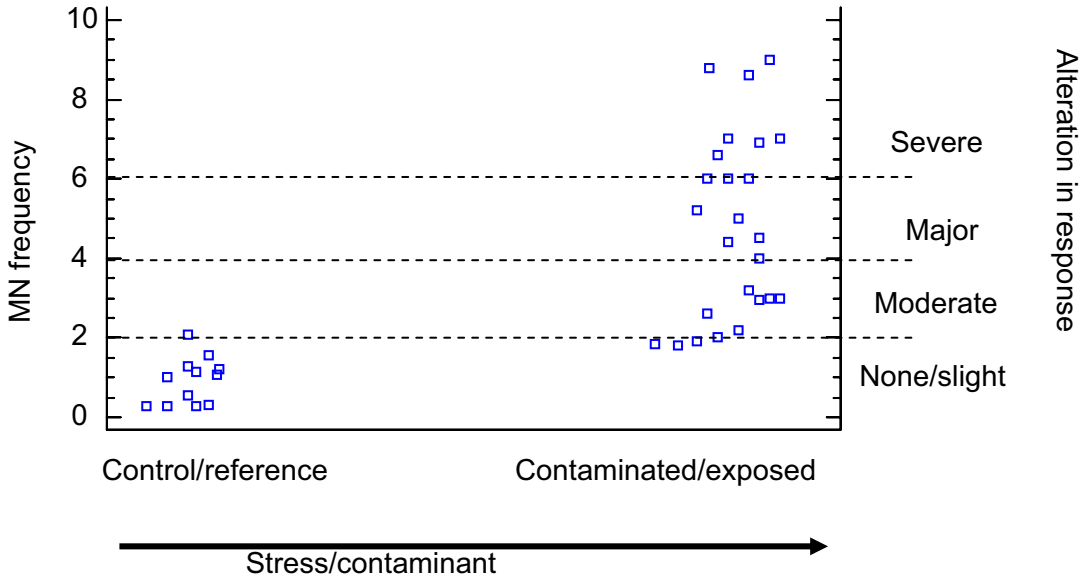


Figure 9 An example of the development of the biomarker grading system. The diagram represents studies demonstrating micronuclei frequency with each data point representing the mean of approx 8-12 individual values. The data are divided into four range categories to provide arbitrary indications of alteration to control/reference responses (See Sections 3.15 – 3.32)

3.33 A Biomarker Response Index (BRI) was developed following modification and refinement of the Bioeffects Assessment Index (BAI) and the Health Assessment Index (HAI) that had been developed previously for fish species (Adams and others 1993; Broeg and others 2005). In an adaptation to the BAI and HAI, the biomarker ranks were weighted in order to account for important variations in responses at different levels of biological organisation. The biomarker ranks were weighted as follows, physiological = 3, cellular = 2 and molecular = 1.

3.34 For example, mussels with a feeding/clearance rate of 0.85 L/h would be assigned a biomarker ranking of 1 as the feeding/clearance rate is less than 0.99 L/h as illustrated in Table 14, as this is a physiological biomarker and in order to correct for weighting the score would then be times by 3 to create a weighted rank value of 3. In comparison, mussels with a feeding/clearance rate of 4 L/h would have a rank of 4, as per Table 14, and following weighting would become 12.

3.35 The final BRI value for each species was then calculated by summing all the weighted biomarker ranks and dividing the total sum by the sum of weighing in order to provide a BRI value of 1 - 4 as illustrated below:

3.36 Biomarker Response Index (BRI) $= \sum (\text{biomarker}_1 \text{ rank} \times \text{biomarker}_1 \text{ weighting}) + (\text{biomarker}_2 \text{ rank} \times \text{biomarker}_2 \text{ weighting})_n / \sum (\text{biomarker}_1 \text{ weighting}) + (\text{biomarker}_2 \text{ weighting})_n$

3.37 An example of how the BRI is determined is illustrated in Table 15.

Table 15 An example of the steps needed to establish the Biomarker Response Index (BRI) in mussels (a) summary of individual biomarker data, (b) allocation of ranks and (c) allocation of weighting and BRI calculation

(a) Site	Heart rate	Feeding rate	ache	Frap	NR	Phag	Protein	MT	MN
1	19.43	2.54	60.13	19.26	4.73	8.17	0.93	23.03	1.00
2	30.34	2.88	96.08	6.89	3.52	10.35	0.86	21.10	1.60
3	23.78	2.37	84.54	11.62	3.50	9.86	0.67	19.18	3.17
4	26.84	1.13	62.58	6.72	3.07	16.57	0.93	21.83	2.71
5	24.82	0.74	72.25	40.54	2.61	26.82	0.58	29.02	2.14
6	24.10	2.03	168.55	21.18	3.08	34.27	0.41	20.33	2.14
7	24.53	1.07	134.06	17.70	2.88	23.46	0.76	21.87	1.80

(b) Site	Heart rate	Feeding rate	MN	NR	Phag	Protein	Frap
1	3	4	4	4	1	2	4
2	3	4	4	3	2	2	2
3	4	4	3	3	1	2	3
4	4	2	3	3	2	2	2
5	4	1	3	2	3	2	1
6	4	4	3	3	4	1	3
7	4	2	4	2	3	2	4

(c)									Sum of total biomarker rank	Final biomarker	Overall health rank
Weighting	3	3	2	2	2	1	1		Min = 14	Value (1-4)	
site	Heart rate	Feeding rate	MN	NR	Phag	Protein	Frap				
1	9	12	8	8	2	2	4	=	45	3.21	3
2	9	12	8	6	4	2	2	=	43	3.07	3
3	12	12	6	6	2	2	3	=	43	3.07	3
4	12	6	6	6	4	2	2	=	38	2.71	2
5	12	3	6	4	6	2	1	=	34	2.43	1
6	12	12	6	6	8	1	3	=	48	3.43	4
7	12	6	8	4	6	2	4	=	42	3.00	3

Table 16 The resulting BRI (1-4) is then classified according to the following table to provide a degree of alteration from normal reference responses

BRI	Biological status and colour code	
3.01-4.0	No or slight alteration from normal response	Green
2.76-3.00	Moderate alteration	Yellow
2.51-2.75	Major alteration	Orange
1-2.5	Severe alteration	Red

3.38 This classification system has been validated through a comparative assessment with the transitional water body classification applied to ten separate transitional water bodies in the South West of England (Hagger and others 2007).

4 Results

Examples of individual biomarkers

4.1 Some of the biomarkers deployed were chosen to represent a means of assessing biological responses to specific contaminants or classes of contaminants. For example, changes in acetylcholinesterase activity signal exposure to neurotoxic chemicals such as organophosphate (OP) pesticides and metallothionein induction is expressed as a result of excess metal exposure. These biomarkers of exposure may be used as tools to explore cause-effect relationships and impacts from specific contaminant types (identified as a potential threat by chemical analysis) and hence to identify gradients or hotspots. Some of the more pertinent results are discussed below.

Metallothionein (MT) induction and metal partitioning

4.2 Metallothionein (MT) is a metal-binding protein specifically induced in most living organisms in response to heavy metal exposure and has been measured directly in mussel tissues in the current project using differential pulse polarography (see Appendix III A-34). In conjunction with this biomarker, investigation of metal partitioning in tissue extracts has been used to identify the most significant bioavailable metal(s) and to indicate whether the organism is adapting successfully to increased body burdens (see Langston and others 2002, for full methodology and Appendix III A-34 for outline).

MT induction: Plymouth Sound & Estuaries

4.3 In mussel gills, both winter and spring data indicate an increase in MT induction, generally, in an upstream direction (Figure 10). This trend matches gradients in metal contamination (see Figure 24), significantly so for Zn in spring ($P<0.05$, $r = 0.9896$, compared with r values of 0.6855, 0.5209 and 0.4145 for Cu, Cd and Ag, respectively). At Torpoint (sampled in spring only) there are indications of a secondary peak in MT levels (Figure 10).

4.4 Thus, MT concentrations in samples from within the Tamar Estuary [at Neil Point and Town Quay (winter), and Torpoint and Jupiter Point (spring)] were significantly higher than at the reference site (Whitsand Bay). Note, however, that ‘basal’ MT levels at Whitsand were slightly higher than those at Maenporth, the corresponding control site in Falmouth Bay. This may be a reflection of the anomalously high Zn burdens in Whitsand mussels.

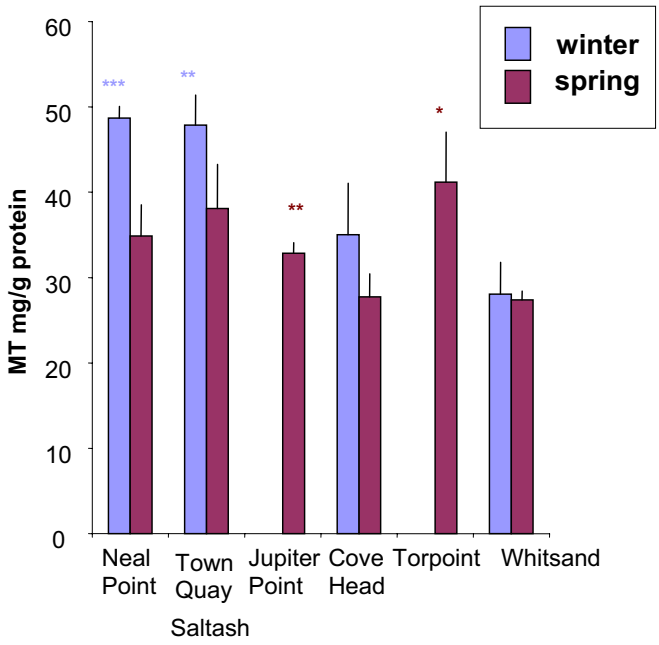


Figure 10 Metallothionein concentrations (m ± 1SE) in gills of *Mytilus edulis* from Plymouth Sound & Estuaries SAC (*P<0.05, **P<0.01, ***P<0.001 values significantly different to reference site, Whitsand)

4.5 Where samples were collected at the same Tamar sites on both occasions, comparison of mean values indicates that concentrations were slightly lower in spring ($p<0.05$ Neal Point). Though seasonal variation in gill tissue appears to be relatively small in this data set, sampling during a fixed season is advisable.

MT induction: Fal & Helford

4.6 In the spring survey, MT induction in mussel gills was most marked at Weir Point - and was significantly higher here than at the reference site at Maenporth (Figure 11). As indicated in Figure 24 and Appendix II, several metals including Cu, Zn and Cd exhibit enhanced bioavailability at this site, presumably because of its proximity to Restronguet Creek. The most significant correlation between tissue metal concentration and MT induction in the spring sample was for Cu ($p<0.05$, $r=0.8253$, compared with r values of 0.7413, 0.447 and 0.4309 for Ag, Cd and Zn).

4.7 The overall pattern for MT in the autumn survey was similar to that in spring and was again dominated by Weir Point, with lowest values at Maenporth (Figure 11). MT concentrations in autumn were higher than in spring, generally, at most sites. The largest seasonal increases were at Helford and King Harry Ferry, Falmouth Marina and Malpas in the Fal (significantly higher than corresponding samples in spring, $P<0.05$).

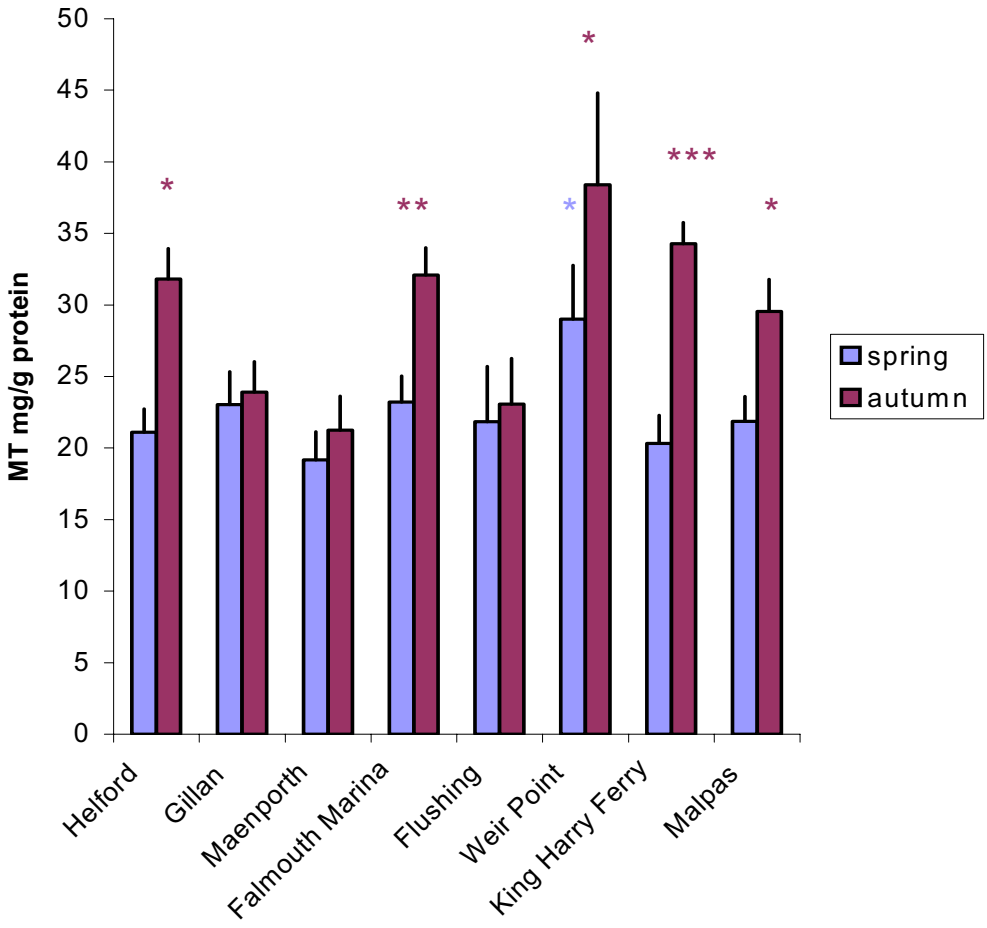


Figure 11 Metallothionein (m ± 1SE) in gills of *Mytilus edulis* from Fal & Helford SAC (*P<0.05, **P<0.01, ***P<0.001 values significantly different to reference site, Maenporth)

4.8 MT induction in the gill tissues of *M. edulis* is, therefore, a viable biomarker of response to metal contamination and an aid to site condition assessment. As indicated above, this can be achieved at the basic level by assessing the differences in MT (protein) concentration between sites. Total metal analysis in mussel tissues points to those elements which are likely to be responsible for responses. More detailed examination of metal-partitioning characteristics, as described below, can sometimes provide further insight in to the degree of metal-induced stress.

Metal partitioning characteristics

4.9 Additional diagnostic information on the metals responsible for MT induction, and their impact, has been obtained by investigation of metal partitioning behaviour. Metals were determined in various subcellular fractions, including MT, following chromatographic separation of tissue extracts (gills and digestive glands). The appearance of atypically high metal concentrations in non-detoxified (non-MT) pools – the 'spillover effect' – signifies that the homeostatic capacity of MT is reaching saturation and heralds the likelihood of deleterious effects (Langston and others 1998).

4.10 The clearest evidence of abnormal metal partitioning obtained in the current samples concerns Cu in mussels from Weir Point in the Fal, (series in green, Figure 12). At this site, Cu increases were observed in all subcellular fractions, including the high molecular weight (HMW) protein pool, metallothionein (MT) fraction and, most noticeably, in the low molecular weight (LMW) fraction of gill extracts (left hand figure). HMW proteins include enzymes and other essential macromolecules and the increase in Cu binding could eventually lead to impaired functioning of these proteins. The LMW pool includes more labile forms and excess free metal – ie potentially toxic species. The appearance of Cu in this pool in significant amounts (nb in gills) suggests the

capacity of MT may have been surpassed and toxicological effects are likely from this metal. In acute exposures previously undertaken in Restronguet Creek, the appearance of Cu in LMW fractions heralds the onset of mortality. The Weir Point population sampled would therefore be considered to be stressed by Cu in particular.

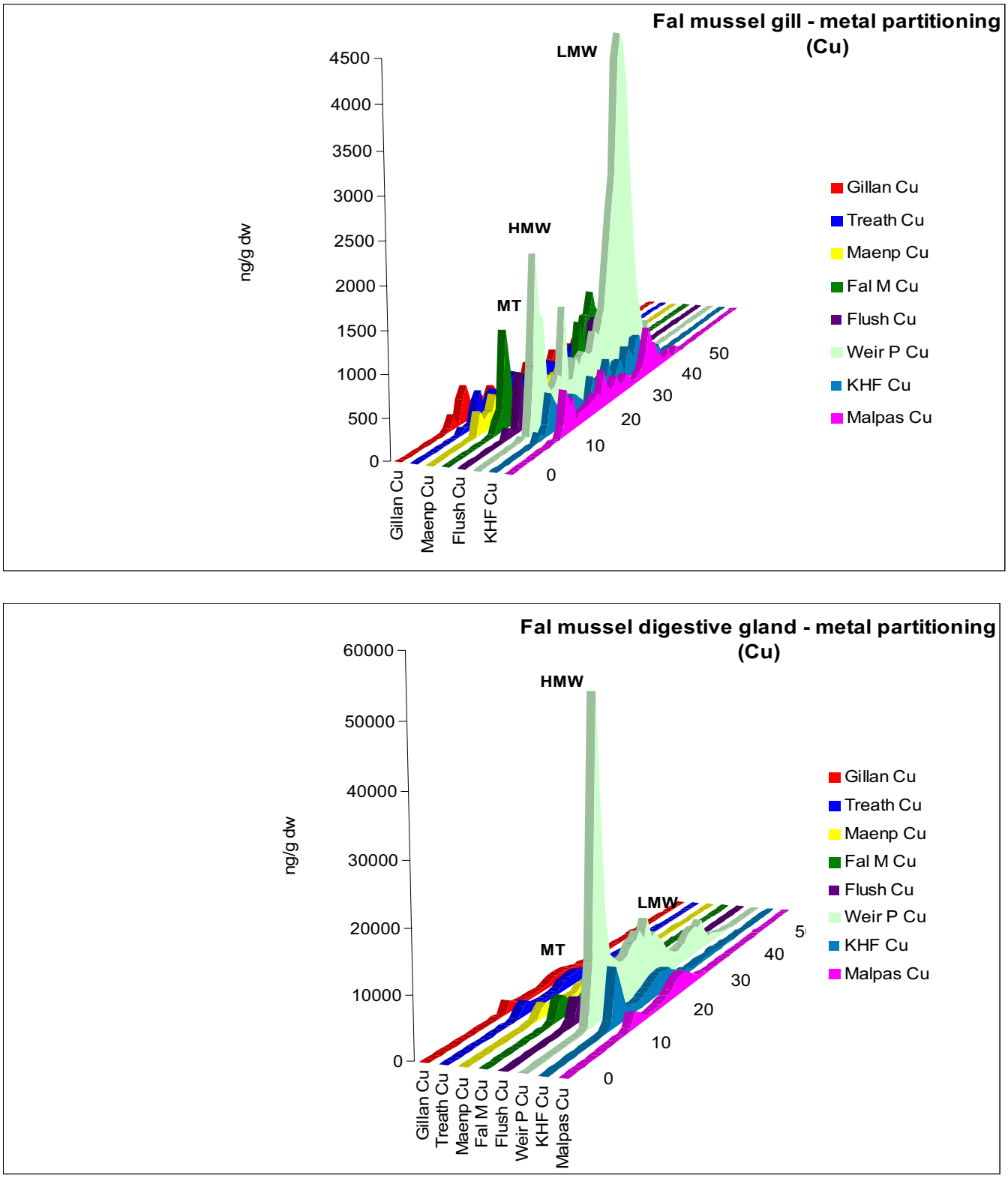


Figure 12 *Mytilus edulis* Fal & Helford SAC spring 2007. Copper partitioning among cytosolic ligands. *Top:* Gill; *Bottom*: Digestive gland

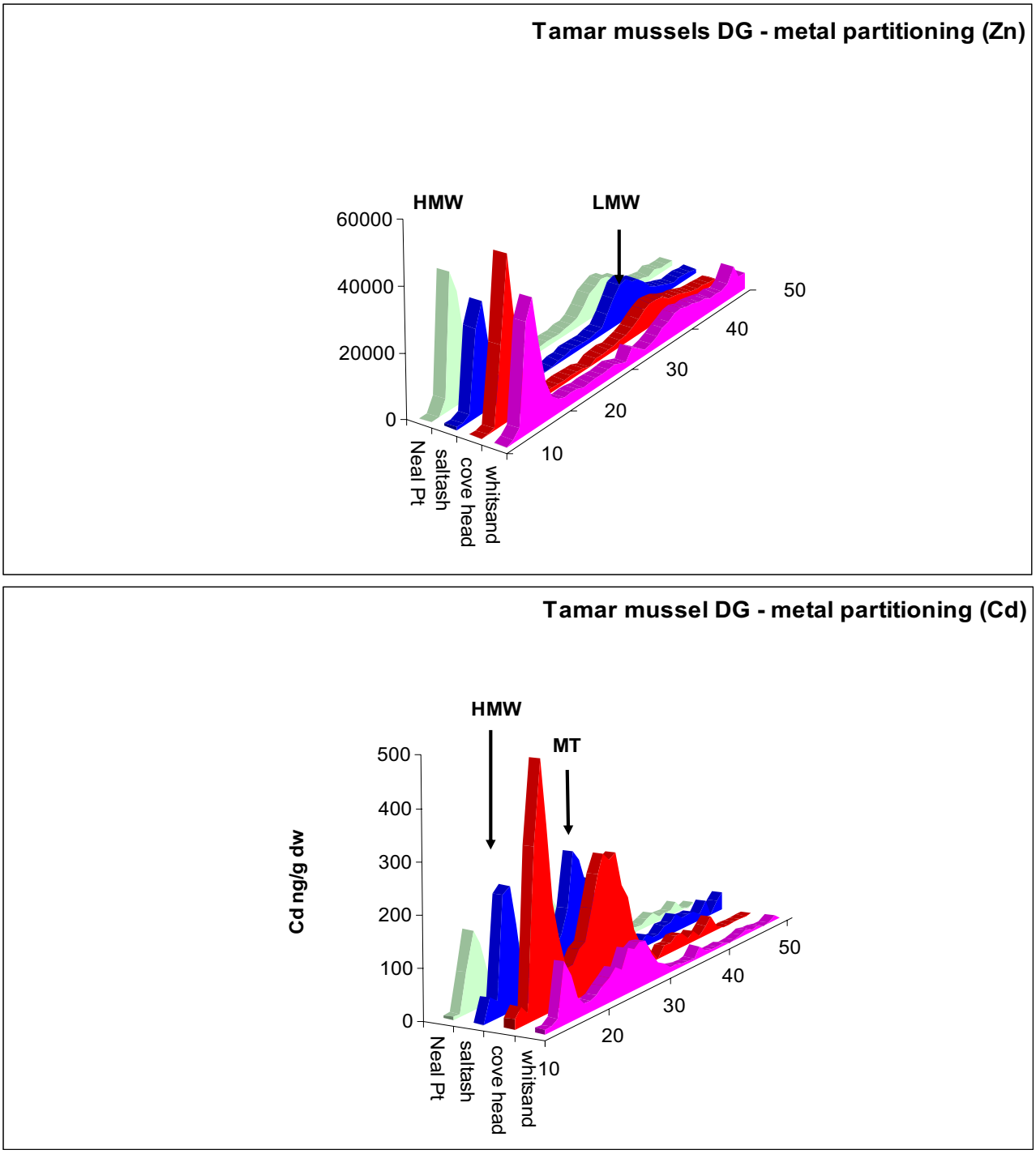


Figure 13 *Mytilus edulis*, Plymouth Sound & Estuaries SAC. Partitioning of *Top:* Zn and *Bottom:* Cd among cytosolic ligands of the digestive gland

4.11 Changes in metal partitioning on this scale were not seen in gills of Tamar mussel samples, suggesting effects were less acute than in parts of the Fal. Amongst Tamar sites, some of the biggest increases in subcellular metals, relative to Whitsand controls, were for Cd in digestive gland, with increases occurring in both MT and HMW pools in the Hamoaze, particularly at Cove Head (Figure 13, left, series in red), but with no disproportionation to indicate saturation of MT. There was also a slightly higher proportion of Cu and Ag bound to HMW proteins at Cove Head, though any 'spillover' effect was much less marked than in the Weir Point (Fal) samples. Zn concentrations, and partitioning in tissue extracts, was similar across all sites in the Plymouth Sound & Estuaries SAC (Figure 13, right), consistent with the notion that this essential metal is partially regulated by mussels. MT involvement in Zn binding was relatively small in comparison to the dominant association of Zn with larger functional proteins (HMW).

Comparison of MT responses in gill and digestive gland

4.12 MT concentrations in digestive gland exceeded those in gills by approximately five-fold. The high level of 'constitutive' MT in digestive glands reflects involvement in the high natural turnover of metals in this organ and appears to mask changes brought about by contamination. This explains why metal-binding patterns in the digestive gland of mussels from Weir Point do not show the same level of disturbance as gills (Figure 12): even though metal concentrations in the digestive gland are higher than in gills, Cu appears to be more effectively detoxified by the higher basal levels of MT. By contrast, metallothionein levels in gill tissues are more responsive to contamination (and less susceptible to seasonal changes in condition) and are therefore preferred as a biomarker for metal exposure.

4.13 To summarise, MT is a specific biomarker which provides information as to whether or not protective mechanisms are being upregulated because of metal exposure (presumably at some cost to the organism's energy budget). The study of metal partitioning is useful as a complementary diagnostic tool to indicate which metals are responsible and to determine whether thresholds of metal homeostasis are being compromised. Results from the current project indicate that MT induction in mussel gills is occurring in both the Tamar Estuary and in parts of the Fal & Helford, particularly near Restronguet: here the capacity to detoxify Cu appears to be near saturation, threatening the condition of this population.

Total oxyradical scavenging capacity (TOSC)

4.14 Total oxyradical scavenging capacity is an indicator of the ability of an organism to cope with oxidative stress. The extent of damage to antioxidative systems can be determined by measurement of a reduction in TOSC as a general marker of oxidative stress (reduced capacity to neutralise various reactive oxygen species). Under certain chronic exposure circumstances, however, an increase in TOSC may be seen as a counteractive measure by the organism to pro-oxidant challenge. It is, therefore, crucial to have appropriate reference samples.

Plymouth Sound & Estuaries

4.15 Results for the Tamar winter survey are shown in Figure 14 (left). Although Whitsand is considered a suitable reference station, TOSC values in gills were, in fact, slightly higher at Neal Point and might indicate a slight induction of the antioxidant system here. Cove Head shows the greatest lowering in TOSC (significantly different to Neal Point and Whitsand, $P<0.05$, t test). Other comparisons were not significantly different.

4.16 The spring survey covered a greater range of sites and showed that, compared to the Whitsand reference population, TOSC was suppressed increasingly in an upstream direction, implying that oxidative stress increased with distance up estuary (Figure 14). It is apparent that seasonal factors, or other temporal variables, are important in the application of this biomarker of general health. Results for the Tamar suggest that stress levels were higher in spring than winter.

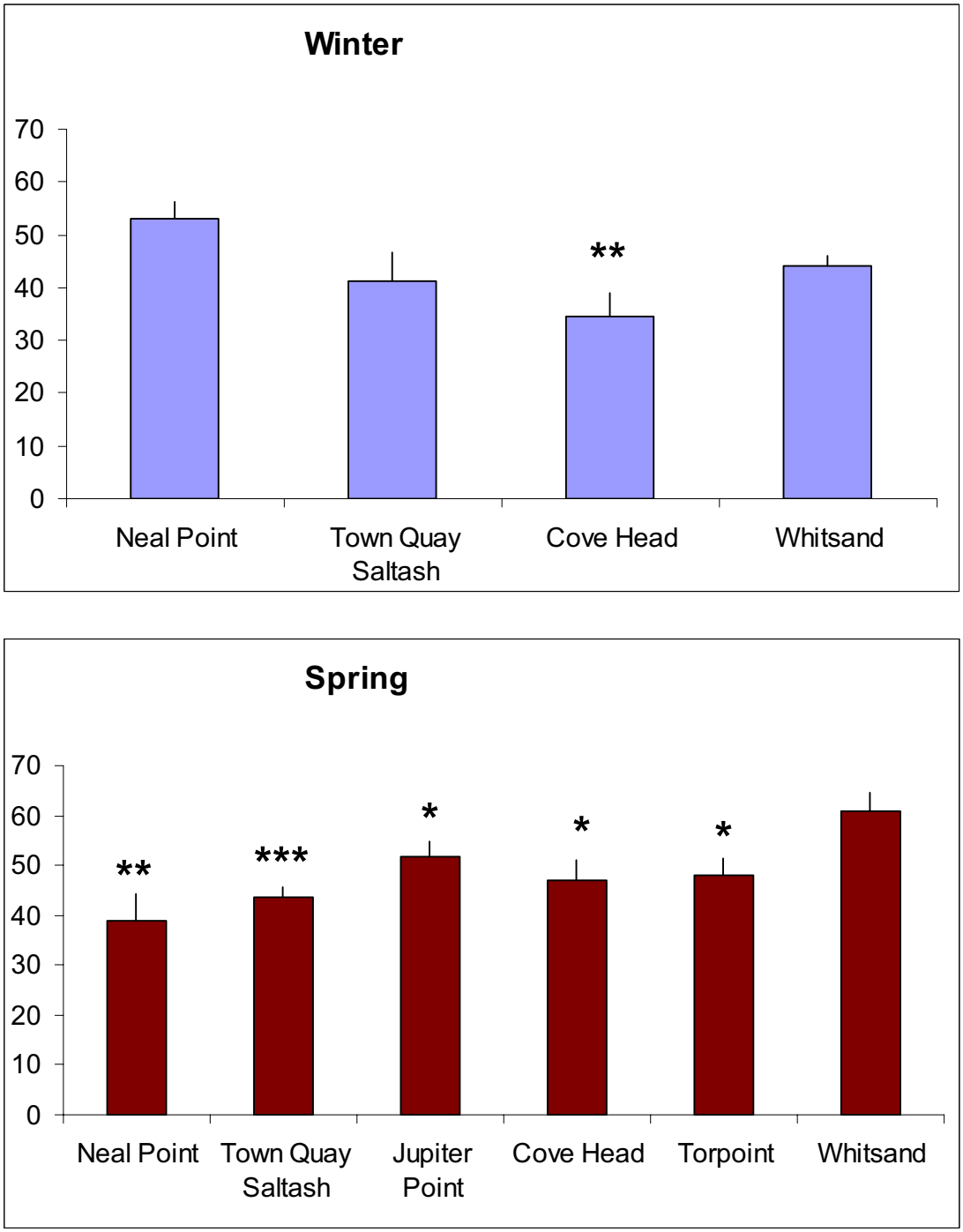


Figure 14 Total oxyradical scavenging capacity (TOSC) in gills of *Mytilus edulis* from Plymouth Sound & Estuaries SAC during winter and spring (*P<0.05, **P<0.01, ***P<0.001, signifies significant difference compared with Whitsand reference)

Fal & Helford

4.17 Results for the Fal & Helford survey also imply different responses between seasons. In spring (Figure 15), the ability to scavenge damaging oxyradicals is reduced to the greatest extent at Weir Point near the mouth of Restronguet Creek (highest in metals) and also at other sites in the upper part of Carrick Roads - at King Harry Ferry and Malpas [essentially, where nutrients and DO fluctuations are highest –including periodic oxygen depletion in the past). These reductions, relative to Maenporth reference mussels, were of marginal statistical significance (test)].

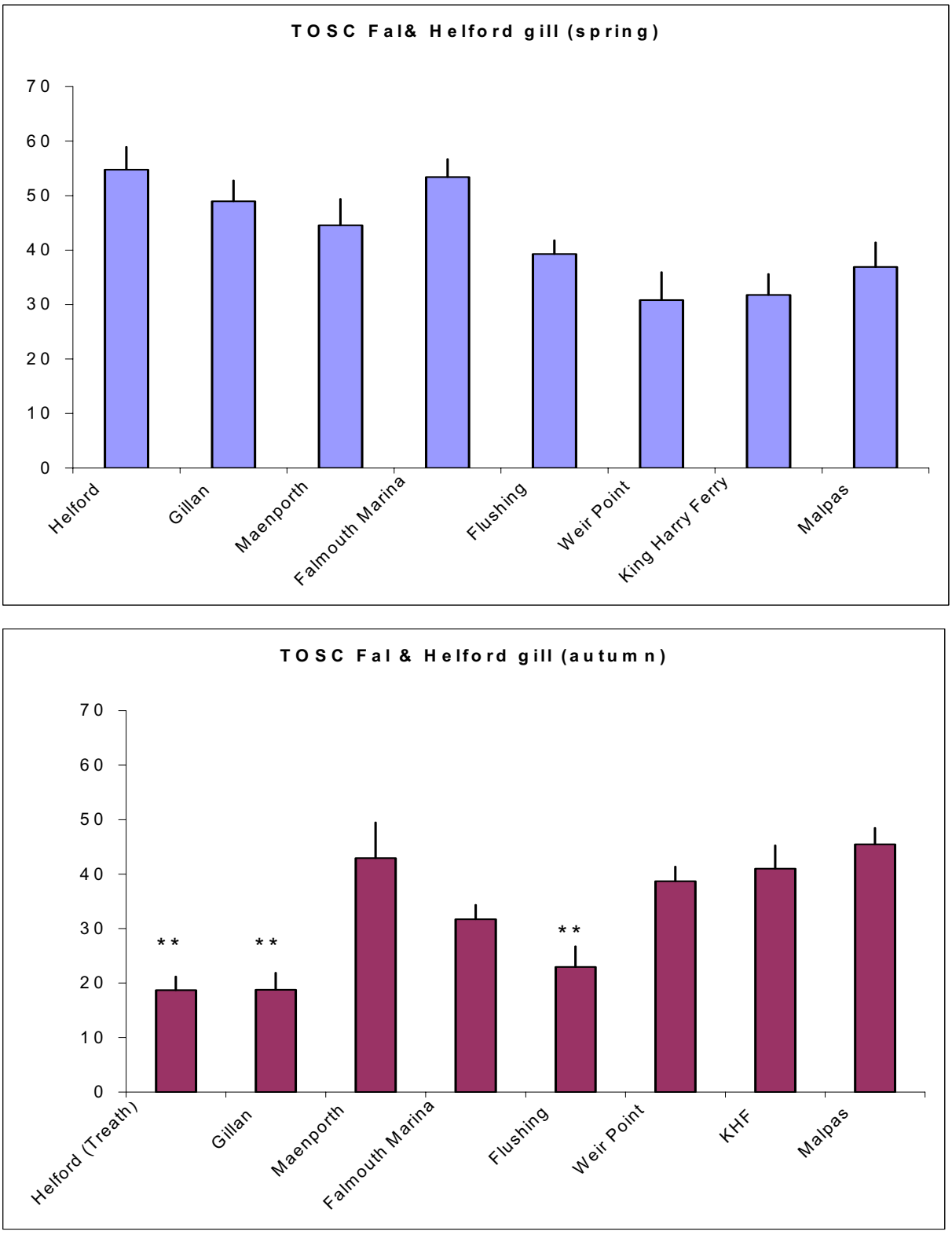


Figure 15 Total oxyradical scavenging capacity (TOSC) in gills of mussels *Mytilus edulis*, Fal & Helford SAC, spring and autumn 2006. [**signifies values significantly lower than the Maenporth reference site (P<0.01)]

4.18 In autumn samples, the pattern was substantially different (Figure 15). Although TOSC values at the Maenporth reference site and in upper Fal sites were broadly comparable with those in spring, substantial temporal decreases were apparent at sites in the Helford, Falmouth Marina (P<0.001) and Flushing (P<0.01), implying a significant reduction in capacity to overcome

oxidative stress. TOSC levels in the Helford and at Flushing were significantly lower than at the reference site at Maenporth ($P<0.01$).

4.19 The cause of this seasonal reaction is difficult to prove and highlights the requirements for additional contextual information on water quality. Given their locations and timings, the most obvious candidates are TBT and/or eutrophication-related pressures.

Polyaromatic hydrocarbon (PAH) metabolites

4.20 The detection of PAH compounds in the biological fluids of marine organisms provides a measure of their environmental exposure to PAHs. When PAHs are ingested they are converted by the organism to water-soluble metabolites which are excreted via the urine. Both the parent compounds and the metabolites are strongly fluorescent due to the presence of multiple aromatic rings in their chemical structure, and this property can be exploited to provide a quantitative assessment of exposure.

4.21 There are two main sources of PAHS in the marine environment, petrogenic (derived from oil) and pyrogenic (derived from combustion). The petrogenic PAHs are typically the alkylated homologues of the two and three ringed PAHs (naphthalene and phenanthrene) and when organisms are exposed to oil spills, the biological fluids will show a fluorescence profile with an excitation wavelength of around 290nm and 256nm, due to naphthalene of phenanthrene type metabolites respectively. The fluorescence spectrum resulting from exposure to the larger 4-6 ringed combustion-derived PAHs typical of harbour and urban areas, often has an excitation peak of between 360-420nm, characteristic of benzo-a-pyrene type metabolites. By measuring the fluorescence at these different wavelengths, it is possible to calculate a ratio of naphthalene to benzo-a-pyrene equivalents as an indicator of the relative amount of metabolites derived from petrogenic and pyrogenic sources.

4.22 From Figure 16, it is apparent that all sites are dominated by pyrogenic PAH equivalents. This indicates that the source of the PAH exposure is less likely to be from ingestion of oil (such as might be expected after a fresh spillage) and more typical of the larger 4-6 ringed pyrogenic products that accumulate in urban settings and harbours from the combustion of petrols, tyres and road traffic. This is particularly evident at sites extending downstream from the Tamar Bridge in the Plymouth SAC, and at Malpas, King Harry Ferry and Falmouth marina and docks sites in the Falmouth SAC. The relationship between PAH exposure and general health is explored further in the next section.

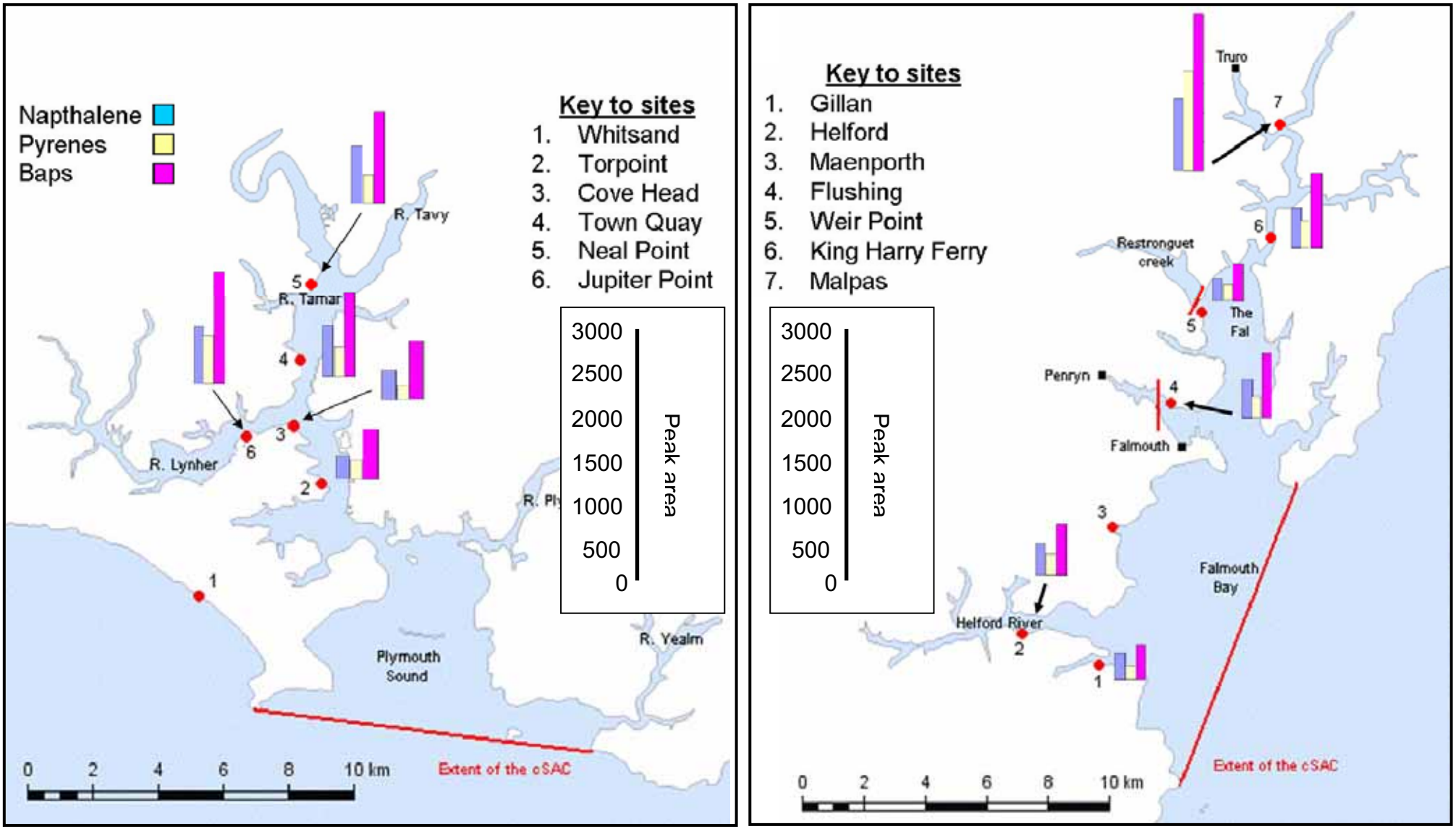


Figure 16 PAH metabolites in the urine of Carcinus maenas collected from *Left:* Plymouth Sound & Estuaries and *Right:* Fal & Helford Estuaries SAC sites. Sites with no data represent sites where no crabs were collected

TBT

4.23 Populations of TBT-sensitive neogastopods were not available at many of the 'biomarker' sites. Nevertheless the results from samples taken at adjacent locations show that TBT is still an issue in the vicinity of Falmouth and, to a lesser extent, the Tamar Estuary and could well contribute to the general stress response at some of these sites.

4.24 The current sampling confirms that *Nucella lapillus* continues to be eliminated from the Fal because of high TBT levels (TBT body burden data in mussel samples illustrate high TBT bioavailability at Penryn Creek/Flushing see Appendix II). The Castle Drive dogwhelk population, outside the estuary mouth, represents the limit of distribution of the species to the west of the Fal Estuary. Indices of imposex have remained high in neogatropods from Castle Drive (100% of females affected at the start of this project). To the east of the estuary mouth, a small number of individuals was also found at St Anthony. These have not been sacrificed for imposex determination so as not to impede recovery of these populations

4.25 In *Nucella lapillus* populations at Renny Rocks, near Wembury (Plymouth Sound & Estuaries SAC), imposex is still detectable, though breeding does not now appear to be compromised. In a sample taken in November 2005, at the start of this project, approximately 50% of females showed imposex, of which less than 2% were sterile (compared with 78% immediately after TBT legislation in 1988).

4.26 Inshore, nearer to the entrance to the Tamar, *Nucella* populations have yet to become re-established. In sublittoral populations of the (less-sensitive) mud snail *Nassarius* (=*Hinia*) *reticulata* from Plymouth Sound, imposex was still evident in 2005, though at lower levels than in the 1980s/early 1990s (Langston and others 1994). This coincides with a progressive reduction in TBT in water from the Sound from a maximum of ~ 8 ng l^{-1} TBT (as Sn) in 1988 to current levels of <1 ng l^{-1}. In the current project, imposex severity in Nassarius, expressed as the Relative Penis Size index, decreased offshore, from ~20% at Drakes Island Near the mouth of the Tamar to ~5% at Cawsand, near Rame Head.

4.27 These results confirm that the contribution of TBT to biological effects is likely to be highest in the region of Penryn Creek (Fal) and upstream in the Tamar estuary.

Microtox rapid toxicity bioassay

4.28 Figure 17 shows the results of the Microtox rapid toxicity bioassay, which was used to determine the toxicity of water samples collected from sites in the Fal and Plymouth Sound SACs. The Microtox bioassay uses a luminescent bacterium to measure toxicity from environmental samples. Luminescent bacteria produce light as a by-product of their cellular respiration and any inhibition of cellular activity (toxicity) results in a decreased rate of respiration and a corresponding decrease in the rate of luminescence. The more toxic the sample, the greater the percent light loss from the test suspension of luminescent bacteria.

4.29 There was no measurable toxicity in any of the water samples tested, apart from a small level of toxicity at the King Harry Ferry site, Falmouth.

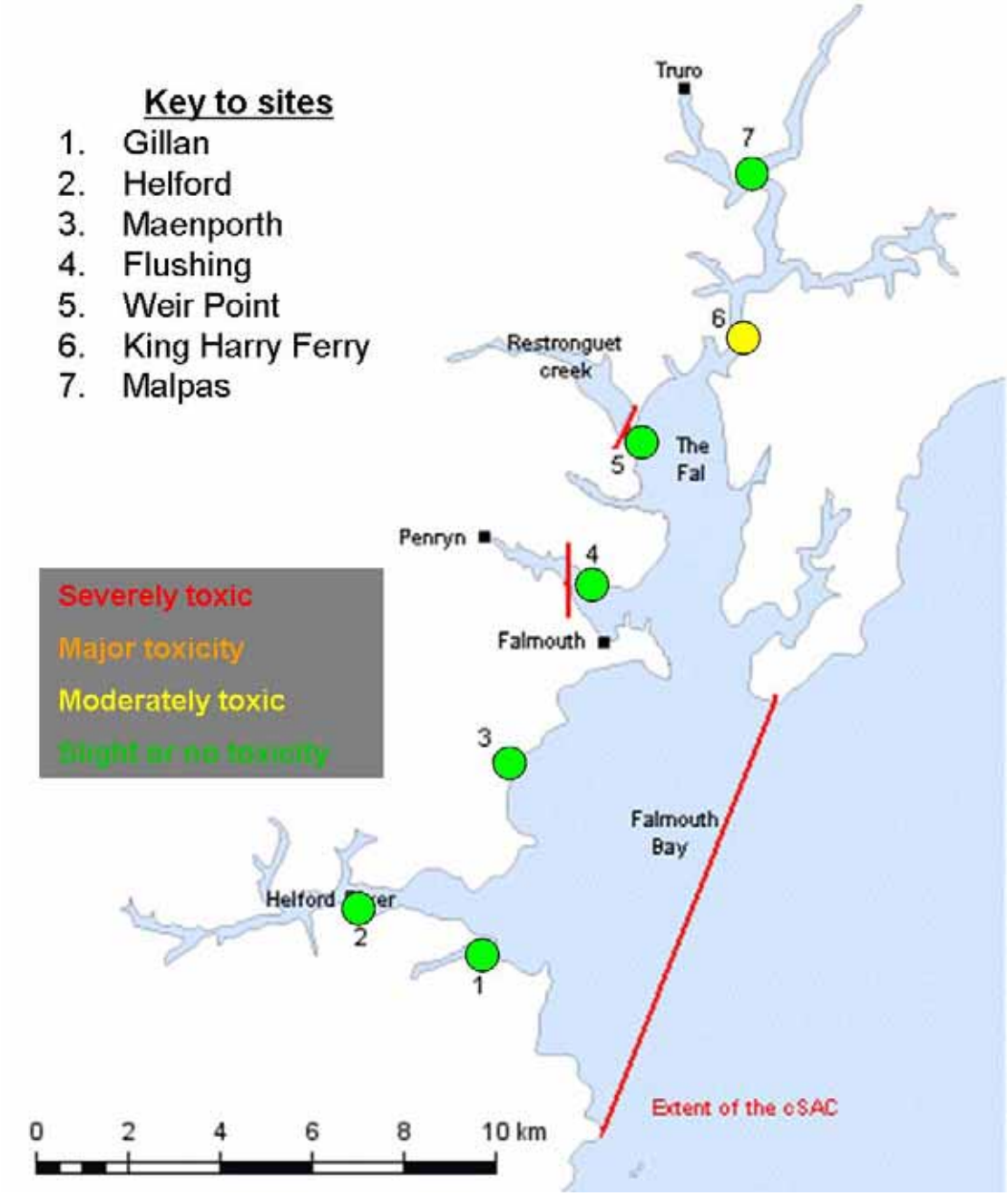


Figure 17 Microtox results for water samples collected in the Fal & Helford SAC during spring. Red = severely toxic (76-100% inhibition); orange = major toxicity (51-75% inhibition); yellow = moderately toxic (26-50% inhibition); green = little or no toxicity compared to normal baseline levels (0-25% inhibition)

Summary: Individual biomarkers

4.30 The results in this section have presented data for individual biomarkers that discriminate gradients or specific impacts. The conclusions are listed below.

- **Metals:** The metal-binding protein metallothionein was measured in the gills and digestive gland of mussels. In the gills, induction of this protective response occurred in both the Tamar Estuary (increasing in an upstream direction, generally) and in parts of the Fal and Helford, particularly near Restronguet Creek.
- **Oxidative stress:** Total oxyradical scavenging capacity (TOSC) is an indicator of the ability of an organism to cope with oxidative stress. Results imply that oxidative stress increased with distance up the estuary towards Torpoint in the Tamar. In the Fal (spring), the greatest impact was apparent at Weir Point near the mouth of Restronguet Creek (highest in metals) and at King Harry Ferry and Malpas (where nutrients and DO fluctuations are highest). However, in autumn TOSC was also significantly reduced at sites in the Helford and at Flushing.
- **Polyaromatic hydrocarbons (PAHs):** PAH metabolites measured in the urine of crabs are an indication of exposure to PAHs. The pattern of exposure at all sites was typical of urban/harbour pyrogenic PAH contamination (dominated by larger 4-6 ringed compounds). Exposure was greatest at Malpas in the Fal, with significant exposure at King Harry Ferry and Falmouth docks, whilst in the Tamar, sites around Torpoint were most affected.
- **TBT:** A combination of imposex determination and population observations, coupled with body-burden data, is a unique and powerful tool for determining threats from TBT. Nucella lapillus is still absent throughout the Fal as a result of TBT pollution. The body burden data for mussels indicate bioavailability is particularly high at Falmouth (Penryn Creek) as a consequence of past and present releases from the docks and marinas. Sensitive neogastropods remain affected by imposex in Plymouth sound, and are largely absent close to and within the Tamar. TBT body burdens in Tamar mussels increase upstream, peaking at Saltash, but are an order of magnitude lower than those at Falmouth.
- **Water toxicity:** None of the water samples collected during the field surveys was acutely toxic, as measured using the Microtox rapid toxicity bioassay.

Integrated biomarker assessment

4.31 The results for individual biomarkers in the preceding section are for those that discriminate gradients or specific impacts from metals, TBT and PAHs. The results suggest that there are sites within each SAC that experience multiple contaminant impacts from PAHs and metals (and possibly, indirectly, from nutrient enrichment). TBT remains a concern, for the integrity of the Fal in particular. In this section, the complete dataset is examined (biomarkers of exposure, effect and general health) to further characterise the relationship between contamination and sublethal effects. We sought to address the following questions:

- Are there significant differences in biomarker responses between sites?
- If so, do the differences persist with time?
- Can the different patterns of response at each site offer a potential means of identifying the types of pollutant contributing most to the observed effects?

4.32 Initially, univariate analysis was used to examine each biomarker for each species individually, against both sampling site and sampling time, using ANOVA where variances were homogenous, or Kruskal Wallis for heterogenous variances (see Appendix IV for all raw data and graphs representing all biomarker results for both species from both SAC sites). Then, the complete dataset was analysed using multivariate tests using the PRIMER (Plymouth Marine Laboratories) software package, to summarise the patterns and relationships between all of the variables. The completed statistical tables for each of these multivariate tests are also presented in the Appendix IV.

4.33 Previous data using univariate analysis was used to calculate the BRI as illustrated in section 3.15-3.33. The BRI classification system provides an alternative way of viewing the full biomarker dataset, providing an essentially similar picture to the integrated assessment based on multivariate analysis. Temporal variability was taken into account in the development of the BRI which, for each biomarker end point, is based on a range of measurements from each test species from laboratory and field studies. It is well known that some biomarkers show temporal and biotic variability, and that to be confident that assessments of environmental health based on biomarkers represent a true measurement, it is vital that the full range of inherent variability in the measurements is known. However, temporal variation is an unavoidable fact of life with any sort of monitoring including water quality and to minimise any seasonal contribution to this effect, it is recommended that sampling is conducted at a fixed time of year. Current results are adding to our confidence in setting the full range of the endpoint measurements for our selected biomarkers but, at this stage, the pattern of seasonality in the data is not clearly defined.

Plymouth Sound & Estuaries results overview

4.34 Multivariate analysis of the data from the Tamar (using ANOSIM, a similarity matrix) revealed distinct patterns in the biomarker responses of mussels and of crabs from the Torpoint and the Neal Point sites. Examination of the individual biomarker results showed a statistically significant increase in both oxidative (FRAP; $F_{4,50} = 5.83$; C=0.24; P<0.001) and genetic stress (micronuclei; $F_{4,60} = 19.96$; C=0.22; P<0.001) in the mussels collected from Torpoint. These results were consistent over time, as shown in Figures 18 and 19. The oxidative status of haemolymph from mussels sampled from Torpoint was significantly higher compared to all other sites. Across all sites, winter values of FRAP were significantly higher than spring, although the overall pattern was consistent. The number of micronuclei within haemocytes from mussels sampled in winter and spring from Torpoint was significantly higher compared to all other sites. At the same time, the immune function (phagocytosis; $F_{1,40} = 4.91$; C=0.20: P<0.001) of crabs from Torpoint was significantly different from all other sites.

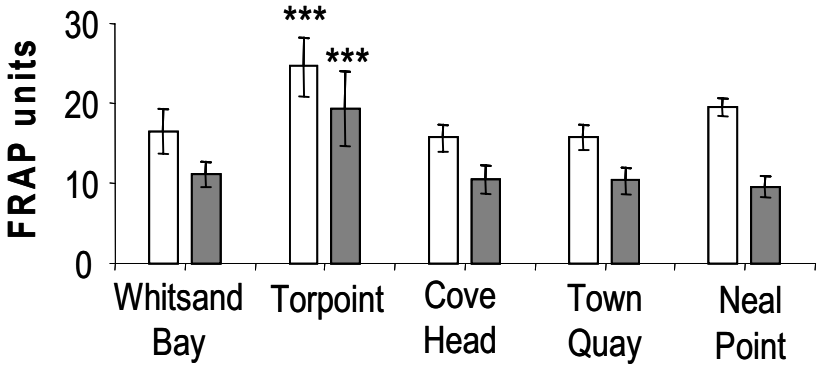


Figure 18 Ferric reducing antioxidant potential in *Mytilus edulis* haemolymph from Plymouth Sound & Estuaries SAC (*** = p<0.001). White bars = winter; grey bars = spring

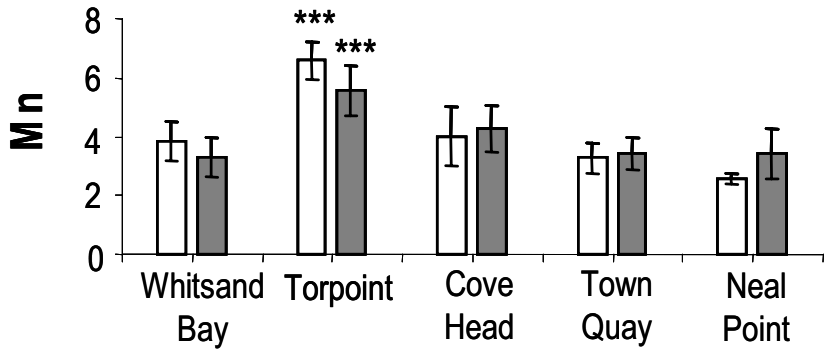


Figure 19 Micronucleus induction in *Mytilus edulis* haemolymph from Plymouth Sound & Estuaries SAC (*** = p<0.001). White bars = winter; grey bars = spring

4.35 At Neal Point, the overall health status of crabs and of mussels was distinct from all other sites. The cardiac rate of crabs (Figure 20) from Neal Point was significantly reduced compared to all other sites ($F_{5,48}$ = 4.20; C = 0.22; p <0.05). Metallothionein induction in mussels at Neal Point and Town Quay was significantly elevated [see also section 4.31 – 4.33] ($F_{3.56}$ = 8.5; C = 0.27; p<0.001)

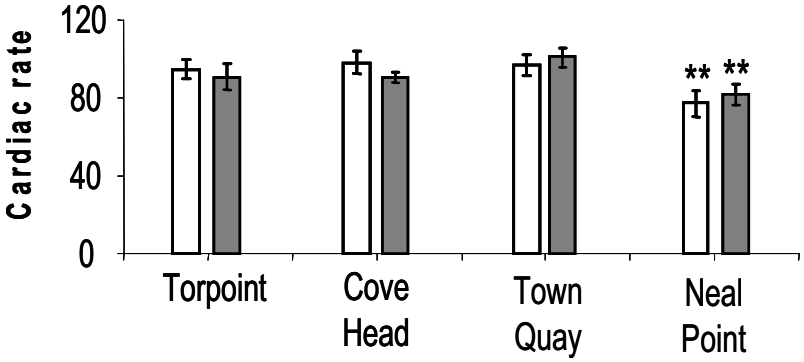


Figure 20 Cardiac rate in *Carcinus maenas* from Plymouth Sound & Estuaries SAC (** = p<0.01). White bars = winter; grey bars = spring

Fal & Helford Estuaries results overview

4.36 Analyses of the data collected from the Fal Estuary indicated distinct patterns in the health of crabs and mussels at Gillian and Weir Point. Pair-wise comparisons using ANOSIM confirmed that the health of these organisms is significantly different at these two sites. Gillian point is considered a clean site and the cardiac rate of crabs from Gillian was significantly lower than all other sites ($F_{5,48}$ = 4.2; C = 0.22; p <0.05) (Figure 21). At Weir Point, levels of metallothionein within mussels were significantly higher at Weir Point compared to all other sites ($F_{6,84}$= 5.2; C = 0.4; p <0.001) and levels were also higher in winter than spring for all sites ($F_{1,84}$= 9.5; C = 0.4; p <0.01).

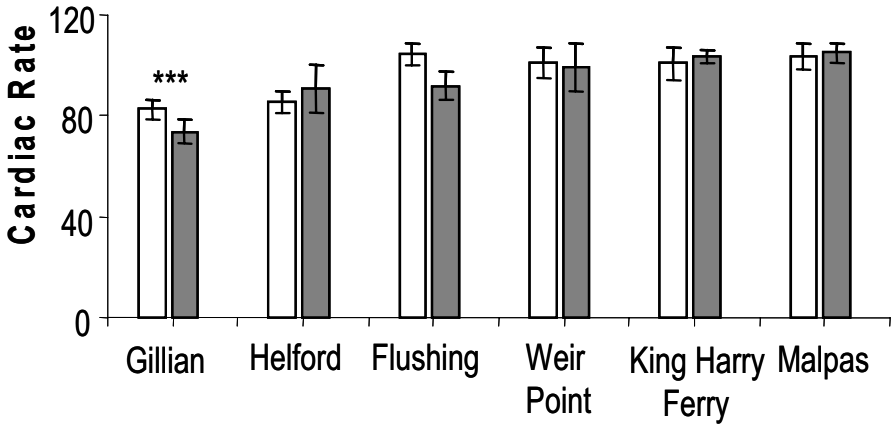
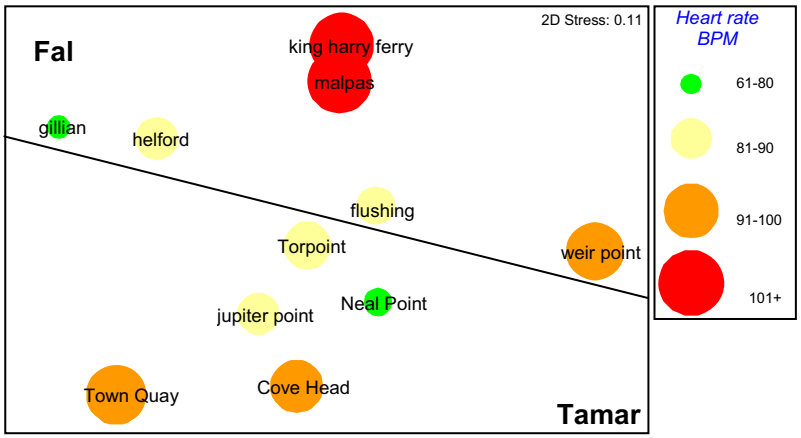


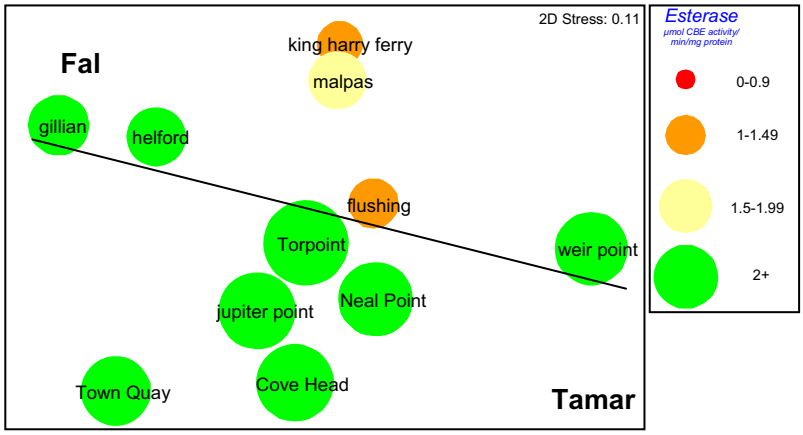
Figure 21 Cardiac rate in *Carcinus maenas* from the Fal & Helford SAC (*** = p<0.05) White = spring; grey = autumn

How can these data be interpreted in terms of organismal health?

4.37 Multidimensional scaling (MDS) is a useful statistical tool that provides a graphical illustration of 'statistical difference'. The further apart the points in the two dimensional MDS graph, the greater their statistical difference. In the MDS plots shown in the next figures (22 and 23), all the biomarker data have been included to produce a 2D plot of the study sites. Then, bubble plots have been superimposed, with the mean value for each biomarker illustrated depicted by the size of the bubble, ie the greater the value, the larger the bubble. From these plots, it is possible to view the distinct patterns of response for each site and animal.

4.38 For example, it is clear from Figure 22 that both the physiological and metabolic condition of the crab population in the upper reaches of the Fal is impacted, and that this coincides with their highest level of exposure to PAH metabolites. Nutrient levels and DO fluctuations are also highest here. In Figure 23, the mussels from the Tamar are distinguishable by their poor physiological condition, higher rate of genetic damage and exposure to metals.





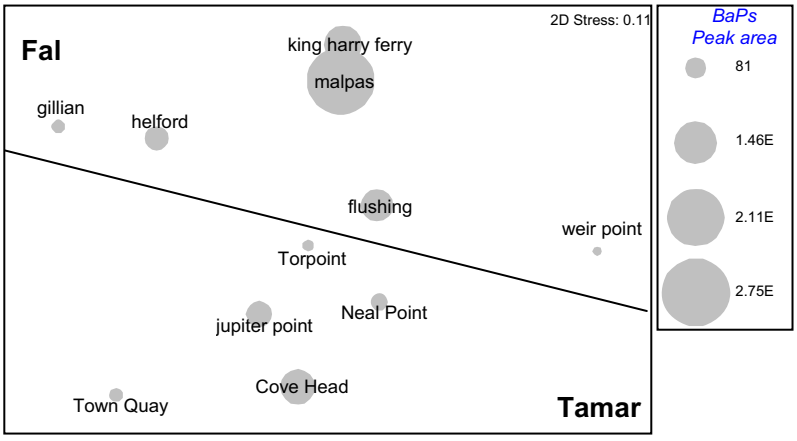
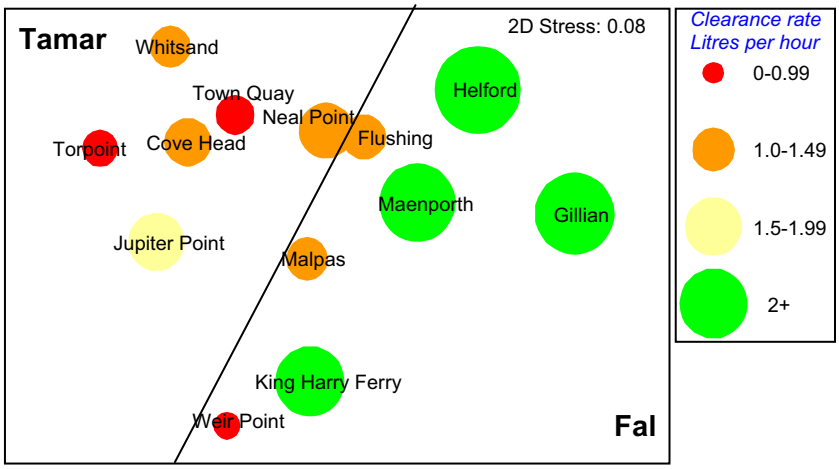
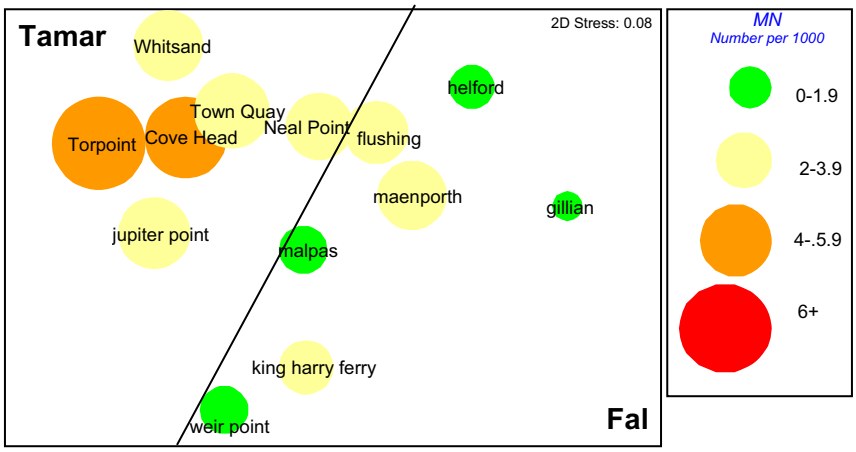


Figure 22 MDS Bubble plots depicting selected biomarker responses in crabs, *C. maenas* from the Fal and Plymouth SAC sites. The colour coding system is described in more detail in the next section, with green representing low risk and red, high risk





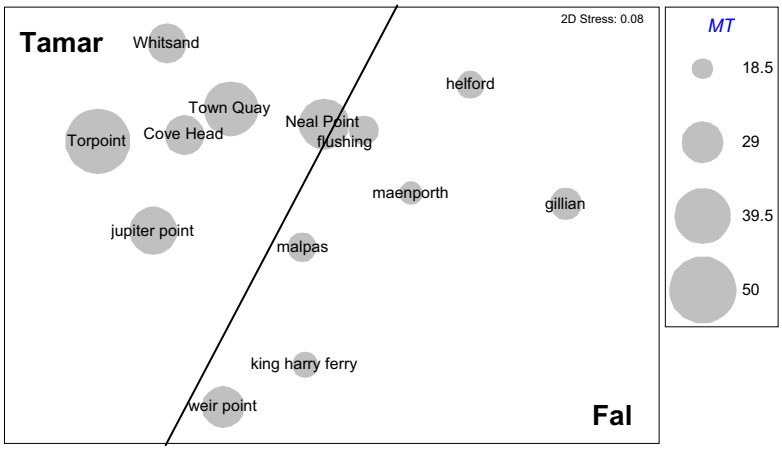


Figure 23 MDS Bubble plots depicting selected biomarker responses in mussels, *M. edulis* from the Fal and Plymouth SAC sites. The colour coding system is described in more detail in the next section, with green representing low risk and red, high risk

5 Discussion

Assessment of the health status of each SAC based on biomarkers

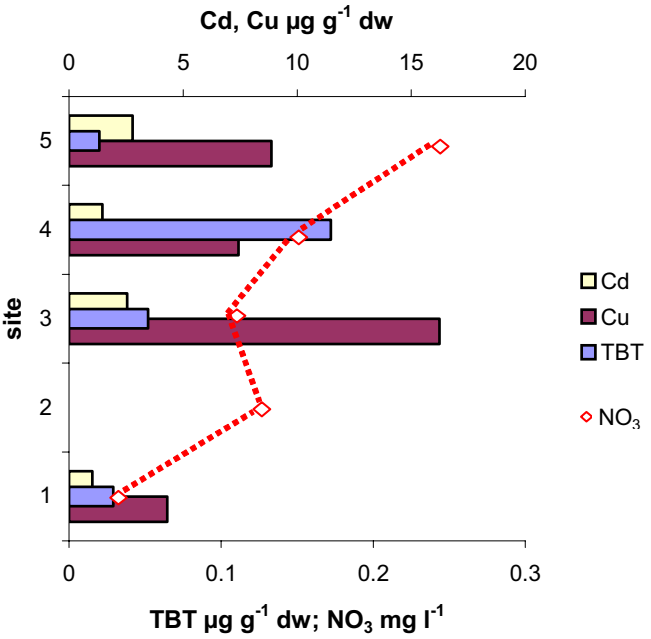
5.1 Biological-effects monitoring suggests that there are sites within each SAC that experience multiple contaminant impacts from PAHs and metals (and possibly, indirectly, from nutrient enrichment). TBT remains a concern, for the integrity of the Fal in particular.

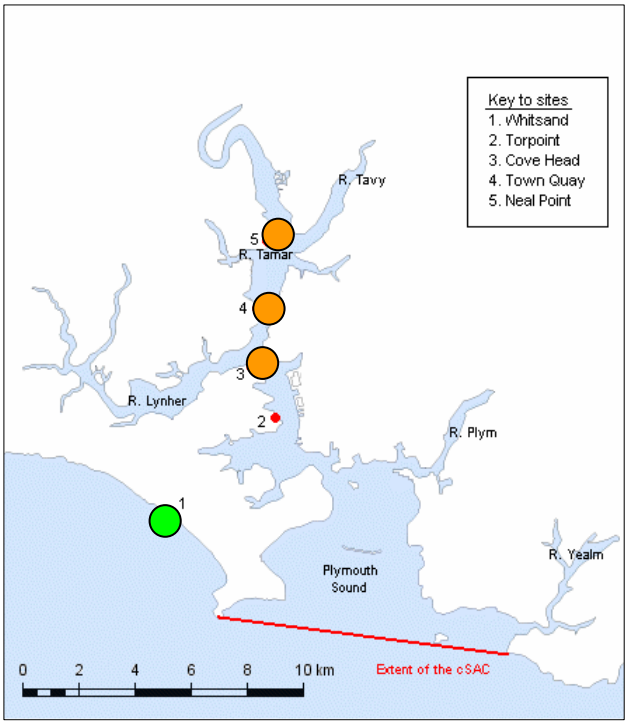
5.2 Apart from mussel body burdens (TBT and metals) information on water quality gradients and other pressures has been derived largely from published literature and the 'site characterisation' reports compiled in 2003. There are a number of caveats over the use of these sources in the context of the current project results, in terms of mis-match of the data, both spatially and temporally. The advantages and recommendations for complementary chemistry, bioassays and biodiversity indices are discussed in Appendix I.

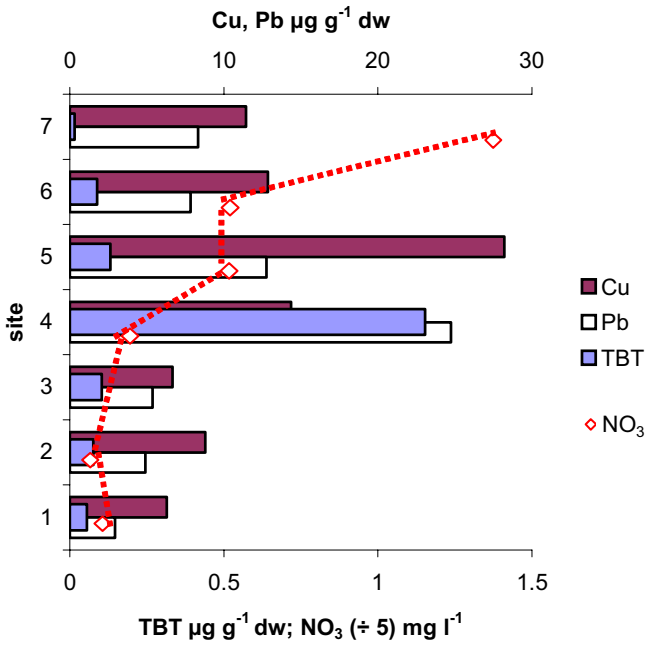
5.3 Despite these caveats, biomarker responses often relate significantly to chemical hotspots, sources and gradients. Previous studies where biomarkers have been carried out in conjunction with chemical analysis have shown gradients of detrimental impacts to biota in coincident with high sediment concentrations of heavy metals, PCBs, PAHs and biocides (Galloway and others 2002; 2004). Although detailed contemporary chemistry was not undertaken in the current study there are a number of instances where biomarkers mirror established water quality gradients (reflecting, for example, Restronguet Creek as a source of metals in the Fal and the reduced water quality in the upper Fal and parts of the Tamar). A case in point is the link between MT and metals in mussels (Figure 24) apparent when viewed alongside selected examples of chemical gradients in the two SACs. These chemical gradients also provide a useful comparison with trends in integrated responses shown in Figure 25.

5.4 Equally important, there is usually consistency (between biomarkers and chemical data) in characterising good water quality/reference conditions (Whitsand and Maenporth in the Plymouth and Fal SACs, respesctively). Overall, therefore, the current project has produced encouraging results which highlight the potential value of biomarkers in the overall scheme of site assessment.

5.5 The application of the BRI classification to the Plymouth and Fal SACs is shown in the maps in Figure 25. The BRI classification system provides an alternative way of viewing the full biomarker dataset, providing an essentially similar picture to the integrated assessment based on multivariate analysis. For the Tamar, mussels and crabs were impacted along a general gradient from Neal Point (highest impact) to Torpoint, possibily mirroring chemical gradients identified from historical data and recent chemistry. For the Fal, the health status of crabs in the upper estuary was impacted, as was the health of mussels from Weir Point (compare with chemical trends summarised in Tables 6 and 8, and examples in Figure 24).







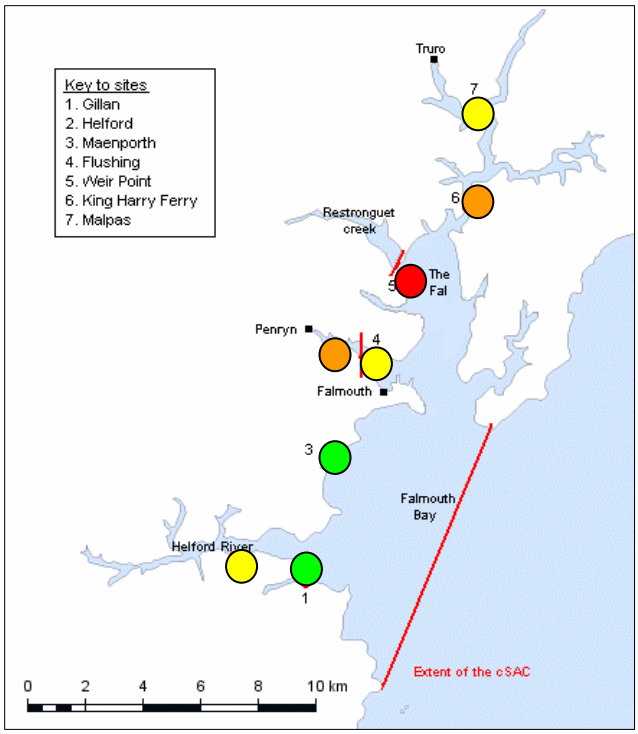


Figure 24 Examples of chemical gradients (left) and MT biomarker response (right) for the Plymouth Sound and Estuaries SAC (top) and Fal and Helford SAC (bottom). Cu, Pb, Cd and TBT are mussel body burden data from the current project. Nitrate values are average dissolved concentrations in tidal waters (see Langston and others 2003a, b)

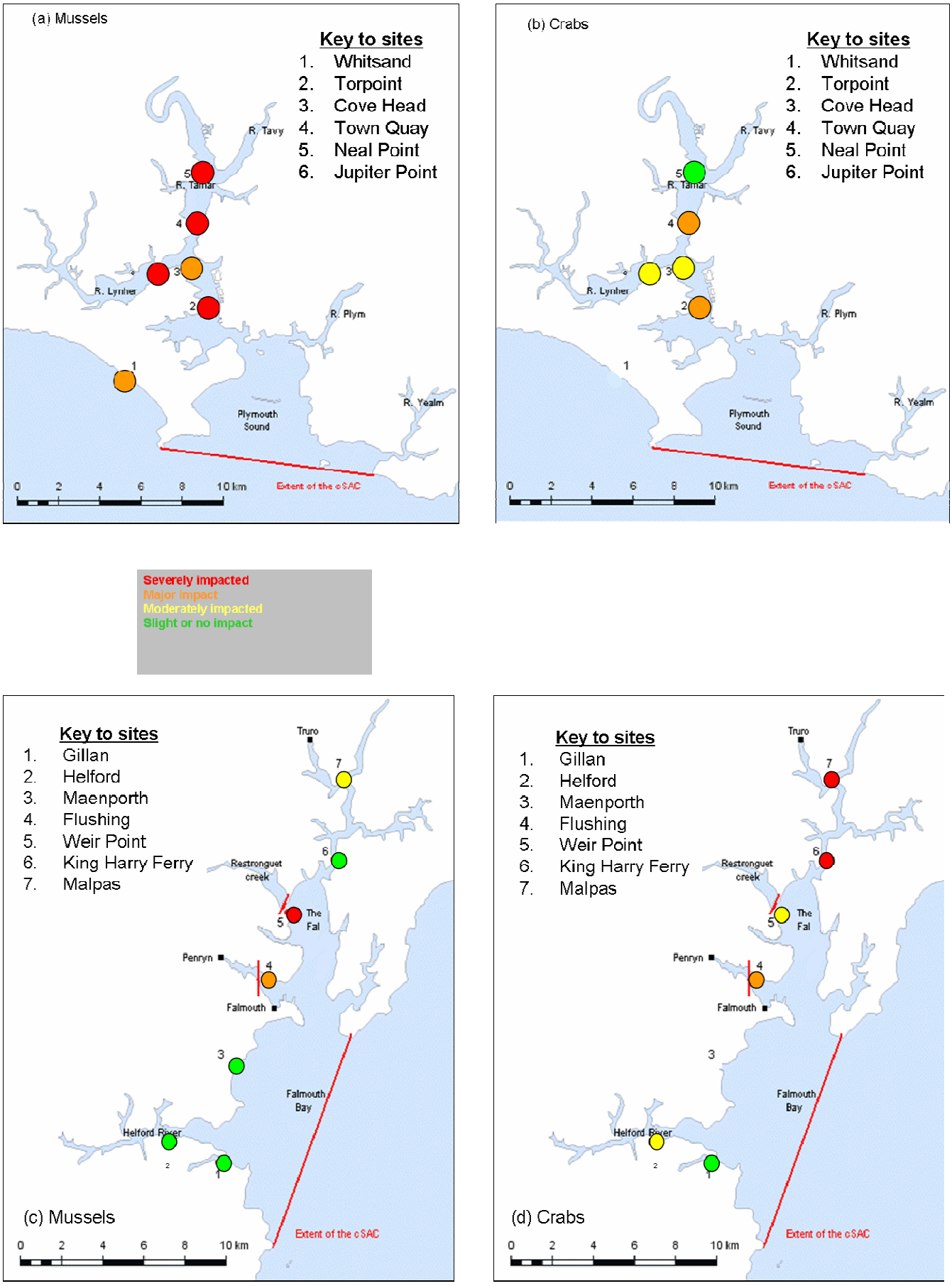


Figure 25 Overall biological status of mussels (a and c) and crabs (b and d) located in the Plymouth Sound & Estuaries SAC and Fal & Helford SAC sampled during spring

5.6 At both the Fal & Helford and the Plymouth Sound & Estuaries SACs there was a difference between the responses of the two species examined. This reflects the different abilities of species to metabolise and excrete chemical compounds. Ideally, as illustrated in Sections 3.1 and 5.3 action step 2, invertebrates with different feeding requirements are sampled to ensure several elements of ecosystem function are covered in the measurements. No single species will provide information to accurately represent all others because each has its own physiology and sensitivity. To be pragmatic, at least two species with different feeding strategies will help fill the void to some extent and highlight the variability between ecological types. Until we have sufficient understanding of whether omnivores are more sensitive than filter feeders, results can only be seen as a guide and trigger for further investigation and a precautionary approach is recommended which bases decisions of impact on the results from the most sensitive species (or tissue).

Assessment of the potential value of using biomarkers in Natural England's routine condition assessment monitoring

5.7 This section provides an overview of examples where biomarker evidence may support (or refute) Natural England's assessments of Favourable Condition. This is based on the Fal & Helford SAC. Tamar Condition Tables were not available. Table 17 indicates where conditions appear favourable (green), appear to be compromised (red) or where there is doubt (orange) (derived from Natural England's condition tables; see Appendix V). Relevant contextual evidence is also added to the table where this helps in the interpretation of cause and effect. A final column also includes suggestions as to how this might help focus management efforts in future.

5.8 It should be remembered that the biomarker results are primarily a representative indication of the biological 'health' status of a given geographical part of the system (and the features and sub-features therein) based on data for a standardised bioindicator organism(s). As such, the results cannot be expected to be an accurate measure of the condition of every species, population or community. Also, it must be emphasised that individual biomarker responses do not give the full assessment of condition and we advocate a multibiomarker approach as part of a broad weight-of-evidence condition assessment (Section 1.17).

5.9 Furthermore, for the Fal & Helford SAC (Appendix V), some of the Features tentatively suggested to be in unfavourable condition [showing as 'red' in the Condition Table (Appendix V)] are subtidal and not covered specifically per se in the current survey. Subtidal sampling or deployment of caged mussels would have been prohibitively expensive given funding levels and the Natural England guidelines of ~£12 K per site. It is felt, however, that the coverage of the SAC afforded by the sampling regime at intertidal locations does encompass much of the total area albeit at course resolution. This may not be adequate to address all features accurately but does at least ensure a rudimentary assessment of threats. Moreover, the data presented in this report place a quantifiable bench mark against which changes can be measured (to assess whether site condition is improving or deteriorating). A summary of the relative costs for applying biomarkers during the current study is shown in Tables 18 and 19.

5.10 There are a number of cases in the current Natural England condition assessment which are based largely on the precautionary approach as there are little available data to assess risk condition; application of the biomarker/bioassay approach would clearly help to firm up this type of assessment.

5.11 For example, current results provide evidence in the following areas of uncertainty:

1) 'Lack of baseline data to demonstrate TBT impacts'. As highlighted in 'Rocky shore communities of large shallow inlet and bays', TBT is the likely cause of the lack of dogwhelks in Fal Estuary component of the SAC and imposex levels remain high at Castle Drive.

Measurement of high TBT levels in mussel flesh from Penryn Creek/Flushing in the present study confirm the threat is considerable here and, although levels decline away from this source, they are still sufficient to prevent recolonisation by sensitive species such as dogwhelks. A precautionary approach in this case is justifiable as is the 'unfavourable' designation at least for features close to the mouth of Carrick Roads and Falmouth Docks. Recently supplied data for sediments in the Helford also suggest localised hotspots near Porth Navas in the Helford at concentrations which would affect benthic molluscs such as clams.

The results would support arguments for a more detailed baseline study of TBT effects near hotspots and better characterisation of trends.

2) The Condition Table indicates problem sites associated with periodic eutrophication around Carrick Roads and parts of the Helford. This is supported by measurements of oxidative stress (TOSC) in mussels, though responses tend to be seasonal (particularly in the Helford). Our biomarker results also highlight that crabs in the upper reaches of the Fal are impacted; this coincides with the highest level of exposure to PAH metabolites, widest DO fluctuations and highest nutrient levels. These results highlight a potential water quality problem for all features and sub-features in the upper Fal and Helford, and that further research is required to investigate cause-effect linkages.
3) Metals have been considered as a possible contribution towards toxic threat to some features (large shallow inlet and bay) and subfeatures (subtidal mud intertidal mudflat and sandflat communities). Metallothionein induction indicates that this threat is probably restricted to the mouth of Restronguet though seasonal increases in response are also evident elsewhere.

5.12 The relative lack of other biomarker responses, and lack of toxicity using the Microtox Bioassay, maps on well with the general good condition of other sections of the SAC [often supporting 'favourable' status in the condition Table 17 (Appendix V)].

5.13 As indicted by the gaps in Table 17, however, not all attributes are addressed by biomarkers in the context of the current project and would require special deployment and better water quality data (eg water clarity issues surrounding dredging and discharges). Others require physical observations: for example, topography and the sediment character of sandbanks. Atlantic Salt meadows are also probably more at threat from physical regimes and would require a more specialised series of biomarker responses than those characterised here in intertidal mussels and crabs.

5.14 Although many caveats are applicable to the current evaluation (mainly due to financial constraints), there is clearly much potential value in using biomarkers in Natural England's routine condition assessment monitoring, to quantify the extent of water quality impacts and changes over time. The ECOMAN philosophy uses an evidence-based approach to assess the effects of contaminant exposures on the general health of estuarine and coastal invertebrates. This assessment is not designed to be definitive, but rather to contribute to the hierarchical process of risk assessment.

Table 17 Favourable Condition Table for the Fal & Helford European marine site: examples where biomarkers provide (or could provide) supplementary evidence to address Natural England's assessment of favourable (green) unfavourable (red) or uncertain (orange) condition. 'Contextual' evidence also added. Note: Only those attributes likely to be affected by water quality issues are included. +++, ++ high or medium priority for further investigations

Attribute/ subfeature	Measure (summary)	Target (summary)	Comments (Natural England)	Biomarker results	Contextual evidence	Further investigation?
Feature: Large shallow inlet and bay						
Nutrient status - phytoplankton concentration	Average phytoplankton concentration in summer measured annually	No significant increase in phytoplankton concentration from the established baseline	Evidence of eutrophication problems with toxic algal blooms in Carrick Roads and outer sections of Helford (Site characterisation report). Site put forward for Sensitive area status and nutrient reductions under AMP 4. Believed to be unfavourable	TOSC in winter survey indicated some suppression in winter in upper Fal, consistent with oxidative stress. In the autumn survey the most suppression in TOSC was observed in the Helford.	Past evidence of high nutrients in upper Fal/Truro. Little recent data, particularly in the Helford but probably still high . Some evidence of larger dissolved oxygen swings in upper Fal.	Priority +++ justification for further investigations (ecological, chemical and biomarkers)
Water clarity	Average light attenuation measured periodically	Average light attenuation should not deviate significantly from the established baseline	Possible localised turbidity associated with Black Rock discharge, dredging activities. No evidence for condition assessment – presumed no changes	Not specifically tested in current project	No baseline data on water clarity.	++ Could deploy caged mussels. Possible forthcoming dredging issue re: Fal Dock

Table continued…

Attribute/ subfeature	Measure (summary)	Target (summary)	Comments (Natural England)	Biomarker results	Contextual evidence	Further investigation?
Feature: Large shallow inlet and bay						
Distribution of characteristic rocky shore communities	Distribution of intertidal rocky shore communities measured using littoral extent	No decrease in littoral extent of range of rocky shore communities from an established baseline value	Baseline established by biotope mapping of whole site. No repeat survey to demonstrate change, but no evidence of substantial change. Presumed favourable although substantial areas impacted by TBT and other contamination. Favourable	Impact of TBT on gastropods extensive in area, focusing on Falmouth. Little indication of recovery of affected neogastropod populations within the Fal. Even Castle Drive populations have high imposex levels	TBT analysis confirms levels still above EQS; mussel body burdens high in Penryn Creek. Sediment also. Localised TBT hotspots in Helford	+++ Need better baselines around entrance to Carrick Roads, particularly in view of possible forthcoming dredging issue for Fal Dock and Penryn Creek
Species composition of low-shore boulder communities	Presence and abundance of composite species from biotope MLR.Fse.Bo.	Should not deviate significantly from baseline	No baseline data to demonstrate change in TBT impacts so unfavourable	Little indication of recovery of affected neogastropod populations within the Fal. Even Castle Drive populations have high imposex levels	TBT analysis confirms levels still above EQS; mussel body burdens high in Penryn	+++ Need better baselines particularly in view of possible forthcoming dredging issue for Fal Dock
Species composition of rockpool communities	Presence and abundance of composite species	Should not deviate from baseline	No baseline data to demonstrate change. Potentially favourable	Not specifically tested in current project		Could deploy biomarkers but likely to reflect adjacent shores

Table continued…

Attribute/ subfeature	Measure (summary)	Target (summary)	Comments (Natural England)	Biomarker results	Contextual evidence	Further investigation?
Kelp forests communities Algal species composition	Presence and abundance of algal species from kelp zone.	Should not deviate from baseline	Baseline data established by ASM surveys. No evidence of change	Not specifically tested in current project		
Characteristic species - Laminaria hyperborea & L. ochroleuca population size	Relative proportions and density of each species in kelp forests at representative series of sites. .	Average ratio of Laminaria hyperborea: L. ochroleuca and density should not deviate from baseline	Baseline data established by ASM surveys. No evidence of change	Not specifically tested in current project		Could deploy algal biomarkers if impacts suspected
Feature: Large shallow inlet and bay						
Characteristic species - Distomus variolosus population size	Average abundance on kelp stipes.	Should not deviate significantly from baseline	No baseline data? potentially favourable?	Not specifically tested in current project		Could deploy biomarkers if impacts suspected
Subtidal rock and boulder communities Species composition	Presence and abundance of composite species from characteristic biotopes.	Should not deviate significantly from an established baseline	ASM report provides baseline –Favourable	Not specifically tested in current project		Could deploy biomarkers if impacts suspected

Table continued…

Attribute/ subfeature	Measure (summary)	Target (summary)	Comments (Natural England)	Biomarker results	Contextual evidence	Further investigation?
Feature: Subtidal sandbanks						
Eelgrass bed communities Characteristic epiphytic species - density of Zostera marina	Average density, measured during peak growth period twice during reporting cycle.	Average density should not deviate significantly from an established baseline	Baseline data provided by monitoring of Black Rock discharge and Helford VMCA monitoring. Some evidence of heavy overgrowth of epiphytic algae in Fal beds. Locally unfavourable	Not specifically tested in current project	Monitoring of nutrients and related parameters required	++ Could deploy more specific biomarkers and selected chemistry to target this issue (also applies to algal mats)
Maerl bed communities Extent of maerl Characteristics of maerl communities	Area (ha) of maerl (live & dead maerl),.	No decrease in extent of maerl	Concern over declines of live maerl, and associated biota potentially through water quality issues (ASM data). Locally unfavourable, declining but extraction now stopped. Also applies to associated communities	Not specifically tested in current project		++ Could deploy biomarkers and selected chemistry to target this issue
Sand/gravel & Mixed sediment communities Species composition	Frequency and occurrence of characteristic species	Presence and abundance of composite species	Likely to be favourable since most seds contaminants are in mud areas.			Could deploy biomarkers to confirm favourable status

Table continued…

Attribute/ subfeature	Measure (summary)	Target (summary)	Comments (Natural England)	Biomarker results	Contextual evidence	Further investigation?
Feature: Intertidal mudflats and sandflats						
Nutrient status - green algal mats	Extent of green algal mats, measured during summer,	Extent of green algal mats should not increase from baseline,	Evidence of significant risk, potentially improved due to AMP 3 changes at Truro, but still risks from diffuse inputs. Potentially favourable, but not meeting management objectives.	Indirect evidence of eutrophication related issues in upper Fal denoted by several biomarkers (egTOSC)	Some evidence of nutrient enrichment and related issues Upper Fal and periodically in Helford	++ Detailed biomarker deployment could assist in apportioning sources
Extent and distribution of characteristic mud communities	Extent and distribution of listed biotopes.	Should not deviate significantly from baseline	Believed to be largely favourable, although concerns over sediment contaminants at above PEL. Locally unfavourable on PEL data.	Biomarker data (MT, TOSC.) indicate area at mouth of Restronguet still impacted by metals: indirect evidence that mud communities are also likely to be affected	Sediments in Restronguet known to elicit effects in bivalves and communities may be modified	+++ Detailed biomarker deployment may help map extent

Table 18 Cost of a selection of biomarkers per hour per species

	Heart rate	Feeding rate	Cellular viability	Phagocytosis	Micronuclei	Protein	Frap	AChE	PAH metabolites	Total hours
Per site (animals = eight)	2	2	5.5	4	4	1	2	2	3	25.5
Per trip (max four sites)	8	8	6.5	5	12	2	3	3	6	53.5
Stats/analysis	0.5	0.5	1	1	0.5	0.5	1	1	1	7
Per SAC (max eight sites)	16	16	12.5	10	24	4	6	6	12	106.5

Table 19 Cost of a selection of biomarkers per hour for two species per SAC

	Heart rate	Feeding rate	Cellular viability	Phagocytosis	Micronuclei	Protein	Frap	AChE	PAH metabolites	Total hours
Per site (animals = eight per species)	4	4	11	8	8	2	4	4	6	51
Per trip (max four sites)	16	16	13	10	24	4	6	6	12	107
Stats/analysis	1	1	2	2	1	1	2	2	2	14
Per SAC (max eight sites)	32	32	25	20	48	8	12	12	24	213

213 hours = approx 27 days for analysis of two species at six to eight sites

Sampling per SAC (six to eight sites) = four days for two species (crabs and mussels)

Total hours per SAC = 31 days

Proposed methodology for routine use of biomarkers which can be taken forward by Natural England on a longer term basis

5.15 We propose an evidence-based approach in which suites of easy-to-use, cost-effective and environmentally valid biomarkers, encompassing a range of sub-lethal endpoints, be evaluated in marine invertebrates that inhabit areas incorporating different designated features. The examples shown during the current study illustrate how this may go some way towards providing what seems an elusive goal, a definition of the health status of SACs and providing supporting evidence for Natural England's Condition Assessment. In the framework described here, contextual information has guided the choice of biomarkers and species, and in some cases, helped to link responses with likely causes providing a practical means of protecting the environment.

5.16 Figure 26 is a simple model which illustrates how a testing regime using a biomarker approach might be assembled for a particular SAC. In this scheme, the identification of sites of concern is based upon existing information relating to specific environmental issues, impacts and pressures (Figure 1). The selection of ecologically relevant species is made bearing in mind the trophic level of the species and their availability at both study and reference sites. Suites of biomarkers, in conjunction with contextual information, are then used to provide a rapid assessment of the extent of contamination and biological degradation. The intention is for this weight-of-evidence approach to be used in conjunction with, not in place of, existing assessment procedures, providing a means of prioritising among sites of concern or to follow the progress of remedial action. A detailed breakdown of each step of the process is shown in Figures 27 to 30 and described below.

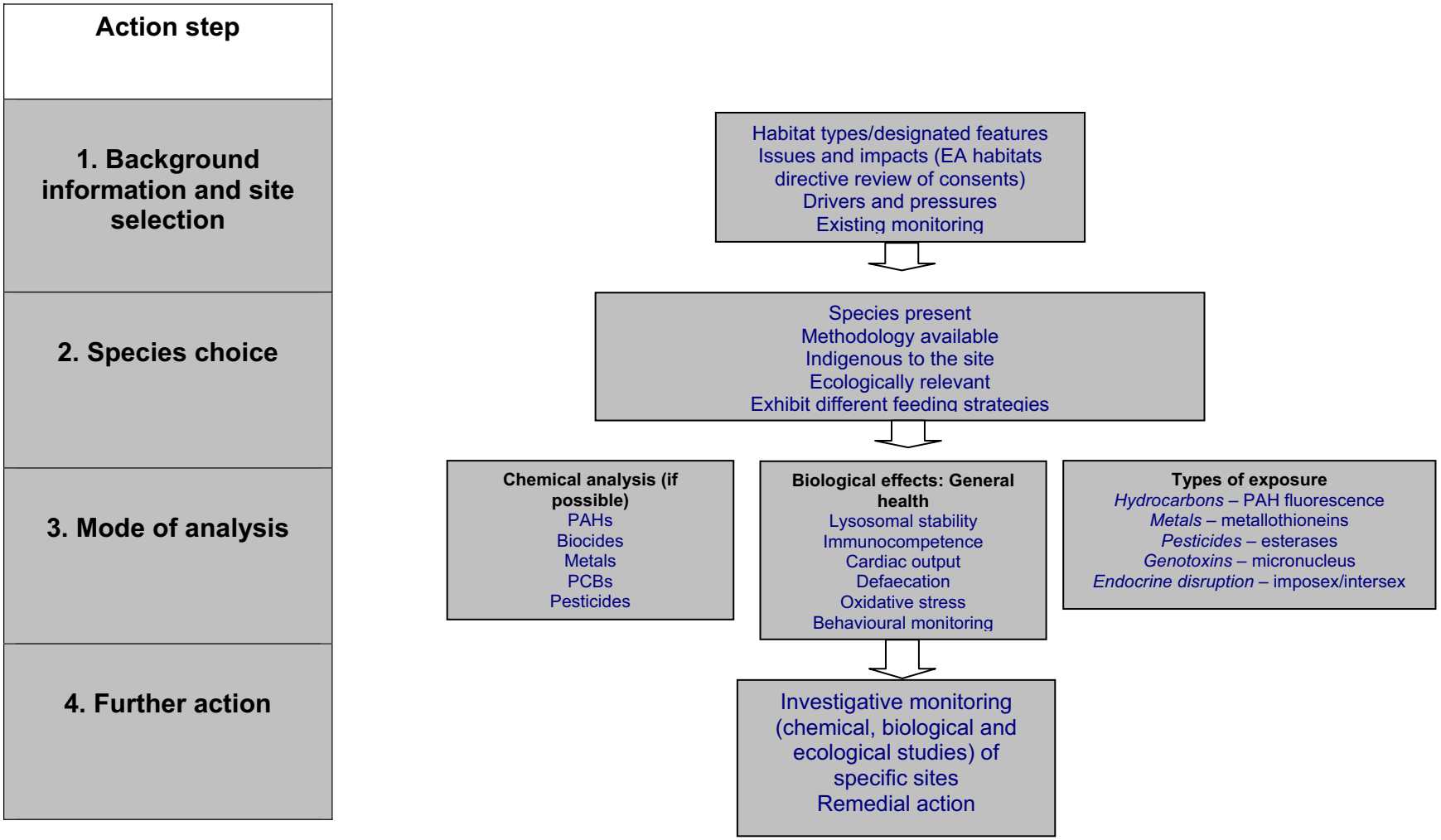


Figure 26 Proposed methodology to assess designated features within a SAC

Action Step 1: Background information and site selection

5.17 This step enables identification of specific sites for potential monitoring and determines whether a biomarker approach is required (Figure 27). For a given location, the main habitats, ecosystems and interest features are identified, together with the main issues and environmental impacts. In addition, the driving forces and pressures that cause specific issues are identified [eg contaminant discharge information (Figure 3), planned developments]. This information is generally available but may need to be adapted for this purpose.

5.18 The outcome of the first step of this process is to prioritise a list of vulnerable habitats or sites within a SAC that require further examination. The next step is to determine current and historical monitoring that has occurred at each site. If the site is (or has been) monitored, then the type, purpose, duration and outcomes of monitoring should be examined and a decision regarding the application of biomarkers is required. If a biomarker approach is to be implemented at a particular SAC site, then the user should progress to Action Step 2.

Action Step 2: Species selection

5.19 This step enables selection of appropriate ecologically relevant species, as described in Section 3.1. The species present at a given designated feature should be established, based on the list presented in Table 1. For each of the species present on this list, a series of questions needs to be asked that determine their relevance. It is important to establish that each species is present at all sites to enable comparability. If species are not present because they have been impaired as a result of a significant impact or stress, this should be noted. Ideally, each species should be indigenous, ecologically relevant and exhibit different feeding strategies. At all steps in this decision-making process, the user should review the relevance of each species chosen using a pragmatic approach. For example, if a species is not indigenous, but is considered ecologically important, it should be reviewed to assess relevance and accepted or rejected accordingly. Finally, a species should only be accepted if there is an appropriate validated biomarker available. If this is the case, the species should be selected for monitoring, as in the present study (see Tables 9 and 10), then the user should proceed to Action step 3 as highlighted in Figure 28.

Action Step 3: Mode of analysis

5.20 This step enables the user to select appropriate biomarkers and chemical techniques for the species of interest (Figure 29), according to the ECOMAN approach described in Section 3.8-3.9.

5.21 Biomarkers of general condition, biological effect and exposure to specific classes of contaminant should be chosen, based on the pressures and impacts affecting the site, if they are known (Action Step 1). In addition, biomarkers should be selected at different levels of biological organisation (molecular, cellular and physiological). The selection of appropriate bioassays (eg ECOBOX or early life stage) and chemical analyses (eg in sediment and biota) should complement this process. After an initial selection process, the biomarkers, bioassays and analytical chemical techniques should be reviewed in their entirety to make sure they are consistent with the ECOMAN approach. In particular, do they examine the health condition of a range of organisms occupying different trophic levels? If this is the case, then the monitoring process (sampling, analysis, data management and reporting) can be undertaken. If not, the selection procedure should be reviewed.

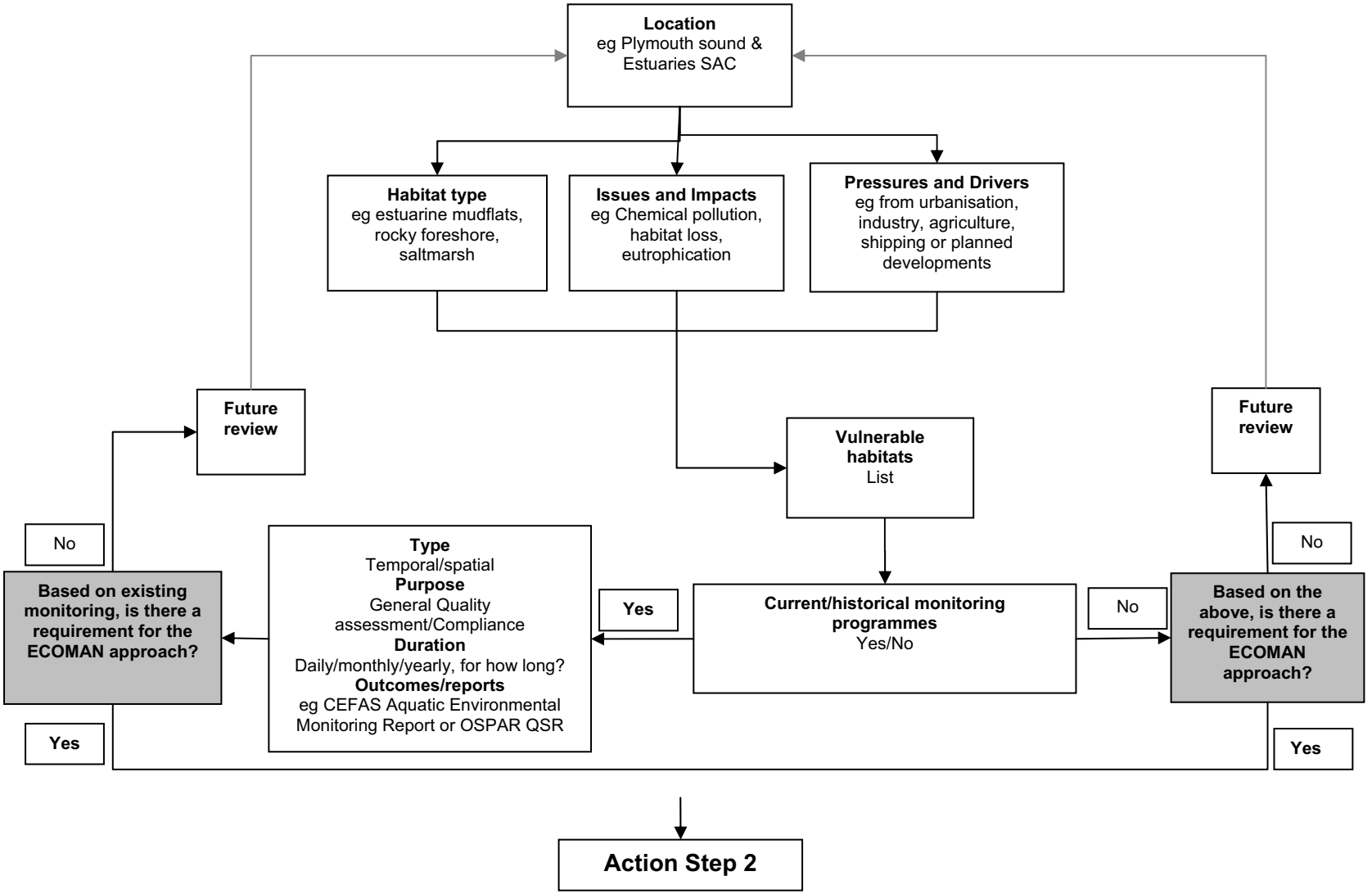


Figure 27 Action step 1: Background information and site selection

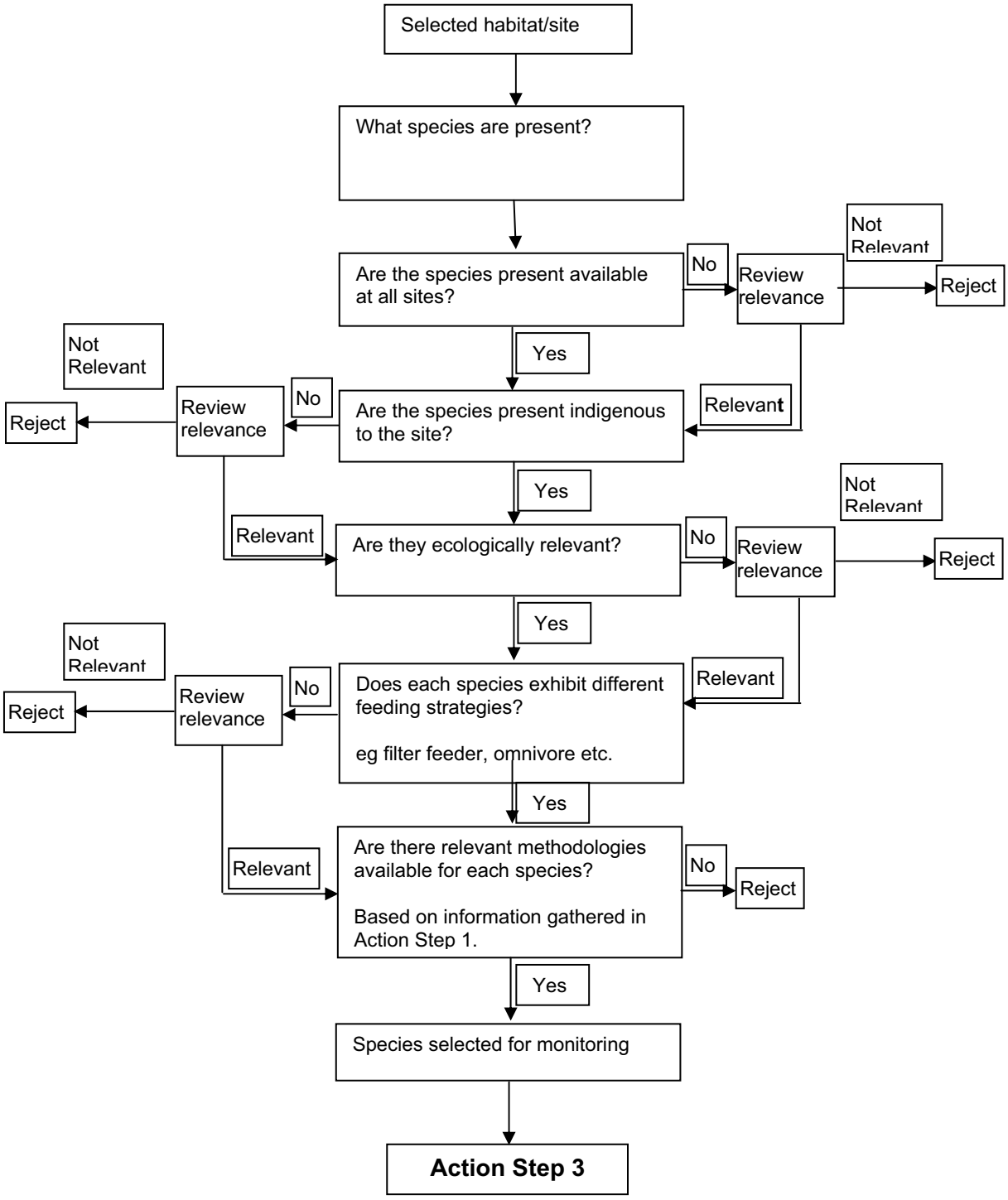


Figure 28 Action step 2: Species selection

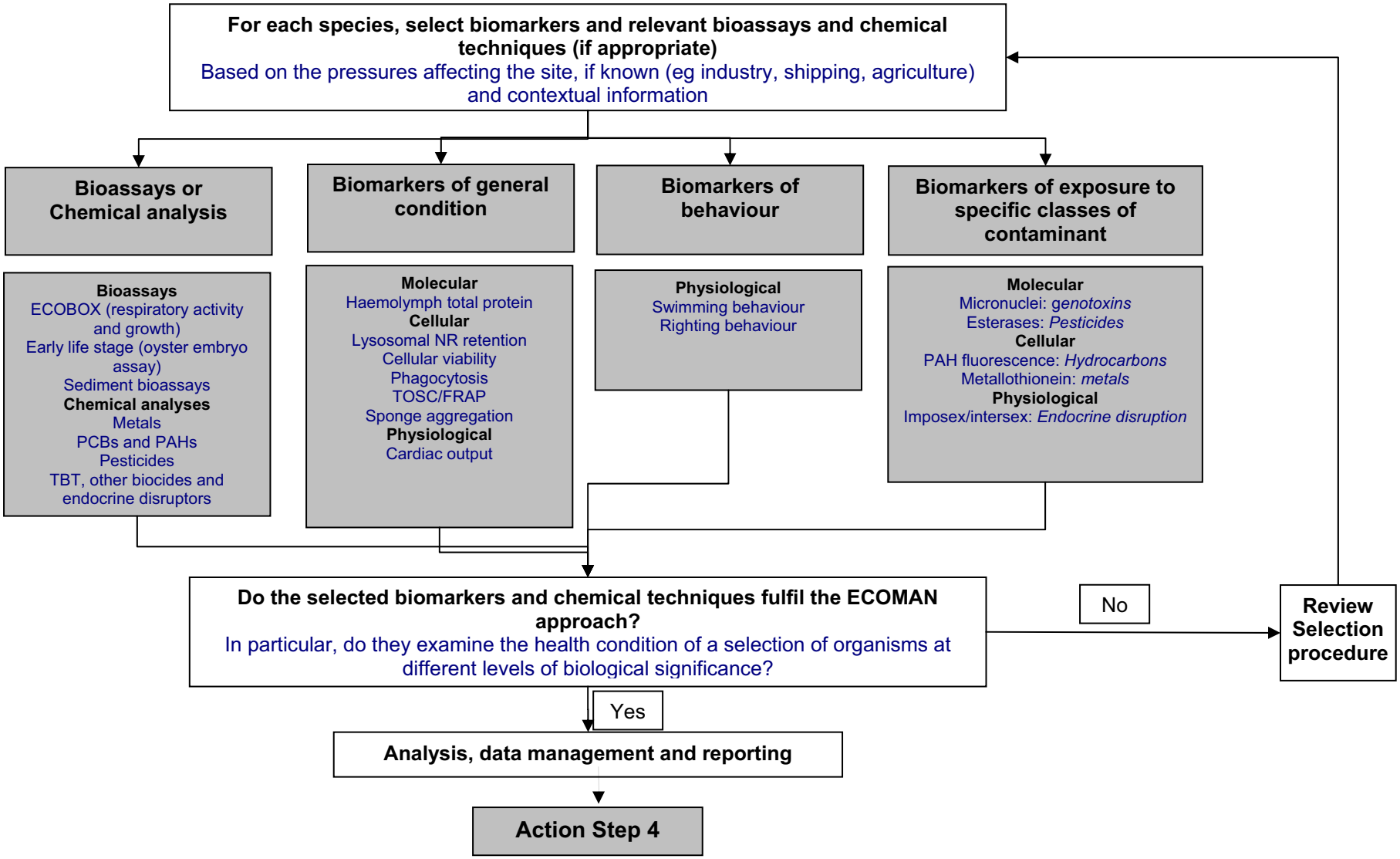


Figure 29 Action step 3: Mode of analysis

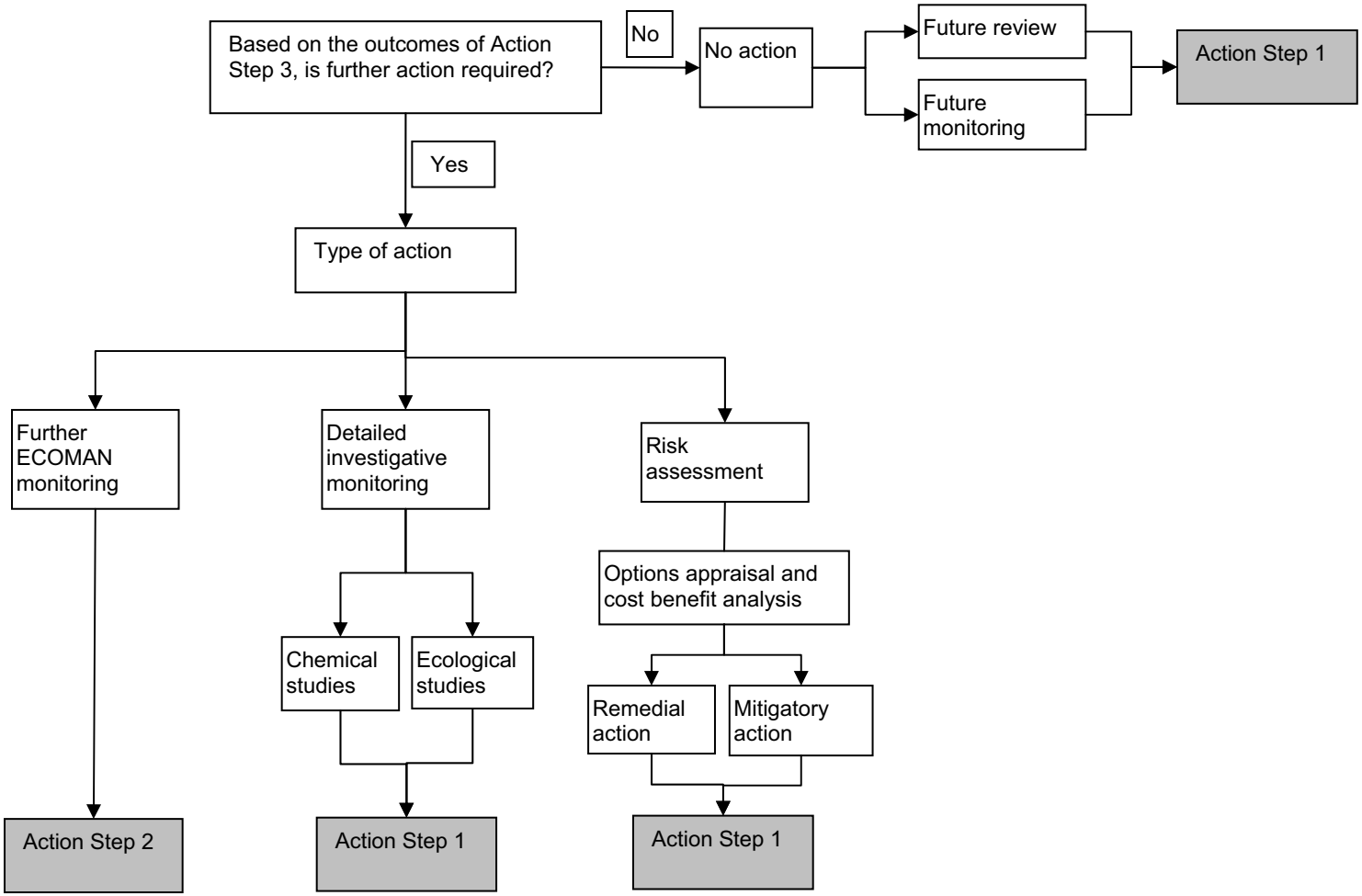


Figure 30 Action step 4: Further action

Action Step 4: Further action

5.22 This step determines what further action is required (Figure 30) and is based on the outputs of the monitoring process. However, the outputs from this approach should not be used in isolation. The intention is for this information to be used in conjunction with, not in place of, existing assessment procedures (refer to Action Step 1). Three types of action are listed:

- Further monitoring could be required (eg seasonal variation may need to be considered or other species occupying different trophic levels may require examination).
- Based on the weight of evidence determined during the assessment step, detailed investigative monitoring (eg detailed chemical analysis or ecological surveys) may be necessary.
- Risk assessment, including options appraisal and cost-benefit analysis may be required. Upon completion of this appropriate remedial action (eg counteracting the effects of pollution) or mitigatory action may be recommended (eg. rectifying an impact by repairing, rehabilitating, or restoring the impacted environment).

5.23 If no action is required, the site under investigation should still be subjected to future review including future reconnaissance monitoring. This would be compatible with the 6-year time frame indicated by Natural England.

5.24 Proposed methodology for routine use of biomarkers might, therefore, be based on the suite of techniques which have been piloted in the current study (see Appendix III for detailed protocols) and could be taken forward by Natural England on a longer term basis to help support their condition assessments for European Marine Sites.

5.25 It is recommended, however, that careful thought be given to the design of the deployment if used at other sites. The final selection of biomarkers should be based on our updated understanding of responses (including seasonal variability) from the current study and also, importantly, in the context of what is known about the perceived water quality issues on the site (ie from site characterisation). This would help to apportion the available resources towards the most important priorities. For similar reasons, it would also be beneficial to ensure the sampling programme at any given site is constructed after consultation of Natural England's condition tables so that biological effects monitoring may address the major uncertainties. To maximise benefits, efforts should be made to provide appropriate contemporary information on water and sediment quality at the selected sites.

5.26 Based on the BRI approach, we would suggest that sites classified as 'green' (no to slight alteration from baseline responses) and 'yellow' (moderate alteration) could be monitored on a 6-year cycle; whereas those classified as 'orange' (major alteration) and 'red' (severe alteration) will require immediate remedial action and more frequent visits to monitor recovery. Sites of concern identified in the present study requiring immediate investigation are Flushing, Weir Point, King Harry Ferry and Malpas for the Fal & Helford SAC; and all sites within the Plymouth Sound & Estuaries SAC. These sites correspond to sites of known point-source pollution as well as historically contaminated locations as initially highlighted in the contextual information, thus emphasising the value of using biological endpoints to confirm and validate current risk assessment of designated interest features.

6 Conclusions

6.1 A suite of biomarkers, incorporating molecular, biochemical, cellular and physiological endpoints, has been applied to two common invertebrates (*Mytilus edulis* and *Carcinus maenas*) to inform the condition of two European Marine sites (Fal & Helford SAC and Plymouth Sound & Estuaries SAC).

6.2 Results were very encouraging and showed how an integrated biomarker approach identifies spatial patterns of sub-lethal responses in relation to water quality trends. This approach offers Natural England a rapid, cost-effective methodology of biological effects-based sampling to inform condition assessment of European Marine sites. Some individual techniques provide useful diagnostic information on the activity of certain classes of contaminants (eg PAH metabolites, metallothionein). However, it must be emphasised that biomarkers are not designed to be used in isolation to assess environmental condition, but rather to form part of a weight-of-evidence approach in an integrated risk assessment. More robust cause-effect relationships might have been achieved with better supporting water and sediment chemistry, and other higher-order biological measurements (biodiversity indices, community structure).

6.3 To further aid Natural England's ability to inform Government on the condition assessment of these European Marine sites, we propose the use of an integrated Biomarker Response Index (BRI). The method of establishing this Index is outlined in detail and, although the confidence for the thresholds of cut off for the different categories of health status will be improved by further field application, the potential value of the BRI is clear for the data collected at the two SACs studied here.

6.4 On current evidence, the strategy adopted here could be deployed on a wider basis to compare the status of Marine European sites and to inform the monitoring needs at individual sites on a cyclic basis, as envisaged in Natural England's original specification. For example, sites classified as 'green' (no or slight alteration from normal response) and 'yellow' (moderate alteration) could be monitored on a six-year cycle; however, those showing as 'orange' (major alteration) and 'red' (severe alteration) may require additional investigative and remedial action and more frequent visits to monitor recovery.

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Appendix 1 - Recommendations to help rectify identified limitations

To help inform condition assessment and ensure a greater level of environmental protection it is important to integrate biological effects-type monitoring with information on chemistry and other pressures. Ideally, contemporary water/sediment chemistry analysis should also be undertaken in tandem with higher level responses. Ideally it would be good to include a wider array of bioassay studies, together with ecological assessments based on quantitative sampling of benthic invertebrate communities.

In particular:

Analytical chemistry

At the sites studied here, there is a lack of appropriate contemporary chemical measurements in sediments, tissues (and water) for metals, TBT, PAHs, and nutrients including, where possible, relevant diagnostic chemistry (eg dissolved vs particulate forms; defining bioavailable biochemically- relevant fractions in sediments; in situ measurements of nutrients). Sampling should co-incide as closely as possible with the 'biological effects' sites, both spatially and temporally, if we are establish cause and effects and prioritise which of the pressures, if any, may be affecting the condition of the site and needs remediatory action. The existing chemical information is often of insufficient extent or resolution to attempt such specific interpretation.

At other European Marine Sites different determinants may be important and priorities should be guided by previous 'site characterisation'. Careful consideration will need to be given to the coverage of interest features and whether known anthropogenic sources need to be targeted.

Biodiversity indices

Estuarine/marine benthos should be sampled (quantitatively) to co-incide as closely as possible with the 'biological effects' sites and to examine their links.

At each site, a minimum of four samples should be taken for the analysis of the community structure of the macrofauna. These samples should be dispersed so that their location matches as closely as possible that of samples taken for biomarkers. For soft substrates, sediment samples should be sieved over a 0.5mm mesh. The sieve residue is fixed in formalin for a minimum period of a week before being washed in fresh water, searched under a binocular microscope and the biota extracted, identified to the lowest practical taxon and counted. If any taxa are particularly abundant sub-sampling may be used in their quantification.

Patterns in the biota should be investigated and compared to patterns of biomarkers (and contaminants) using multivariate techniques.

Additional bioassays

A number of bioassays are available for rapid screening of sediment toxicity, including cast production and survival in *Arenicola marina* and burrowing responses in *Corophium volutator*. These would be useful to trial alongside biomarkers and higher order biodiversity indices.

The threat of endocrine disruption (other than TBT) is largely unknown in many European Marine Sites. In additional to previous techniques requiring the sampling of flounder, it is possible to screen populations of clams *Scrobicularia plana* for evidence of oestrogenic effects. This could be used to trigger further investigation.

Appendix 2 - TBT and metals in mussels

To assist in the interpretation of biomarker data, chemical analysis has been performed on mussels (body burdens minus gills and digestive glands) for two key groups of toxic contaminants - TBT and metals - using methods described elsewhere (Langston and Pope 1995 and Langston and others 2002). Results of organotin analysis (TBT and DBT) are shown in the following table.

Table A TBT and DBT concentrations (µg/g Sn dw) in *Mytilus edulis*

Site	Date	µg/g Sn TBT	µg/g Sn DBT	Total	%TBT
Town Quay	31/01/2006	0.172	0.120	0.292	58.8%
Neal Point	31/01/2006	0.020	0.072	0.092	22.0%
Freathy	14/02/2006	0.029	0.020	0.049	58.8%
Cove Head	31/01/2006	0.052	0.063	0.116	45.4%
Helford (Treath)	14/03/2006	0.076	0.031	0.107	71.1%
Gillian	14/03/2006	0.055	0.055	0.111	50.0%
Maenporth	14/03/2006	0.104	0.100	0.204	51.0%
Falmouth Marina	14/03/2006	1.070	0.282	1.353	79.1%
Malpas	28/03/2006	0.152	0.115	0.268	56.9%
King Harry Ferry	28/03/2006	0.088	0.094	0.182	48.4%
Weir Point	28/03/2006	0.131	0.154	0.285	46.0%
Flushing	28/03/2006	1.153	0.391	1.545	74.7%

Key points with regards to TBT are the enhanced bioavailability of TBT in Penryn Creek (Flushing and Falmouth Marina), presumably arising from a combination of inputs from Falmouth Docks, sediments and possibly some illegal usage on leisure craft. The high proportion of TBT suggests some inputs may be recent. Elsewhere, TBT concentrations in both systems appear to be an order of magnitude lower. Lowest concentrations were those in mussels from Whitsand Bay (Freathy) and Neal Point in the Tamar. Within the latter system, highest body burdens were at Town Quay. As a whole, however, sites near Falmouth are distinctively contaminated with TBT.

Results of metal analysis in mussels from Plymouth Sound & Estuaries SAC and the Fal & Helford SAC are presented in the following table.

In the Plymouth Sound and Estuaries samples, burdens of most metals in mussels were, for the most part, highest in the Hamoaze and upstream, compared with the reference site at Freathy, Whitsand Bay (with the notable exception of Zn). This trend is particularly obvious for Cd (bioavailability in the Tamar generally higher than in the Fal & Helford). At Cove Head, concentrations of Fe, Mn, Co, Cr, Ni exhibited a pronounced peak, probably indicative of particulate contaminants.

Cu and Zn bioavailability was generally highest in the Fal, notably at Weir Point (reflecting inputs from Restronguet Creek). The latter site also appears to be a regional hotspot for bioaccumulation of Cd, Co and Ni. It should be noted however that competition for uptake from Cu and Zn (present at higher levels) appears to suppress bioaccumulation of Cd in the Fal (Langston and others 2003a).

Table B Metal concentrations (µg/g dw) in *Mytilus edulis*

Sample	Date	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Town Quay	31/01/2006	1.457 ± 0.015	0.86 ± 0.13	1.83 ± 0.56	7.79 ± 0.36	396.76 ± 31.08	0.10 ± 0.00	12.35 ± 0.38	1.64 ± 1.04	13.00 ± 0.85	108.87 ± 6.54
Neal Point	31/01/2006	2.744 ± 0.071	1.30 ± 0.09	1.30 ± 0.13	9.91 ± 1.24	383.37 ± 56.96	0.27 ± 0.21	12.20 ± 0.76	2.99 ± 1.74	11.05 ± 1.10	127.10 ± 2.35
Freathy	14/02/2006	1.129 ± 0.109	0.67 ± 0.07	3.33 ± 2.17	4.90 ± 0.88	520.10 ± 54.24	0.17 ± 0.01	13.20 ± 0.24	1.30 ± 0.34	11.63 ± 1.16	326.50 ± 23.21
Cove Head	31/01/2006	2.756 ± 0.278	2.14 ± 0.30	8.16 ± 6.23	16.12 ± 0.59	1,381.92 ± 70.93	0.20 ± 0.05	110.24 ± 2.84	5.35 ± 1.00	15.38 ± 0.14	171.82 ± 14.13
Helford (Treath)	14/03/2006	0.578 ± 0.040	0.22 ± 0.03	2.21 ± 0.35	8.45 ± 0.43	277.31 ± 19.56	0.14 ± 0.01	9.07 ± 0.63	0.93 ± 0.75	4.48 ± 0.45	131.18 ± 2.19
Gillan	14/03/2006	0.803 ± 0.088	0.29 ± 0.05	5.17 ± 4.31	5.87 ± 0.50	353.11 ± 75.59	0.35 ± 0.01	6.87 ± 1.22	1.59 ± 0.59	2.86 ± 0.08	128.61 ± 12.18
Maenporth	14/03/2006	0.633 ± 0.093	0.22 ± 0.07	1.77 ± 0.19	8.46 ± 1.55	360.63 ± 97.82	0.32 ± 0.18	10.87 ± 3.45	1.02 ± 0.60	6.86 ± 1.33	156.36 ± 23.78
Falmouth Marina	14/03/2006	0.575 ± 0.054	0.18 ± 0.02	1.19 ± 0.31	16.74 ± 0.55	232.65 ± 31.15	0.27 ± 0.01	6.35 ± 0.41	1.11 ± 0.42	23.13 ± 1.82	210.17 ± 18.46
Malpas	28/03/2006	0.642 ± 0.006	0.22 ± 0.03	1.73 ± 0.83	11.49 ± 0.23	325.79 ± 60.30	0.15 ± 0.03	6.79 ± 1.89	0.59 ± 0.20	9.12 ± 1.00	151.29 ± 11.78
King Harry Ferry	28/03/2006	0.720 ± 0.014	0.46 ± 0.10	1.04 ± 0.08	13.01 ± 1.45	260.03 ± 22.13	0.20 ± 0.00	10.03 ± 0.32	1.59 ± 0.64	8.11 ± 1.29	175.29 ± 15.67
Weir Point	28/03/2006	1.152 ± 0.152	1.56 ± 0.45	4.34 ± 5.01	31.47 ± 3.43	264.33 ± 29.53	0.32 ± 0.02	11.41 ± 4.10	2.69 ± 2.49	14.91 ± 2.44	469.42 ± 67.83
Flushing	28/03/2006	0.613 ± 0.031	0.41 ± 0.02	1.47 ± 0.15	13.67 ± 0.59	234.68 ± 26.03	0.46 ± 0.01	7.52 ± 0.74	2.52 ± 2.68	22.97 ± 1.54	287.40 ± 19.69

Appendix 3 - Procedural guidelines

Haemolymph extraction

Background/purpose

The haemolymph of bivalve molluscs such as *Mytilus edulis* is relatively easy to withdraw without causing irreparable damage to the organism. Haemolymph extraction from the shore crab *Carcinus maenas* is also relatively simple and non-destructive. Numerous assays can be conducted on a haemolymph sample allowing for the application of a suite of cellular and molecular biomarkers on a single organism without the need to sacrifice the individual.

Methods

Equipment

- 21 gauge needles;
- 1 ml syringes;
- siliconised 1.5 ml eppendorfs; and
- scalpel.

Personnel
One person experienced in haemolymph extraction on the selected species.

Time
To extract haemolymph from 8 individuals requires approximately 0.5 hours.

Protocol
Mussel haemolymph extraction (Figure A):

1) Carefully prise apart the shell valves by the insertion of a fixed scalpel blade, once open keep the blade in position to maintain the opened valves.
2) Drain out seawater using absorbent paper towelling.
3) Using a 1 ml syringe, fitted with a 21 gauge needle, locate the posterior adductor muscle; insert the needle towards the posterior end of the shell close to the valve opening and draw the needle down horizontally until it meets resistance by touching the muscle.
4) Withdraw the needle slightly and then push gently into the muscle and slowly extract the haemolymph.
5) Having obtained the haemolymph sample, ensuring the needle is removed (to prevent the cells from bursting), transfer the syringe contents to a siliconised eppendorf on ice.

Figure A Haemolymph extraction from the bivalve mollusc, *Mytilus edulis*. The red circle indicates the position of the adductor muscle

Crab haemolymph extraction:

1) Carefully place the crab dorsal side down and restrain by holding down gently with absorbent paper towelling.
2) Insert the 21 gauge needle into the haemocoel, through the arthodial membrane at the base of the third walking leg (Figure B) and slowly withdraw the haemolymph.
3) Remove the needle from the syringe and transfer the extracted haemolymph to a siliconised eppendorf on ice.

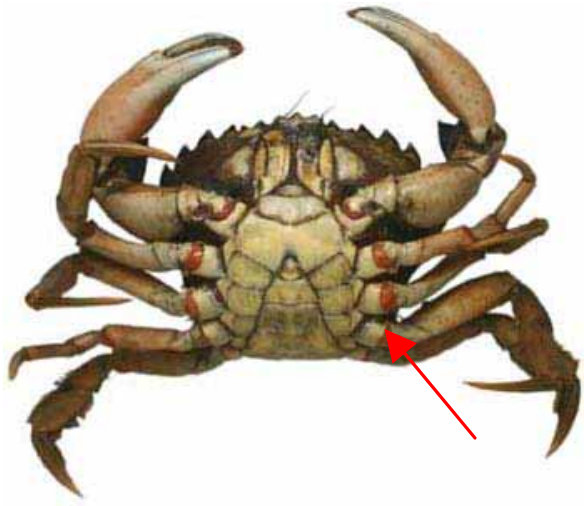

Figure B Ventral view of the common shore crab *Carcinus maenas*. The red arrow indicates the arthodial membrane at the base of the third walking leg, through which the needle is inserted for haemolymph extraction

Health and safety
Take care when using scalpels and needles. Dispose of used sharps appropriately. Wear gloves when handling organisms, especially if organisms originate from a contaminated or unknown field site, or have been used in a laboratory exposure.

Cellular viability (neutral red method)

(Babich & Borenfreund 1992)

Background / purpose

Cell membranes are essential in maintaining the integrity of a cell. The main lysosomal response to a range of pollutants involves alteration in lysosomal membrane stability (Domouhtsidou and others 2004). The cell viability assay therefore provides a sensitive indicator of the general condition of the organism. Neutral Red (NR) is a cationic dye that rapidly passes across the cell membrane through non-ionic diffusion and binds to anionic sites in the lysosomal matrix, accumulating in the lysosomal compartment (Pipe and others 1995). Alterations in the lysosomal membrane result in a reduced uptake and binding of NR, and therefore optical density of NR can be used to give an indication of cell viability (Babich & Borenfreund 1992).

NR cellular viability is a biomarker that has been successfully applied across species with different functional feeding groups, suggesting that membrane stability is one of the first parameters to be disrupted by contaminant exposure reflecting both direct (contaminants that have been taken up by the cells) and indirect contaminant toxicity (Brown and others 2004). NR cellular viability in *M. edulis* has been used to demonstrate a concentration dependent decrease in lysosomal viability following 96hr exposure to the organophosphorous insecticide Chlorfenvinphos at concentrations ranging from 0.007-0.03 ppm (Rickwood & Galloway 2004). Dose-dependent reductions in NR cellular viability have also been reported in Mytilus edulis exposed to cadmium (Coles and others 1995) and pyrene (Okay and others 2006), and in the earthworm Lumbricus rubellus exposed to copper (Weeks & Svendsen 1996). Field studies involving NR cellular viability in *Mytilus spp*. have successfully used this biomarker in Norway (Camus and others 2000) and Southampton waters (Galloway and others 2004), as well as in highly contaminated regions including Venice Lagoon (Lowe and others 1995).

Methods

Equipment

- pipettes and tips (suitable for 5, 50, 100 µl);
- siliconised 1.5 ml eppendorfs;
- mylar plate sealer;
- flat bottom microtitreplate; and
- multi-well plate reader.

Chemicals

- neutral red stock solution (1 ml DMSO, 28.8 mg neutral red CAS no. 553-24-2);
- molluscan physiological saline (0.02 M HEPES, 0.4 M $NaCl_2$, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$; pH 7.4) OR Phosphate Buffered Saline (0.01 M, 1 PBS tablet in 200 ml distilled water) for analyses on crab haemolymph;
- acidified ethanol (1 % glacial acetic acid, 50 % analytical grade ethanol);
- BSA (Bovine Serum Albumin) protein standard (CAS no. 9048-46-8); and
- Bio-Rad reagent (Bio-Rad laboratories GmbH).

Personnel

- One person experienced in the neutral red retention microtitreplate assay.

Time

- To perform this assay on 8 samples (in duplicate) requires approximately 5.5 hours (this includes haemolymph extraction) + 1 hour for protein determination.

Protocol

1) Pipette 200 µl haemolymph in triplicate into flat-bottom microtitreplates pre-treated with poly-L-lysine.
2) Cover with Mylar plate sealer and incubate at room temperature for 45 minutes to allow cells to adhere to the wells.
3) Following this period, discard non-adhered cells through plate inversion onto absorbent paper towelling.
4) Add aliquots of 200 µl neutral red working solution (0.2% neutral red stock solution in physiological saline) to each well and incubate at room temperature for 3 hours.
5) After the incubation period tip away excess NR through plate inversion and carefully wash the wells several times (x 3) with physiological saline.
6) Add 200 µl of acidified ethanol (1% acetic acid, 50% ethanol) to breakdown to cellular membrane and resolubilise the NR dye.
7) Measure the optical density of NR spectrophotometrically at 540 nm.

Standardise NRR as a function of protein content.

Protein concentrations are determined using a modified microtitreplate method of Bradford (1976).

1) Pipette 5 µl blanks (physiological saline) and 5 µl protein standards (0-1 mg ml-1 Bovine Serum Albumin) in duplicate into empty wells on the NR microtitreplate.
2) Add 200 µl diluted Bio-Rad reagent (1:5 in distilled water) to the blanks, standards and each well used for the neutral red assay.
3) Following incubation at 25oC for 20 minutes, read the absorbance of the samples at 595 nm.

Health and safety

Wear gloves when handling NR (in both the original powder form and the stock/working solutions). Do not inhale/ingest NR. Dispose of waste NR solution via appropriate waste collection services.

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Phagocytosis

(Pipe and others 1995)

Background / purpose

Animals have developed an internal defence system capable of eliminating potential pathogens. Invertebrates rely on innate immunity (Livingstone and others 2000), of which one of the most common non-specific defence mechanisms is phagocytosis (Pipe & Pulsford 1995). This mechanism is the cellular ingestion of particulate matter by either nodulation (response to small particles) or encapsulation (response to larger foreign material) and involves the 5 stages of recognition, chemotaxis, attachment, ingestion and destruction (Pipe & Coles 1995; Pipe and others 1995). The phagocytic capability of bivalve molluscs can be measured directly though quantification of the uptake of particles into haemocytes (Pipe & Coles 1995). Zymosan particles are consistently uniform in size and are readily ingested by bivalve haemocytes (Pipe and others 1995).

The ingestion of stained zymosan particles assessed spectrophotometrically has been widely applied as a biomarker of immune function. Laboratory studies indicate a concentration-dependent phagocytic response in bivalves following exposure to cadmium (Coles and others 1995), zinc and mercury (Sauvé and others 2002), and chlorfenvinphos (Rickwood & Galloway 2004) whereby phagocytosis is enhanced at low exposure levels but inhibited at higher concentrations. Such dose-responses suggest that there is a phagocytosis range above and below which phagocytic activity is either stimulated or inhibited as a result of contaminant exposure. Field studies have successfully applied this immunological biomarker, with phagocytic activity in *M. galloprovincialis* from the Bay of Biscay reduced in contaminated sites compared to a non-polluted reference site (Cajaraville and others 1996). Reduced uptake of zymosan particles in natural populations of *M. edulis* has also been observed following the 1996 Sea Empress oil spill indicating modulations in cell mediated immunity after exposure to PAHs (Dyrynda and others 1997).

Methods

Equipment

- pipettes and tips (suitable for 5, 50, 100 µl);
- spatula;
- balance;
- water bath;
- haemocytometer;
- microscope;
- siliconised 1.5 ml eppendorfs;
- flat bottom microtitreplate;
- mylar plate sealer;
- centrifuge; and
- multi-well plate reader.

Chemicals

- zymosan particles, *Saccharomyces cervisae* (CAS no. 58856-93-2);
- neutral red dye (CAS no. 553-24-2);
- phosphate Buffered saline (0.01 M, 1 PBS tablet in 200 ml distilled water);
- molluscan physiological saline (0.02 M HEPES, 0.4 M $NaCl_2$, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$; pH 7.4);
- Bakers Formol Calcium (2 % NaCl, 1 % $C_4H_6CaO_4$, 4 % H_2CO);
- acidified ethanol (1 % glacial acetic acid, 50 % analytical grade ethanol);

- BSA (Bovine Serum Albumin) protein standard (CAS no. 9048-46-8); and
- Bio-Rad reagent (Bio-Rad laboratories GmbH, cat no. 500-0006).

Personnel

- One person experienced in the phagocytosis procedure.

Time

- To perform this assay on 8 samples (in duplicate) requires approximately 4 hours (this includes haemolymph extraction) + 1 hour for protein determination.

Protocol

Preparation of dyed zymosan suspension.

1) Make up 1 % neutral red stock solution (0.03 g in 3 ml distilled water).
2) Add 1g of zymosan particles to 3ml of 1 % neutral red solution in a 100 ml beaker.
3) Add 7 ml of distilled water and mix thoroughly with a spatula to ensure each zymosan particle is exposed to the neutral red dye and allow to stand for 15 minutes.
4) Heat fix in a boiling water bath for 15 minutes.
5) After cooling, centrifuge the suspension at 2000 rpm for 5 minutes.
6) Remove supernatant and resuspend mixture in Phosphate Buffered Saline (PBS); repeat this process until the supernatant is clear.
7) Determine zymosan particle concentration using a haemocytometer and resuspended in physiological saline at a concentration of 5 x 108 particles ml^{-1}.

Phagocytosis assay.

1) Pipette 50 µl haemolymph in duplicate into ploy-L-lysine treated, flat-bottomed microtitreplates and 50 µl of haemolymph to a further two wells to act as negative controls.
2) Cover with Mylar plate sealer and incubate for 1 hour at room temperature to allow cell adhesion to occur; 10 minutes before the end of incubation add 100 µl of Baker's formol calcium (2 % NaCl, 1 % $C_4H_6CaO_4$, 4 % H_2CO) to the negative controls to kill the cells.
3) Remove supernatant by plate inversion and wash with physiological saline to remove non-adhered cells.
4) Add 50 µl of dyed zymosan suspension to each well and incubate for 30 minutes at room temperature.
5) After this period, add 100 µl of Baker's formol calcium to all wells to stop the reaction for 10 minutes.
6) Centrifuge the plate at 1000 rpm for 5 minutes. Remove supernatant through plate inversion and wash with physiological saline. Repeat until the negative controls are clear.
7) Add 100 µl duplicate zymosan standards at concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.56 (x 107) zymosan particles ml^{-1} to produce a standard curve.
8) Resolubilise the dye by adding 100 µl acidified ethanol to each well and read the absorbance at 550 nm.
9) Quantify the number of zymosan particles phagocytosed against the zymosan standard curve.

Standardise phagocytosis as a function of protein content.

Protein concentrations are determined using a modified microtitreplate method of Bradford (1976).

1) Pipette 5 µl blanks (physiological saline) and 5 µl protein standards (0-1 mg ml^{-1} Bovine Serum Albumin) in duplicate into empty wells on the phagocytosis microtitreplate.
2) Add 200 µl diluted Bio-Rad reagent (1:5 in distilled water) to the blanks, standards and each well used for the phagocytosis assay.
3) Following incubation at 25^{o}C for 20 minutes, read the absorbance of the samples at 595 nm.

Health and safety
Wear gloves when handling NR (in both the original powder form and the stock/working solutions). Do not inhale/ingest NR. Dispose of waste NR solution via appropriate waste collection services.

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Heart rate

(Depledge & Anderson 1990)

Background/purpose

Cardiac activity can provide a potential measure of physiological stress within an organism (Depledge & Lundebye 1996). The development of a computer-aided physiological monitoring system (CAPMON) has enabled application of this biomarker for long-term, non-invasive, continuous recording of cardiac activity (Depledge & Anderson 1990). Heart rate in the crustacean *Carcinus maenas* is sensitive to environmental contaminants with laboratory exposures demonstrating dramatic changes in heart rate. Both crude oil (Depledge 1984a) and copper (Depledge 1984b; Aagaard and others 1991; Bamber & Depledge 1997) are reported to increase the heart rate in *C. maenas*. In contrast exposure to increasing arsenite concentrations resulted in a reduction in the heart rate of the same species (Bamber & Depledge 1997). Similar concentration-dependent bradycardia has also been observed in *C. maenas* exposed to the organophosphorous pesticide, dimethoate (Lundebye and others 1997). Cardiac activity in bivalves has also been used as a biomarker of physiological competence (Nicholson 1999; Wedderburn and others 2000). Elevated heart rates have been observed in the mussel *Perna viridis* following copper exposure (Nicholson 2003). However, bradycardia was observed in *Mytilus edulis* exposed to the same contaminant (Grace & Gainey 1987; Curtis and others 2001). This variation in cardiac response following contaminant exposure may indicate a range within which heart rate can be categorised as 'normal'. Beyond the upper threshold of this range, heart rate becomes tachycardic, a typical response to short term stress. Whilst below this range organisms express bradycardia, possibly reflecting chronic contaminant exposure.

Methods

Equipment

- superglue;
- 2 litre beakers;
- aluminium foil;
- CAPMON leads;
- CAPMON sensors with collars;
- CAPMON unit/software; and
- laptop.

Personnel

- One person experienced in the CAPMON procedure and software.

Time

- To perform this assay on 8 samples (in duplicate) requires approximately 2 hours (this includes haemolymph extraction – see procedural guideline).

Protocol

Loading software.

1) Go into *My Computer* and create a new folder and call it, for example, CAPMON.
2) Insert the disk containing the CAPMON software and copy all files from the disk to the new folder.
3) Ensure that the files **Capmon** and **Egavga.bgi** appear together in the new directory.

Setting up the CAPMON unit.

1) Connect the CAPMON unit to the computer parallel port using the lead provided.
2) Connect the sensors to the back of the machine.

3) Plug in and switch on the computer and CAPMON unit.
4) Go into *My Computer* in Windows and open the folder called CAPMON.
5) Double click on the CAPMON icon.
6) Follow the instructions on the screen.
7) You will be asked to enter whether you have a colour monitor Y/N. Enter your response.
8) You will then be asked if you wish to save all parameters. Type **N**.
9) You will then be asked if you want to load scan rate, trigger and bandwidth. Type **N**.
10) An introductory screen will then appear. Press **return**.
11) You need to select your LPT port. (This is the route of communication between the CAPMON and the computer). Type **1**.
12) A page showing specifications and key functions will then appear. Press **return**.
13) Eight traces will appear running across the screen from left to right.
14) Check that each of the sensors is working by tapping the end of each of them in turn and observing the traces on the computer screen. If all is well you should see a clear deflection appearing as a wave, coincident with your tapping.
15) In operation the heartbeat signal derived from the test animal appears on the screen as a repeating cycle. It is necessary to be able to count each of these cycles for each individual test animal and store the resulting data. In order to achieve this, a pair of lines appears on the screen for each of the eight plots. For a complete cycle to be recorded the trace from the animal must pass up through these lines and then return back down through them. The gap between the lines on the screen is called the bandwidth and it is possible to adjust this to account for complex waveforms. Adjustment of this parameter for individual channels is more typical when using molluscs. When using crabs, with their much stronger and predictable signals we can set the bandwidth of the lines at a standard distance apart. It is only necessary to set the bandwidth at the start of the experiment. To do this you will need to put the program into manual mode by typing **M**. Automatic mode is designed for use on molluscs and is not suitable for use with crabs. To set the bandwidth type **B** you will be asked to enter a value here for each of the eight channels. Type in **500** and press the return key in each case.

You are now ready to connect up your crabs to the CAPMON unit.

Setting up the CAPMON equipment.

1) Attach one of the plastic collars (supplied with the machine) over the heart of the organisms using a very small amount of ‘Superglue’ (this facilitates removal once the experiment has finished).

Locating the heart.

Crabs: The heart is positioned just beneath the cuticle (shell) towards the back of the top of the crab. There is typically a series of indentations on the crab surrounding the heart forming a square shape.

Mussels: the heart is usually located near the edge of the shell, just forward of the shell hinge (towards the anterior, more pointed end).

2) Once the collar is firmly in place the sensor can be mounted. After ensuring that the retaining screw is not blocking progress, slide the sensor inside the collar. Ensure the end of the sensor is tight up against the surface of the cuticle. Now gently tighten the screw in the side of the collar to hold the sensor in place.
3) Examine the trace generated by the sensor on the screen. It should appear as a rough sine wave with clear cyclic repetition of pattern. This is the heartbeat of the organism.
4) As stated previously, for the software to register a complete heart cycle the trace must pass up and then down through both of the dashed lines. If the pattern produced by the sensor is quite complex it is possible to generate two peaks within a single cycle and thus generate an incorrect count. It is important to avoid this. If the wave appears to be complex (having more than one significant peak per cycle) then it is possible to loosen the collar screw and gently

twist the sensor so that the emitter and detector are repositioned to give a smoother output. Persist in the set up until you are happy you have a good clear signal that represents the heartbeat of the organism.

5) If the trace shape develops no clear repetitive pattern on the screen check that the sensor is functioning correctly by removing it from the collar and tapping the end of it with your finger. Does it respond correctly? Use a second sensor on the same organism to see what sort of output you get from it. Once a sensor develops a fault it should be discarded with a replacement then made up (it pays to have several spare sensors at the start of an experiment).
6) Ensure each individual is returned to suitable holding conditions (2 litre foil wrapped beaker containing enough seawater to cover the organism) prior to starting the experiment.

Adjusting the signal.

1) The amplitude of the signal (size of the wave) on the screen can be adjusted by using the gain knobs on the front of the CAPMON unit. The key with heartbeat recording is to ensure that the trace passes well clear of the dashed lines on each cycle.
2) The sample speed setting on the computer is now changed to suit measurements taken from crabs. Type **S**, then **5** and finally **return**. This setting ensures a good easily read passage of the signal across the screen.

Recording.

1) Once you are happy with the output of all of your sensors it is time to start recording the data.
2) Data is saved in the form of beats per minute for each channel. A cumulative counter is incremented each time a cycle is detected. The counter runs for one minute then saves its accumulated total, resets to zero and restarts its count for the subsequent minute. Thus at the end of each session there will be eight columns of data, one representing each of the sensors, with each row of data representing the heart rate for that individual crab during a specific minute of time.
3) To start recording type **R** (record) and enter an appropriate file name (ie the date 030602). The CAPMON unit will now continuously monitor and record all the connected channels.
4) At the end of the recording period type **Q** (quit).
5) Once recording is completed loosen the screw to remove the sensor from the crab. The collar is removed by placing the blade of a small flat screwdriver under the edge closest to the carapace and very gently twisting it. If firm resistance is felt try placing the blade in another area around the rim. When removed correctly all that should be left on the crab is a small amount of glue residue.
6) **NOTE**: The principle of crustacean heart rate monitoring is the same of that applicable to monitoring scallop cardiac activity.

Extracting CAPMON raw-data files to Excel.

1) Run Microsoft Excel and go to File, Open.
2) Change file type to All files.
3) Open the CAPMON *.dat file for the recording session to be analysed.
4) The text import wizard will appear.
5) Place a tick in the delimited box.
6) Go to Next page. For delimiter place a tick in the space box.
7) Click on Finish.
8) The Final step is to delete the first empty column. You are then left with the 8 columns of data as described above. You can then save the raw data as an Excel file.
9) It is necessary to manipulate the data to visualise what you have measured.
10) Previously generated template files allow a rapid assessment of the gathered data. Several of these will be included with the software package. They are very simple files created within the spreadsheet. If the experimental regime is predetermined and carried out accurately then the data can be fed directly into these files to generate the final plots.

Health and safety
Take care when using superglue. Ensure CAPMON machine and laptop are kept away from seawater as much as possible.

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PAH metabolite analysis

(Watson and others 2002)

Background/purpose

PAHs absorb ultraviolet light followed by emission of light at a longer wavelength, this UV-fluorescence occurs due to the delocalised π-electrons. The fluorescence properties vary between PAH compounds, as they are dependent on size, structure and corresponding substituents. A general trend is recognised whereby the optimal excitation wavelength increases with increasing PAH molecule size. This variability between compounds has been used in detection techniques for PAHs (Aas and others 2000).

PAH metabolites were first detected in fish bile using HPLC/Fluorescence (HPLC/F) and implemented as a monitoring tool for environmental contamination (Krahn and others 1984). The technique of detecting PAHs by way of spectroscopic fluorescence measurement has been developed for use in decapod crustaceans (Watson and others 2002). Urine samples from male crabs are diluted in ethanol and directly analysed using Synchronous Fluorescence Spectrometry (SFS). These techniques enable pyrogenic and petrogenic sources of contamination to be distinguished. Filed applications have shown these techniques to be rapid, cost-effective biomonitoring tools for detecting PAH contamination in coastal ecosystems (Dissanayake & Galloway 2003; Eickhoff and others 2003; Watson and others 2002).

Methods

Equipment

- pipette and tips;
- cuvette;
- siliconised eppendorfs; and
- fluorescence spectrophotometer.

Chemicals

- analytical grade ethanol;
- 1-OH pyrene (CAS no. 5315-79-7).

Personnel

- One person experienced in the extraction of urine from crabs and in the use of a fluorescence spectrophotometer.

Time

- To perform this assay on 8 samples (in duplicate) requires approximately 3 hours (this includes haemolymph extraction – see procedural guideline 1).

Protocol

Urine extraction – Figure C (technique taken from Bamber & Naylor 1997).

1) Restrain crab, ventral side up, on a plastic board using elastic bands.
2) Move aside third maxillipeds and dry the epistome to prevent seawater contaminating the urine.
3) Using a hooked seeker, lift the operculum of the antennal gland bladder and collect the urine using a 200 µl pipette.
4) Transfer urine sample to siliconised eppendorf and store at 80^{o}C until subsequent analysis.

Standards.

Standards are used to take into account instrumental drift over time and allow samples to be compared despite fluctuations in instrumental response. 1-OH pyrene standards are used rather than the parent compound; this takes into account the conjugation of a polar group thereby facilitating metabolism and excretion. 1-OH pyrene serves as an internal standard but does not quantify peaks in terms of μg l^{-1} of 1-OH pyrene; therefore results from standards are reported in terms of pyrene metabolites.

1) Scan a 1 ml blank (50% ethanol) by using synchronous fluorescence spectrophotometry (SFS) prior to sample analysis. This provides a measure of the background fluorescence contributed by the solvent.
2) Analyse 1-OH pyrene standards using SFS for each standard concentration (200, 100, 50, 25, 10, 5 μg l^{-1}).

Fluorimetric sample analysis.

1) Dilute urine samples 1:20 with 50 % ethanol solution (50 μl sample + 950 μl ethanol).
2) Transfer 1 ml of the diluted sample into a cuvette.
3) Analyse samples using SFS with excitation and emission slit widths of 2.5 nm. Perform SFS analysis using a wavelength difference (Δλ) of 37 nm with a spectrum taken from 240-500 nm.
4) Measure the peak area on resulting spectra to compare relative levels of PAH metabolite fluorescence; 310-370 nm peak area indicates naphthalene type metabolites, whilst peak areas between 370-400 nm and 400-460 nm indicate pyrene and benzo[a]pyrene type metabolites respectively.

Figure C Urine extraction from the shore crab *Carcinus maenas*

Health and safety
Wear gloves when handling 1-OH pyrene. Dispose of waste 1-OH pyrene standards through appropriate waste disposal services.

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Feeding rate (clearance rate) of mussels

(Widdows & Donkin 1989)

Background/purpose

The feeding rate in mussels can be determined by measuring the rate of clearance of algal particles from solution. Studies have shown that the clearance rate is the component of the energy budget most sensitive to contaminants (Gilfillan 1975; Howell and others 1984; Axiak & George 1987; Widdows & Donkin 1989 1992; Bourdelin 1996). The feeding rate in Mytilus edulis is reported to be inhibited following exposure to PAH contaminated sediments with increased body burdens of both fluoranthene and benzo[a]pyrene correlating with reduced feeding rates (Eertman and others 1995). Metal exposure has also been shown to decrease feeding rate in bivalves, with exposure to 100 µg l^{-1} copper and above significantly inhibiting the clearance rate in the mussel *Perna viridis* (Nicholson 2003).

Alterations to components of the energy budget (such as feeding rate) can impact the organism's scope for growth (SFG) through disruption of the energy balance (Newman 1998). Previous work has shown that lower feeding rates in the mussels *Mytilus galloprovincialis* and *P. viridis* results in reduced SFG (Widdows and others 1997; Wang and others 2005). Since SFG reflects the energy available for growth and reproduction (Newman 1998), changes in feeding rate, which in turn alters the energy budget upon which SFG is based, will clearly impact the fitness of the organism. Both microcosm and field studies confirm that long-term consequences to growth and survival can be predicted from measured effects on the energy balance observed at the level of the individual (Widdows & Donkin 1991) making feeding rate an important and sensitive biomarker.

Methods

Equipment

- 400 ml glass beakers;
- fleas;
- forceps;
- pipette and tips (suitable for 500 µl);
- 20 ml glass syringe;
- plastic vials; and
- coulter counter.

Chemicals

- Isochrysis galbana concentrate (Varicon Aqua Solutions, Malvern, UK).

Personnel

- One person experienced in the use of a coulter counter.

Time

- To perform this assay on eight samples (in duplicate) requires approximately two hours (this includes haemolymph extraction – see procedural guideline 1).

Protocol

1) Prepare algal solution by diluting 1-3 ml *Isochrysis galbana* concentrate in 100 ml seawater.
2) Fill 400 ml beakers with 350 ml seawater (15°C), add a flea and place on magnetic stirrer. Ensure a further two beakers are set up as controls (which will contain no mussels).
3) Place one mussel in each beaker, except for the control vessels, using long forceps, ensuring that they are positioned away from the moving stirring bar. Leave mussels *in situ* to allow them to acclimatise and open their valves.

4) To initiate feeding, add 500 µl aliquots of the thoroughly mixed prepared algal suspension to each beaker (this will give an algal concentration of 12-15 000 cells 0.5 ml^{-1}).
5) Immediately (t_0) remove 20 ml of seawater from each beaker using a glass syringe and place into plastic vials ready for analysis on the coulter counter.
6) Take further 20 ml water samples 15 minutes (t_1) and 30 minutes (t_2) after the feeding was initiated and transfer to plastic vials.
7) Gently swirl samples to ensure samples are homogenous, then analyse using a coulter counter (set up to measure particles between 4-10 µm).
8) The feeding rate of the mussels is calculated as follows:

Feeding rate (l hr^{-1}) = (v x 60 / t) (t_o - t_2);

v = volume of seawater in beaker (in litres);

t = duration of assay;

t_o = initial cell count;

t_2 = final cell count.

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Cholinesterase/ carboxylesterase activity

(Ellman and others 1961)

Background/purpose

The extensive use of OPs and carbamates has led to levels in the aquatic environment that are sufficient to pose a potential risk to organisms (Gabrielides 1995; Hai and others 1997). The toxicity of these compounds is primarily through the inhibition of acetylcholinesterase (AChE), a serine hydrolase which degrades the neurotransmitter acetylcholine in cholinergic synapses (Walker 2001). AChE inhibition results in accumulation of acetylcholine at nerve synapses and subsequent disruption of nerve function (Peakall 1992). Measurement of this peripheral enzyme activity can provide a useful means of monitoring OP/carbamate exposure (Carlock and others 1999). Such esterase activity can be determined through the hydrolysis of thiocholine esters or thioacetate derivatives by cholinesterase or carboxylesterase respectively. This produces thiocholine or thioacetate. Subsequent combination with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) yields the yellow anion 5-thio-2nitrobenzoic acid which absorbs strongly at a wavelength of 405 nm (Ellman and others 1961).

Laboratory studies have observed dose-dependent inhibition of AChE in bivalve molluscs following exposure to the organophosphates chlorfenvinphos (Rickwood & Galloway 2004), azamethiphos (Brown and others 2004), chlorpyrifos (Bonacci and others 2004) and paraoxon (Galloway and others 2002). In crustaceans it has been reported that carboxylesterases (CbE) are more sensitive to contaminants compared to AChE (O'Neill and others 2004). CbEs are also subject to inhibition by carbamate and OP compounds, with reductions in CbE activity in *Mytilus edulis* after paraoxon exposure (Galloway and others 2002) and in paraoxon-methyl exposed zebra fish *Danio rerio* (Küster 2005).

Methods

Equipment

- pipette and tips;
- flat bottom microtitreplate;
- siliconised eppendorfs; and
- multi-well plate reader.

Chemicals

- acetylthiocholine iodide (CAS no. 1866-15-5);
- phenylthioacetate (CAS no. 934-87-2);
- Phosphate Buffered Saline tablets (PBS);
- Bio-Rad reagent (Bio-Rad laboratories GmbH); and
- Bovine Serum Albumin (BSA) protein standard (CAS no. 9048-46-8).

Personnel

- One person experienced in the procedure.

Time

- To perform this assay on eight samples (in duplicate) requires approximately two hours (this includes haemolymph extraction) + 1 hour for protein determination.

Protocol

Esterase activity in *M. edulis* is determined through the hydrolysis of acetylthiocholine iodide providing a measurement of AChE, whilst in *Carcinus maenas* CbE activity is measured, using phenylthioacetate as a substrate.

1) Prepare haemolymph samples by centrifuging at 3000 rpm for five minutes.

2) Pipette 50 µl of haemolymph supernatant into a flat-bottomed microtitreplate in duplicate. In a further two wells add 50 µl Phosphate Buffered Saline (PBS) as blanks.
3) Add 150 µl of DTNB (270 µM: 0.0107g in 100 ml PBS) to each well and read kinetically at 405 nm for five minutes.
4) Add 50 µl of acetylthiocholine iodide (3 mM: 0.043 g in 50 ml distilled water) to *Mytilus edulis* samples OR 50 µl of phenylthioacetate (6 mM: 0.046 g in 50 ml distilled water) to *Carcinus maenas* samples and read kinetically at 405 nm for five minutes.

Standardise esterase activity as a function of protein content.

Protein concentrations are determined using a modified microtitreplate method of Bradford (1976).

1) Pipette 5 µl haemolymph supernatant*, 5 µl blanks (physiological saline) and 5 µl protein standards (0-1 mg ml^{-1} Bovine Serum Albumin) in duplicate into empty wells on a microtitreplate.
2) Add 200 µl diluted Bio-Rad reagent (1:5 in distilled water) to the samples, blanks and standards.
3) Following incubation at 25^{o}C for 20 minutes, read the absorbance of the samples at 595 nm.

*NOTE: for *C. maenas* samples carry out protein assay on haemolymph supernatant diluted 1:50 in PBS.

Express enzyme activity as micromoles of substrate hydrolysed per minute relative to protein content (µmol min^{-1} mg^{-1} protein):

$$\text{Esterase activity } (\mu\text{mol min}^{-1}\ \text{mg}^{-1}\ \text{protein}) = \frac{\Delta A_{405} \times Vol_T \times 1000}{(1.36 \times 10^4) \times \text{lightpath} \times Vol_S \times [\text{protein}]}$$

$\Delta A405$ = change in absorbance (optical density) per minute
Vol_T = total assay volume (DNTB + sample in ml)
1.36×10^4 = extinction coefficient of TNB (M^{-1} cm^{-1})
light path = microplate well depth
Vol_S = Sample volume (in ml)
[protein] = protein concentration (mg ml^{-1})

Health and safety
Prepare phenylthioacetate in a fume cupboard. Wear gloves when preparing and handling the phenylthioacetate solution.

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Micronucleus assay

(Countryman & Heddle 1976)

Background/purpose

Evans and others (1959) were the first authors to suggest counting cells with micronuclei (Mn) as a method for the evaluation of cytogenetic damage and Schmid (1975) and Heddle (1973) both independently proposed the micronucleus test as an alternative to the laborious and complex counting of aberrations in metaphases. Micronuclei are expressed in dividing cells that contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term “micronucleus”. Figure D represents the mechanisms involved in the formation of micronuclei.

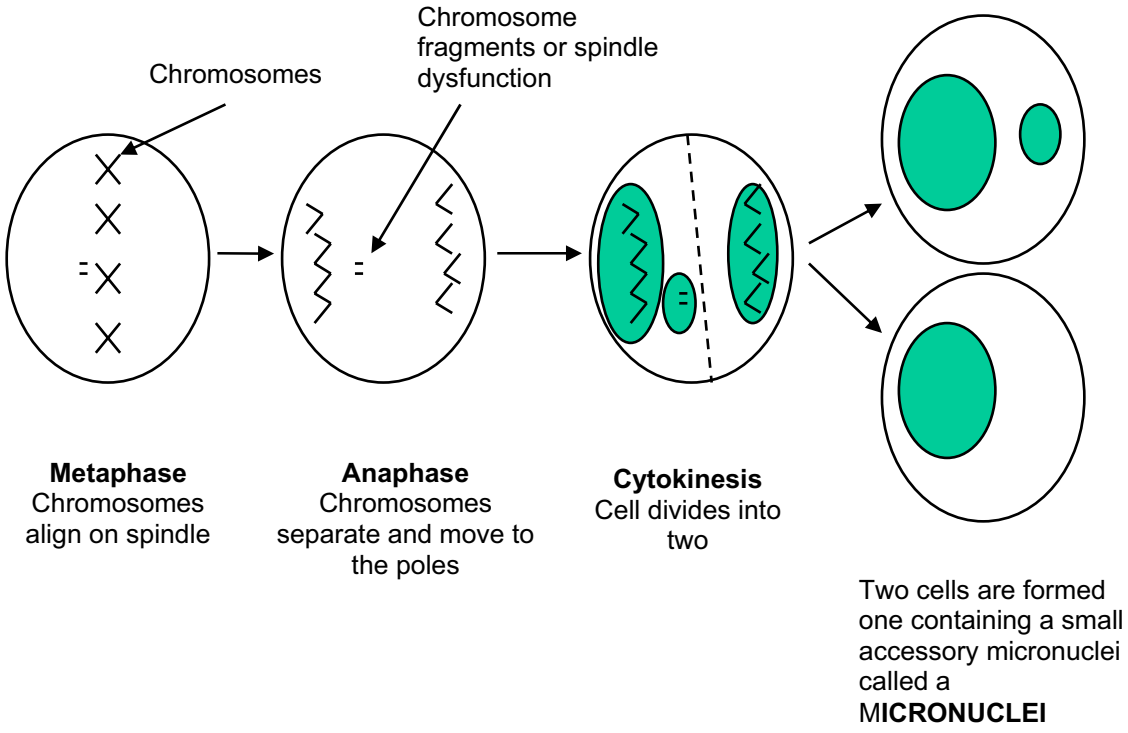

Figure D Formation of micronuclei during cell division

Micronuclei provide a convenient and reliable index of both chromosome breakage and chromosome loss (Fenech 1996). Micronuclei are also known as Howell-Jolly bodies by haematologists and because they are caused by chromosomal aberrations (Countryman and Heddle 1976) are considered to be a sensitive indicator of genotoxic risk of exposure to mutagenic agents (Rodgers and Baker 2000). The micronucleus assay was initially developed in dividing mammalian cells and its use in bone marrow and peripheral blood erythrocytes is now one of the best established in vivo cytogenetic assay in the field of genetic toxicology. Micronuclei are only expressed in dividing cells and the assay can not be used in nondividing cell populations in which the cell division kinetics is not well controlled or understood. In mammalian systems, the cytokinesis-blocked micronucleus assay has been developed to block cells that have completed one nuclear division from performing cytokinesis, using cytochalasin-B. Consequently cells will have a binucleated appearance and the accumulation of virtually all dividing cells will occur regardless of their degree of synchrony and proportions of dividing cells. Micronuclei are then scored in binucleated cells only, which enables reliable comparisons of chromosome damage between cell populations that may differ in their cell division kinetics.

The micronucleus assay has several advantages over other cytogenetic assays. Heddle (1973) stated that the scoring micronuclei were more than ten times faster than scoring chromosomal aberrations at metaphase. Other advantages of the micronucleus assay are that the number of scorable cells is virtually unlimited, little formal training is needed as the end-point is easily recognisable and a suitable karyotype is not required. Problems associated with the interpretation of other cytogenetic tests such as the significance of chromosomal gaps or sister chromatid exchanges are avoided with the micronucleus test. One limitation of the micronucleus assay is when agents cause neither chromosomal breakage or lagging chromosomes, for example, aberrations that involve chromosomal rearrangement without the occurrence of an acentric fragment such as translocation or inversion, these will not be detected (Heddle and others 1983).

As with many other mammalian based assays, the micronucleus test has been successfully adapted to aquatic organisms. Induction of micronuclei in fish has been widely used due to advantages over other cytogenetic assays such as chromosomal aberrations and sister chromatid exchanges. As well as being time consuming these tests are not very effective due to the relatively large number of very small chromosomes (Ayllon and Garcia-Vazquez 2000) and, as in cold water fish, the cells have a low mitotic activity which results in fewer scorable metaphase spreads (Hooftman and De Raat 1982). The micronucleus assay has been extensively used in haemocytes and gill cells of many mollusca species, including the freshwater zebra mussel *Dreissena polymorpha* (Mersch and Beauvais 1997; Pavlica and others 2000), the oyster *Crassostrea gigas* (Burgeot and others 1995), the Mediterranean mussel *Mytilus galloprovinciallis* (Majone and others 1987,1988; Scarpato and others 1990; Venier and others 1997) and the common blue mussel *Mytilus edulis* (Bolognesi and others 1999; Dopp and others 1996; Wrisberg and Rhemrev 1992). Many micronuclei studies have been carried out on molluscs in laboratory conditions but due to sedentary nature of molluscs the micronucleus test has also been proposed as a potential biomarker of pollutant exposure in field studies (Mersch and Beauvais 1997). Although the micronuclei frequency in haemocytes from mussels were shown to be seasonally dependent (Wrisberg and Rhemrev 1992) and many authors have reported high variability between individuals (Mersch and Beauvais 1997). The incidence of micronuclei has also been linked to the induction of leukemia cells in the clam *Mya arenaria*, suggesting that the micronucleus test is a very good indicator of the potentially life threatening consequences of genotoxic exposure (Dopp and others 1996).

Methods

Equipment

- poly-L-lysine slides;
- pipette and tips (50 µl);
- staining trough;
- cover slips; and
- microscope.

Chemicals

- methanol (analytical grade);
- Giesma stain solution;
- Phosphate Buffer Saline tablets; and
- DPX mountant for microscopy (BDH Laboratory Supplies, Poole, Dorset).

Personnel:

- One person experienced in the use of a microscope and the scoring procedure.

Time

- To perform this assay on eight samples (in duplicate) requires approximately four hours (this includes haemolymph extraction).

Protocol

1) Pipette 50 µl of haemolymph onto the slide and spread gently.

2) Leave in a stable environment for at least 30minutes and allow to air dry at room temperature.
3) Fix the slides in methanol for 15 minutes and allow to air dry. The slides can now be stored for staining.
4) Stain the slides using 5% (v/) Giemsa's stain in Giemsa buffer solution for 15-25 minutes.
5) Rinse at least twice in distilled water and allow to air dry.
6) Mount with DPX and a cover slip.
7) Slides are scored blind under x40 and Mn validated under oil immersion.
8) A total of 1000 cells are analysed per mussel.

The Mn were identified according to the following criteria: (1) diameter smaller than one-third of the main nucleus but greater than one-tenth (2) no contact with nucleus (absence of chromatid bridge) (3) colour and texture resembling the nucleus (4) spherical cytoplasmic inclusions with sharp contour (Countryman and Heddle 1976). Figure E represents typical micronuclei observed in haemocytes of *M. edulis* (x100 magnification). Figure F demonstrates micronuclei that are not included in scoring.

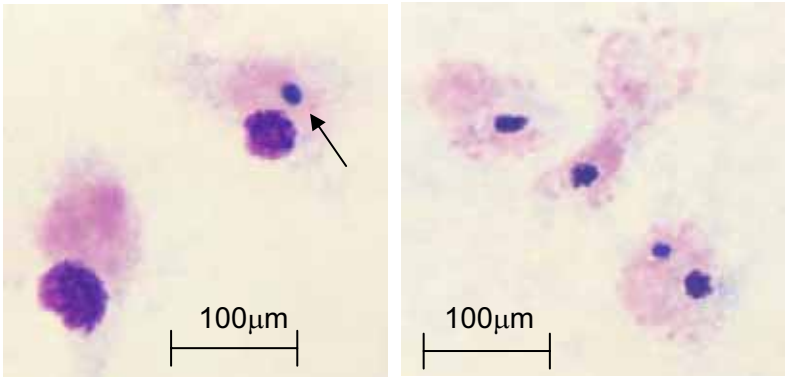

Figure E Micronuclei (→) in haemocytes of Mytilus edulis stained with Giemsa

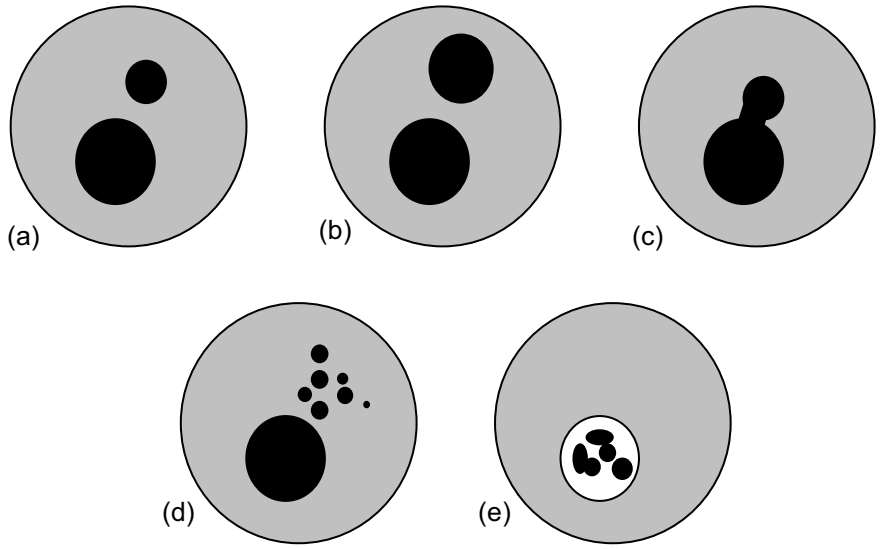

Figure F Criteria adopted to analyse for structures that are not micronuclei

Health and safety
Wear gloves when handling Giesma stain and DPX. Use DPX in a fume cupboard. Dispose of waste Giemsa solution through appropriate waste disposal services.

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Ferric Reducing Ability of Plasma (FRAP)

(Benzie & Strain 1996)

Background/purpose

Antioxidant power can be referred to analogously as reducing ability (Benzie & Strain 1996). This FRAP method is based upon the reduction of a ferric tripyridyltriazine (Fe^{3+} - TPTZ) complex to the ferrous tripyridyltriazine (Fe^{2+} - TPTZ) by a reductant at low pH. Fe^{2+} has an intense blue colour and can be determined by measuring the absorbance spectrophotometrically at 593 nm (Benzie & Strain 1996; Benzie & Strain 1999). Frap values are quantified by comparing absorbance changes (over 10 minutes) in a sample against absorbance change in standards containing ferrous ions in known concentrations (Benzie & Strain 1996). Originally developed for use on human plasma (Benzie & Strain 1996), this simple and inexpensive measure of antioxidant potential has been applied to corals (Griffin & Bhagooli 2004), scallops (Hagger and others 2004) and mussels (Hagger and others 2005).

Methods

Equipment

- pipettes and tips (suitable for 5, 200 µl);
- siliconised 1.5 ml eppendorfs;
- mylar plate sealer;
- flat bottom microtitreplate; and
- multi-well plate reader.

Chemicals

- 300 mM sodium acetate buffer;
- 10 mM TBTZ (2,4,6-Tris(2-pyridyl)-s-triazine CAS no. 3682-35-7) in 40 mM HCl;
- 20 mM $FeCl_3.6H_20$;
- 1 mM $FeSO_4.7H_20$;
- Molluscan physiological saline (0.02 M HEPES, 0.4 M $NaCl_2$, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$; pH 7.4) OR Phosphate Buffered Saline (0.01 M, 1 PBS tablet in 200 ml distilled water) for analyses on crab haemolymph;
- BSA (Bovine Serum Albumin) protein standard (CAS no. 9048-46-8); and
- Bio-Rad reagent (Bio-Rad Laboratories GmbH).

Personnel

- One person experienced in the FRAP assay.

Time

- To perform this assay on eight samples (in duplicate) requires approximately two hours (this includes haemolymph extraction) + 1 hour for protein determination.

Protocol

1) Make up the FRAP reagent: 10 ml 300 mM acetate buffer; 1 ml TBTZ; 1 ml 20 mM iron chloride.
2) Prepare standards (0-500 µg l^{-1}) using Ferris oxidised iron (eg 100 µl $FeSO_4.7H_20$ + 900 µl distilled H_20).
3) Centrifuge haemolymph samples at 3000 rpm for 5 minutes.
4) Pipette 50 µl of haemolymph supernatant and standards into a flat-bottomed microtitreplate in duplicate.
5) Add 200 µl of FRAP reagent to each well.
6) Read the absorbance at 593 nm (T_0).

7) Incubate the plate for 10 minutes at 25°C then read again at 593 nm ($T1_0$).

Express FRAP as a function of protein content.

Protein concentrations are determined using a modified microtitreplate method of Bradford (1976).

1) Pipette 5 µl haemolymph supernatant*, 5 µl blanks (physiological saline) and 5 µl protein standards (0-1 mg ml^{-1} Bovine Serum Albumin) in duplicate into empty wells on a microtitreplate.
2) Add 200 µl diluted Bio-Rad reagent (1:5 in distilled water) to the samples, blanks and standards.
3) Following incubation at 25°C for 20 minutes, read the absorbance of the samples at 595 nm.
4) Express FRAP values as follows:

$$\frac{(T1^0 - T^0)}{\text{protein content of sample}}$$

5) *NOTE: for C. maenas samples carry out protein assay on haemolymph supernatant diluted 1:50 in PBS.

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Metallothionein assays (MT)

(Langston and others 2002)

Background/purpose

The intracellular metal-binding protein metallothionein is reported to occur throughout the animal kingdom, in plants, several eukaryotic micro-organisms, and in some prokaryotes (Engel & Brouwer 1989). Research has to date provided evidence for its role in metal detoxification, homeostasis of Zn and Cu, and protection against radiation-induced DNA damage and oxidative stress (Roesijadi 1992; Olsson and others 1987; Cai and Cherian 2003; Kling and others 1996; Kiningham & Kasarskis 1998).

MT synthesis in bivalve species has been shown to be specifically induced by heavy metals such as Cu, Zn, Hg and Cd present in coastal and estuarine waters, (Amiard-Triquet and others 1998, Hamza-Chaffai and others 2000, Mourgaud and others 2002; Domouhtsidou and others 2004). MT concentrations have been examined in several tissue types in *Mytilus spp*. although measurements of MT induction in gill and digestive gland has been found to be the most reliable biomarker of metal exposure, providing seasonal variation is taken into account (Ivankovic and others 2005: Serra and others 1999; Geffard and others 2005).

Changes in subcellular partitioning of metals can be a valuable additional indication of metal exposure: After transplantation to a metal contaminated site, the proportion of Cd bound to MT in freshwater bivalve *Pyganodon grandishave*, decreased in relation to other metal–containing pools (Couillard and others 1995) indicating saturation of MT followed by metal "spillover". However, Giguère and others (2003) found that the amounts of Cd bound to HMW and LMW ligands in all mussels collected from sites along a metal gradient remained proportional to the amount bound to MT, suggesting that perhaps the concept of "spillover" above a threshold level may not be applicable where organisms are chronically exposed to elevated metal concentrations.

The induction of MT, and its capacity to bind metals, has been applied in biochemical monitoring of environmental contamination in various aquatic ecosystems: for example MT induction was correlated with gradients of metal concentration (eg, Hg, Cd, Pb, Cu, Zn) in mussels *Mytilus edulis* in the southwestern Baltic Sea (Schiedek and others 2006). Giguère and others (2003) found that Cd and MT concentrations in the gill cytosol of mussels *Pyganodon grandis* decreased along a polymetallic contamination metal gradient downwind from a copper smelter north-west of Montréal, Québec, Canada.

Similarly, field studies have examined MT in fish, for example brown trout (*Salmo trutta*) caged in a Cd/Zn contaminated river in Norway displayed higher levels of MT (both protein and mRNA) in liver, kidney and in gills compared to trout from an uncontaminated, reference river (Hansen and others 2006). MT levels correlated with the Cd burden of perch *Perca fluviatilis*, from a Cd-polluted river (Olsson and Haux 1986), and rainbow trout *Oncorhynchus mykiss* caged in lakes with differing metal concentrations showed increases in hepatic MT and a strong correlation between MT and Zn concentrations (Roch and McCarter 1984). On the River Forth Estuary in Scotland, a clear gradient of hepatic MT induction in flounder *Platyichthys flesus* was found, the highest levels coinciding with the most metal-contaminated sediments (Sulaiman and others 1991). In the euryhaline species *Anguilla anguilla* (the European eel) from the Thames Estuary, hepatic MT levels were found to be directly related to metal contamination, Langston and others (2002).

MT induction can also be used to monitor the effects of events such as dredging: concentrations of MT in eelpout *Zoarces viviparus* in metal-contaminated Goteborg harbour (Sweden) were measured both before and during extensive dredging operations. Elevated MT gene expression indicated an increase in metal exposure, showing that the fish were even more affected by remobilized pollutants (Sturve and others 2005).

Examining metallothionein levels and metal binding characteristics in aquatic organisms has thus been successfully applied to field studies and can serve as an early indication of contaminant stress, providing valuable information regarding the extent and possible consequences of chronic exposure.

Methods

Equipment

- acid washed laboratory glassware (vials, measuring cylinder, beakers etc);
- Ultra Turrax tissue homogeniser;
- water bath @ 80°C;
- Hi Spin 21 centrifuge;
- PARC model 174A analyser, and a PARC/EG&G model 303 static mercury drop electrode (SMDE);
- Sephadex G-75 column (1.5 x 60cm) equilibrated at 4˚C with the buffer used for elution (0.02M Tris HCl, pH 8.6);
- Varian® Cary 50 Bio UV visible spectrophotometer; and
- hotplate.

Chemicals

- tris (hydroxymethyl) hminomethane (CAS no. 77-86-1);
- hydrochloric acid (HCl – Analytical grade);
- ammonia (1m NH_4OH – Analytical grade);
- ammoniun chloride (1m NH_4Cl – Analytical grade);
- hexamminecobaltic chloride ($[C_o(NH_3)_6]Cl_3$- Analytical grade);
- triton-X-100 (CAS no. 9002-93-1);
- purified rabbit MT (CAS no. 73767-16-5); and
- nitric acid (HNO_3- Analytical grade).

Personnel

- One person experienced in use of laboratory equipment, and familiar with AAS methods.

Time

To perform this assay on eight samples requires approximately:

- -1 day (8 hours) (MT purification and quantification);
- - 2 days (metals analysis).

Protocol used at the MBA based on method described by Langston and others 2002

- Reagents;
- 0.2M Tris Buffer;
- 0.02M Tris HCl buffer;
- DPP buffer;
- Co-Hex buffer.

Purification of MT

1) Weigh glass vial and centrifuge tube.
2) Cut small pieces of tissue (0.5g), place in glass vial – weigh.
3) Add Tris buffer (2-5 x tissue weight) and re-weigh.
4) Homogenise on ice, pipette of a few mls into centrifuge tube and weigh.
5) Weigh vial and remaining homogenate for W:D.
6) Oven-dry vial and homogenate overnight (80˚C) then re-weigh for W:D.
7) Balance centrifuge tubes and spin at 18000rpm (28000g) 40mins at 4˚C.

8) Pipette off supernatant into clean centrifuge tube, taking approx 0.5mls of 1 sample for column chromatography (weigh and keep on ice).
9) Weigh tube and pellet.
10) Heat treat remaining supernatant for 10 mins at 80˚C then centrifuge again for 40 mins at 18000rpm (28000g).
11) Pour off supernatant for polarography.

Polarography

1) Add an aliquot (10-50 µl) of heat-treated cytosol, together with 300µl Triton-X (0.025% v/v), to 10 mls hexamminecobalt chloride buffer (the electrolyte).
2) Measure the polarographic response of MT during a potential scan between –1.4 and –1.6V.
3) Quantify MT using standard additions of purified rabbit MT (Sigma).

Metal binding

1) Apply aliquot of non heat-treated supernatant to Sephadex G-75 column.
2) Elute with the Tris buffer at 0.5 ml min^{-1} and collect as 3 ml fractions.
3) Measure optical absorbance of the elueate at 254nm (as a general marker for proteins and amino acids).
4) Measure metals (Cd, Cu, Ag and Zn) in the column fractions by air-acetylene flame (AAS) and graphite furnace atomic absorption spectrophotometry using standard addition methods (GFAAS).

Total metals

1) Digest dried homogenate samples with 5 ml HNO_3 on a hotplate until digest becomes a pale straw colour.
2) Evaporated to near dryness before dissolution in 10%HCl.
3) Determine metal concentration (Cd, Cu, Ag and Zn) by air-acetylene flame (AAS) and graphite furnace atomic absorption spectrophotometry (GFAAS) using standard addition methods.

Health and safety

Chemicals and reagents: When making up and handling chemicals and reagents - wear nitrile gloves, lab coat and eye-protection. Make up in fume cupboard where appropriate. Be aware of any specific hazards - consult MSDS sheets.

Vapour and fumes: Ensure that room is well ventilated, and that fume cupboard is switched on.

Spills: If splashed on an individual or in eyes flush for 15 minutes with copious quantities of water.

Sharps: Scalpel blades (and microtome blades), should be disposed of in an approved Sharps Container.

Disposal: Dispose of waste Hg through appropriate waste disposal services.

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Total Oxyradical Scavenging Capacity assay (TOSC)

(Regoli and Winston 1999)

Background/purpose

There is increasing interest in the areas of oxyradical metabolism and oxidative stress, because several classes of environmental pollutants are known to enhance intracellular formation of reactive oxygen species (ROS) and affect the efficiency of antioxidant defences. Variations in the levels or activities of the main antioxidants have often been proposed as biomarkers of contaminant mediated oxidative stress in marine organisms (Di Giulio 1991). Increases in markers of oxidative stress, or cell cultures derived from them, have been shown after laboratory exposure to hydrocarbons and PAHs (Steadman and others 1991; Peters and others 1996; Winzer and others 2000).

Metals, eg Cd, Ni, are also oxidative stress inducers and may interfere with antioxidant enzymatic defence systems (Rodríguez-Ariza and others 1993; Van der Oost and others 2003; Romeo and others 2000). Specific components of the anti-oxidant system (including superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR), and levels of total glutathione) can be measured as biomarkers of pollutant-mediated oxidative stress (Regoli, and others 2002), although their ecological relevance may be restricted (Company and others 2006), as the responsiveness of antioxidants to chemical stress is difficult to predict, especially when considering different species, complexity of chemical mixtures in environmental matrices, duration and intensity of exposure, and various adaptation mechanisms (Regoli and others 2002). Because of this complexity, summarising the overall biological effects in terms of susceptibility to oxidative stress is problematic, and it has been proposed that analyses of individual antioxidants be integrated with a more holistic measurement of the whole capability of a tissue to neutralise specific oxyradicals (Winston and others 1998).

The TOSC assay measures the overall capacity of a tissue to absorb different reactive oxygen species (ROS), and can provide a quantifiable value of the susceptibility of a biological tissue to oxidative stress (Regoli 2000; Regoli & Winston 1999). Reduced TOSC has been shown to correlate with DNA damage (Regoli 2003; Frenzilli and others 2001) and with lysosomal stability (Regoli 2000). In contrast, Regoli and others (2002) found significantly higher TOSC values in red mullet (M. barbatus) trawled from an offshore dredge-disposal site, compared to those trawled from areas remote from the site. Regoli and others (2004) conducted time course experiments using mussels Mytilus galloprovincialis, which suggest a biphasic trend for TOSC. The mussels were caged for four weeks in an industrialised harbour in north-west Italy, and sampled at different time intervals. TOSC values showed no variation or increase during the first two weeks of exposure in the polluted site, followed by a progressive decrease up to a severe depletion in the final part of the experiment (Regoli and others 2004).

Recent field studies also have shown increases and decreases in TOSC: enhanced TOSC values (toward peroxyl radicals) were found in mussels Mytilus edulis caged along a contamination gradient, seaward from the River Elbe in the German Bight, whilst TOSC values in mussels caged along a pollution gradient from an oil rig in Statfjord were characterised by a reduction in the overall ability to neutralise peroxyl radicals in those closer to the platform (Regoli and others 2006). This could be considered to be predictive of the occurrence of oxyradical-mediated damage at various cellular targets, however careful interpretation of such results is essential and TOSC, like almost all biomarkers, appears to be more valuable when measured as part of a multi-biomarker approach.

Methods

Equipment

- functional GC system (N.B. note safety reg. and advice regarding GC operations - read instrument manuals and local SWPs);
- gas-tight syringe with side-port needle;

- shaking water-bath; and
- butyl rubber lined aluminium septum caps (Fisher Scientific Part No. BTS-370-010S) and suitable LSC vials (volume determined and pre-cleaned).

Chemicals

- 1M KPi (CAS no. 7758-11-4, Analytical Grade)- 13.609g KH_2PO_4 into 50ml de-ionised distilled water (DDW) - adjust pH to 7.5 with KOH - make to final volume of 100ml - store at 0 - 4°C;
- peroxy radical generator: 200mM 2,2'-azobis-amidinopropane dihydrochloride (ABAP - CAS no. 2997-92-4) (Fluka 11633, Sigma-Aldrich 44,091-4) azodiisobutyr-amidine dihydrochloride, or 2,2'- azobis(2-methylpropionamidine) dihydrochloride :- 54.25mg per ml of DDW. (Stores up to 2 days at 0 - 8°C in darkness);
- hydroxy radical generator:

  a) 92.5µM/185µM $FeCl_3$/EDTA : 0.5mg $FeCl_3.6H_2O$ (CAS no. 10025-77-1, Analytical Grade)/1.38mg Na_2 EDTA.$2H_2O$ (CAS no. 6381-92-6, Analytical Grade) per 20ml DDW. Adjust pH to approx.7 with small additions of 1M NaOH (use 10µl pipette for 2µl additions). Check with pH paper. Store frozen in a plastic vial. Thaw and mix just before use;
  b) 1.85mM sodium ascorbate (CAS no. 134-03-2, Analytical Grade): 0.367mg per ml DDW, make fresh per day of assay;

- peroxynitrite radical generator:

  a) 5mM diethylenetriamine-pentaacetic acid (DTPA - CAS No. 67-43-6) (Sigma-Aldrich D6518) : 1.97mg per ml in DDW. Make fresh per day of assay;
  b) 800µM 3-morpholinosyd-nonimine hydrochloride (SIN-1 - CAS No. 33876-97-0) (Sigma-Aldrich M5793) : 0.165mg per ml DDW. Make fresh per day of assay; and

- 2mM keto-methiolbutyric acid (KMBA - CAS no. 51828-97-8):- 0.34mg per ml DDW. Keep on ice.

Personnel

One person experienced in the use of GC.

Time

To perform this assay on 10 samples requires approximately eight hours.

Protocol

1) Perform assay incubations in batches in standard liquid scintillation vials (mean capped internal volume of 23.72±0.16ml, n = 21), with butyl rubber-sealed aluminium screw-caps.
2) Make up total incubation mixture volumes to 1ml with use of appropriate additions of nannopure water.
3) Typically ten vials (eight test samples with two control BSA/buffer only samples) can be analysed per assay.
4) Incubation mixtures comprise, 100µmoles KH_2PO_4 (pH 7.5), 200µl of preparation buffer (20mM Tris/HCl - pH 8.6) for control samples, or various balance (make-up) volumes of the same to experimental samples (to bring total Tris/HCl buffer volume added to 200µl – ie. including volume of buffer contributed by the samples that contain 50µg protein, 200nmoles of KMBA in 100µl nannopure water.
5) Loosely cap all vials, place into a shaking water-bath at 35°C and allow to equilibrate.
6) Quick-thaw frozen (-70°C) samples at 35°C and hold at 0°C in an ice bath.
7) Sequentially add volumes containing 50µg of sample protein from these supernatants to the incubation solutions at timed (2 min) intervals, mix and place in water-bath at 35°C.
8) After 1 min equilibration of each sample addition at 35°C, add 20µmoles of 2,2'-azobis-amidinopropane dihydrochloride (ABAP : CAS No. 2997-92-4) radical generating reagent

(100µl in nanopure water) to each vial, screw the cap down tightly and place into the shaking water-bath to ensure mixing of contents (incubation zero-time).

9) At 20 min intervals sequentially withdraw a 0.3ml sample of head-space gas from each vial with a syringe (1ml Vici Precision Sampling Inc. Pressure-Lok series A-2, with side-port needle) and immediately inject into a GC for analysis.
10) Repeat sequence at 40 and 60 min intervals from each zero-time.
11) Analyse the gas on a 2metre x 3mm OD packed column of Porapak Q (80 - 100 mesh) maintained at 75°C in a Varian Star 3400 CX gas chromatograph.
12) Set carrier and fuel gas (nitrogen and hydrogen, respectively) flow rates at 30 ml/min, set the oxidant gas (air) flow rate at 300ml/min.
13) Set injector and detector oven temperatures at 120°C and 200°C, respectively.
14) Detect ethylene gas with a flame ionisation detector (FID) and quantify the signal by measurement of the peak heights plotted on a chart recorder.
15) Plot peak heights against time of incubation (intervals of 20, 40 and 60 min from zero-time) for all samples and controls.
16) Enter data into Microsoft Excel spreadsheet and determine equations and R2 values for each peak versus time curve (normally 3rd order polynomial).
17) Determine the area beneath each curve and calculate sample TOSC as follows:

    Sample TOSC = 100-(area under sample curve/area under control curve)*100; and

    Specific TOSC = Sample TOSC/mg protein in sample.

Health and safety

Chemicals - Wear gloves, lab coats and eye-protection. Use automatic pipettes. Be aware of any specific hazards - consult MSDS sheets.

Acids and $FeCl_3$ are corrosive.

Apply/consult other SWPs (specifically, tissue preparation, centrifugation, compressed gases and GC)

Handle syringe with needle carefully - potential puncture wounding. Be aware of hazard.

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Appendix 4 - Raw data

Table C Raw data for *C. maenas* sampled from the Plymouth sound & Estuaries SAC in 'winter'

Site	Heart rate	Ache	Frap	NR	Phag	Protein	Length
Torpoint	92.9	0.533378	2.466066	2.318526	22.16541	56.301	42
Torpoint	91.8	0.343154	3.312347	2.288396	17.52105	44.982	51
Torpoint	81.13333	0.276257	2.305435	2.074967	19.24029	45.08	46
Torpoint	84.13333	0.55037	2.278729	2.629213	18.75968	51.254	50
Torpoint	108.3	0.558565	2.708326	2.845331	29.26556	47.824	46
Torpoint	108.3333	0.693372	1.406768	2.360343	17.33136	49.441	44
Torpoint	96.26667	0.474017	2.797028	1.792953	13.50396	60.368	40
Torpoint	73.3						
average	**92.02083**	**0.489873**	**2.467814**	**2.329961**	**19.6839**	**50.75**	**45.57143**
stdev	**12.42795**	**0.140886**	**0.586999**	**0.34432**	**4.956834**	**5.762916**	**3.994043**
Cove Head	90.06667		5.000884	0.572392	6.609366		55
Cove Head	99.93333	1.847991	3.68958	2.369048	11.71047	39.6	53
Cove Head	80.66667	1.509078	6.584755	1.356452	11.92816	60.75	57
Cove Head	122.7	1.858005	6.489835	2.78169	4.377395	47.95	51
Cove Head	98.53333	1.312089	4.909053	1.497201	8.375977	63.45	53
Cove Head	86.66667	0.633899	3.514812	4.780423	9.087995	59.65	51
Cove Head	99.3	0.67732	5.97499	2.317844	11.74334	54.55	51
Cove Head	111.9	1.695631		2.065966	5.772053	51.9	54
average	**98.72083**	**1.362002**	**5.166273**	**2.217627**	**8.700595**	**53.97857**	**53.125**
stdev	**13.6103**	**0.519063**	**1.252956**	**1.248399**	**2.94303**	**8.321251**	**2.167124**
Town Quay	93.16667	1.948606	7.27496	10.1125	13.06115	24.8	62
Town Quay	116.2333	2.423615	6.760264	6.46732	8.264128	18.95	50
Town Quay	87.13333	2.129149	6.595276	5.599338	9.302312	19.05	51
Town Quay	102.0333	3.021762	5.243508	3.257143	4.8967	75.55	44
Town Quay	81.96667	4.22219	5.364627	3.959641	10.21618	40.85	45
Town Quay	101.5333	2.842787	2.890966	1.902604	4.144297	65.75	48

Table continued…

Site	Heart rate	Ache	Frap	NR	Phag	Protein	Length
Town Quay	98.36667	1.898702	15.16602	4.145161	5.816049	21.75	47
Town Quay	111.2333	2.303436	8.276447	3.727551	5.113663	37.15	47
average	**98.95833**	**2.598781**	**7.196509**	**4.896407**	**7.60181**	**37.98125**	**49.25**
stdev	**11.51166**	**0.767669**	**3.604463**	**2.524952**	**3.130986**	**21.87371**	**5.650537**
Neal Point	85.8	2.174062	7.709704	7.727273	6.414941	40.6	50
Neal Point	84.83333	2.086394	8.673066	3.042796	6.519742	27.4	47
Neal Point	75.2	1.953192	7.35735	5.228155	7.872669	50.95	55
Neal Point	56	2.056068	6.486273	1.932177	9.245054	38.1	48
Neal Point	100.2333	1.921962	6.633586	2.905102	5.629825	46.15	49
Neal Point	60.83333	1.9728	5.192085	7.472789	8.596597	32.85	48
Neal Point	96.6	2.831788	7.075342	1.908297	6.294118	30.7	53
Neal Point	69.13333	1.883356	6.884798	4.539409	6.020728	39.6	50
average	**78.57917**	**2.109953**	**7.001526**	**4.3445**	**7.074209**	**38.29375**	**50**
stdev	**16.08142**	**0.306754**	**1.008265**	**2.316675**	**1.320955**	**7.882505**	**2.725541**

Table D Raw data for *M. edulis* sampled from the Plymouth sound & Estuaries SAC in 'winter'

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Whitsand	24.13	2.03	121.37	10.61	2.47	23.21	0.55	1.43	48.48	X
Whitsand	21.53	2.90	61.46	10.91	3.48	34.68	0.43	0.62	52.22	5
Whitsand	40.67	1.34	45.81	30.61	0.74	28.73	0.54	1.12	38.17	4
Whitsand	35.10	1.42	53.82	12.43	1.54	24.66	0.61	0.99	39.11	7
Whitsand	21.60	2.49	63.94	13.40	1.85	23.11	0.59	0.79	47.54	2
Whitsand	18.00	1.15	80.15	14.75	1.69	18.15	0.80	1.13	42.86	2
Whitsand	39.10	1.51	126.73	22.97	1.13	35.04	0.41	0.95	42.86	3
Whitsand	35.13	0.47	70.43	12.49	2.50	29.69	0.42	1.16	42.86	X
average	**29.41**	**1.66**	**77.96**	**16.02**	**1.92**	**27.16**	**0.55**	**1.02**	**44.26**	**3.83**
stdev	**9.00**	**0.78**	**30.26**	**7.08**	**0.87**	**5.94**	**0.13**	**0.25**	**4.80**	**1.94**
Torpoint	35.77	1.74	97.85	25.06	0.74	13.17	0.76			4
Torpoint	39.30	2.83	145.26	11.84	3.87	15.35	0.19			9
Torpoint	40.37	3.04	121.42	17.88	3.12	15.90	0.32			7
Torpoint	40.10	2.25	183.69	27.69	10.24	17.63	0.07			8
Torpoint	37.07	2.70	170.38	20.01	3.48	21.28	0.32			5
Torpoint	42.13	2.15	134.70	26.89	4.62	22.36	0.28			7
Torpoint	36.50	2.54	115.60	42.71	3.68	13.57	0.34			6
Torpoint	44.10	0.84	231.58		7.79	15.00	0.10			4.00
average	**39.42**	**2.26**	**150.06**	**24.58**	**4.69**	**16.78**	**0.30**			**6.25**
stdev	**2.88**	**0.71**	**43.39**	**9.76**	**2.97**	**3.41**	**0.21**			**1.83**
Cove Head	50.07	0.21	64.77	11.34	1.14	24.15	0.97	1.03	51.10	1
Cove Head	40.03	0.15	98.61	22.64	2.00	22.55	0.58	1.35	43.77	9
Cove Head	32.47	0.43	88.20	12.15	1.57	18.66	0.89	2.86	27.66	2
Cove Head	30.47	0.03	95.25	21.57	1.34	15.99	1.17	1.53	27.66	6
Cove Head	25.63	0.00	100.09	13.92	1.39	16.59	0.99	1.41	11.98	4
Cove Head	28.77	0.10	89.13	13.83	1.48	16.58	1.10	1.51	45.96	3
Cove Head	28.13	0.18	111.18	14.32	2.04	16.24	0.55	1.65	39.57	3
Cove Head	38.53	0.00	116.25	16.46	2.91	19.76	0.47	2.58	28.94	9
average	**34.26**	**0.14**	**95.44**	**15.78**	**1.73**	**18.81**	**0.84**	**1.74**	**34.58**	**4.63**
stdev	**8.12**	**0.14**	**15.79**	**4.20**	**0.57**	**3.11**	**0.27**	**0.63**	**12.82**	**3.07**

Table continued…

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Town Quay	41.07	1.26	121.67	11.28	1.60	22.67	0.59	2.65	23.23	4
Town Quay	37.07	0.64	120.81	18.92	8.36	20.88	0.16	2.40	16.87	4
Town Quay	35.93	1.21	221.47	19.58	3.24	20.73	0.39	1.83	59.35	4
Town Quay	40.80	2.41	127.24	10.01	1.54	17.24	0.74	2.14	43.09	5
Town Quay	40.23	2.01	89.49	12.56	1.28	16.93	0.49	2.12	57.32	2
Town Quay	50.47	1.79	76.83	17.74	1.55	15.20	0.54	1.94	53.25	1
Town Quay	38.67	2.01	88.13	20.03	1.81	17.97	0.54	1.87	43.09	3
average	**40.60**	**1.62**	**120.81**	**15.73**	**2.77**	**18.80**	**0.49**	**2.14**	**42.31**	**3.29**
stdev	**4.76**	**0.61**	**48.58**	**4.28**	**2.55**	**2.66**	**0.18**	**0.30**	**16.57**	**1.38**
Neal Point	38.70	1.87	93.37	18.98	2.07	23.93	0.38	1.99	60.00	3
Neal Point	34.90	1.04	105.66	21.97	1.49	21.82	0.51	1.32	46.21	2
Neal Point	39.53	1.43	169.56	17.60	2.39	16.03	0.36	1.74	57.78	2
Neal Point	26.20	1.74	161.63	23.43	2.51	20.74	0.28	1.96	60.00	3
Neal Point	31.00	2.02	150.01	16.31	3.07	15.82	0.29	2.13	42.22	3
Neal Point	30.27	0.48	75.33	16.67	4.32	26.39	0.10	1.48	37.78	3
Neal Point	41.63	1.59	203.22	22.30	2.35	21.42	0.40	1.78	64.44	2
Neal Point	32.03	0.69	171.67	26.25	1.43	17.52	0.62	1.27	55.01	9.00
average	**34.28**	**1.36**	**141.30**	**20.44**	**2.46**	**20.46**	**0.37**	**1.71**	**52.93**	**3.38**
stdev	**5.33**	**0.56**	**44.66**	**3.58**	**0.93**	**3.78**	**0.16**	**0.32**	**9.63**	**2.33**

Table E Raw data for *C. maenas* sampled from the Plymouth sound & Estuaries SAC in 'spring'

Site	Heart rate	Ache	Frap	NR	Phag	Protein	MN
Torpoint	83.4	5.895587	7.227975	0.418023	12.66738	64.45	2
Torpoint	76.26667	1.360731	6.951194	0.604034	8.088036	65.3	1
Torpoint	72.3	2.463482	5.758553	0.589318	33.98144	67.05	3
Torpoint	109.5667	2.755902	5.748084	1.187556	24.29971	68.65	3
Torpoint		2.988058	10.38499	0.85061	44.87958	18.25	2
Torpoint	79.83333	4.157932		X	X	12.2	1
Torpoint	99.2	2.403603	5.536273	X	X	69.5	5
Torpoint	102.7667	5.909791	11.61859	X	X	19.15	3
average	**89.04762**	**3.491886**	**7.603665**	**0.729908**	**24.78323**	**48.06875**	**2.5**
stdev	**14.56565**	**1.675324**	**2.433925**	**0.298703**	**15.1181**	**26.24188**	**1.309307**
Cove Head	101.5667	X	X	X	X	X	X
Cove Head	88.06667	1.854675	4.564242	1.622576	12.15337	51.15	X
Cove Head	81.86667	5.355044	31.28213	1.85567	35.74773	10.35	2
Cove Head	94.96667	2.59362	11.78199	0.760152	21.83891	25.1	1
Cove Head	100.0667	1.370666	2.35071	0.39486	11.29333	66.2	3
Cove Head	89.83333	3.256955	3.261054	0.45124	6.34262	72.1	1
Cove Head		X	X	X	X	X	X
Cove Head		X	X	X	X	X	X
average	**92.72778**	**2.886192**	**10.64802**	**1.0169**	**17.47519**	**44.98**	**1.75**
stdev	**7.5507**	**1.555566**	**12.11896**	**0.678823**	**11.65546**	**26.54385**	**0.957427**
Town Quay	100.4333	3.537564	8.699279	1.528266	37.06395	33.3	2
Town Quay	105.9333	2.83237	15.85065	1.235849	66.14596	25.35	3
Town Quay	94	1.473971	4.077797	0.515942	23.03987	59	1
Town Quay	103.8333	1.822048	4.240857	0.636295	13.22666	47.85	1
Town Quay	82.96667	1.6089	8.97367	1.523196	25.73114	36.65	2
Town Quay	94.03333	X	X	X	X	X	3
Town Quay	92.6	2.823406	9.643022	1.317949	17.76586	32.1	2
Town Quay	119.8333	2.49779	6.104708	1.167932	6.206383	56.5	2
average	**99.20417**	**2.370864**	**8.22714**	**1.132204**	**27.02569**	**41.53571**	**2**
stdev	**11.05103**	**0.761718**	**4.052059**	**0.40455**	**19.8256**	**12.98402**	**0.755929**

Table continued…

Site	Heart rate	Ache	Frap	NR	Phag	Protein	MN
Neal Point	80.53333	1.883981	9.508342	0.353496	8.555741	50.95	1
Neal Point	92.66667	2.251887	9.733058	0.329334	11.2669	56.9	0
Neal Point	85.3	2.458035	6.746448	0.594816	19.56562	53.35	2
Neal Point	72.96667	2.258036	6.999892	0.877358	7.313223	46.1	1
Neal Point	65.83333	2.403469	8.937806	1.081414	6.178549	46.5	4
Neal Point	66.2	3.010618	9.955661	0.975295	14.40936	59	1
Neal Point	78.9	2.770378	9.089339	0.90682	18.36876	56	2
Neal Point	70.8	4.306337	8.509082	3.005282	18.33419	51.75	1
average	**76.65**	**2.667843**	**8.684953**	**1.015477**	**12.99904**	**52.56875**	**1.5**
stdev	**9.452824**	**0.744694**	**1.209889**	**0.852068**	**5.398379**	**4.697563**	**1.195229**
Jupiter Point	73.36667	3.790336	5.603579	1.285912	26.18089	57.55	1
Jupiter Point	72.16667	4.189428	5.142044	0.983764	19.06579	29.85	X
Jupiter point	72.83333	2.848514	7.138335	1.183446	24.85615	43.85	3
Jupiter Point	84	2.631658	9.72897	0.563181	34.43495	39.8	2
Jupiter Point		3.36277	9.286457	0.401253	12.95868	31.75	X
Jupiter Point	80	0.874996	13.95064	1.407596	8.168613	36.1	1
Jupiter Point	107.7667	1.839616	9.162748	1.527417	7.898629	60.4	1
Jupiter Point	102.8333	3.390292	10.86971	1.004342	10.7766	53.35	0
average	**84.70952**	**2.865951**	**8.86031**	**1.044614**	**18.04254**	**44.08125**	**1.333333**
stdev	**14.77506**	**1.082346**	**2.887751**	**0.395216**	**9.720736**	**11.77339**	**1.032796**

Table F Raw data for *M. edulis* sampled from the Plymouth sound & Estuaries SAC in 'spring'

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Whitsand	42.10	1.55	47.06	7.19	0.73	45.91	0.94	28.3706	73.3	3
Whitsand	30.10	1.21	75.64	14.41	1.84	24.94	1.07	26.0391	48.7	6
Whitsand	37.27	0.76	59.02	8.18	7.28	34.27	0.36	23.5304	47.7	2
Whitsand	30.17	0.88	45.22	15.19	0.74	16.12	0.51	25.9052	73.3	1
Whitsand		0.90	52.55	12.13	1.38	40.56	0.67	24.2132	60.5	5
Whitsand	25.23	0.87	131.42	5.67	2.27	35.96	0.57	29.0834	53.8	2
Whitsand	50.93	1.07	62.56	14.63	0.26	51.51	0.88	31.7515	66.7	4
Whitsand	23.47	1.03	19.34	4.30	X	X	1.34	30.1801	64.1	3
average	**34.18**	**1.03**	**61.60**	**10.21**	**2.07**	**35.61**	**0.79**	**27.38**	**61.03**	**3.25**
stdev	**9.84**	**0.25**	**32.57**	**4.38**	**2.40**	**12.10**	**0.32**	**2.92**	**10.18**	**1.67**
Torpoint	31.77	0.89	134.81	19.36	1.88	45.00	0.60	34.8067	46.7	9
Torpoint		0.84	142.48	42.80	1.57	35.84	0.33	60.9492	52.0	3
Torpoint	28.77	1.01	84.92	11.42	1.78	25.92	1.11	45.0723	54.7	4
Torpoint	48.43	0.83	50.85	3.65	2.50	23.45	0.95	26.1021	50.9	5
Torpoint	31.57	1.01	160.99	15.26	0.18		0.75	39.0839	36.0	8
Torpoint	19.43	0.93	155.20	16.99	X	X	0.65			4
Torpoint	25.27	1.07	109.53	25.39	X	X	0.79			3
Torpoint	25.17	0.86	77.23	26.93	X	X	0.64			7
average	**30.06**	**0.93**	**114.50**	**20.22**	**1.58**	**32.55**	**0.73**	**41.20**	**48.05**	**5.38**
stdev	**9.17**	**0.09**	**40.27**	**11.77**	**0.86**	**9.88**	**0.24**	**13.02**	**7.33**	**2.33**
Cove Head	36.07	0.79	111.17	4.76	4.90	20.40	0.11	21.8268	27.5	7
Cove Head	40.77	1.74	405.32		3.94	47.85		35.8702	40.0	3
Cove Head	36.77	1.27	140.11	10.49	1.12	48.99	0.50	20.8800	50.0	3
Cove Head	40.37	1.60	34.77		3.76	31.33	0.31	35.8602	69.0	5
Cove Head	30.40	1.58	29.66	12.94	2.69	13.49	0.61	22.3486	49.0	2
Cove Head	25.83	0.74	104.34	18.77	3.09	21.09	0.65	24.8204	50.0	3
Cove Head	29.57	0.91	142.13	4.76	1.73	13.73	0.66	21.7276	46.5	7
Cove Head	42.60	1.16	151.24	11.16	2.86	9.02	0.40	38.6138	45.0	4
average	**35.30**	**1.22**	**139.84**	**10.48**	**3.01**	**25.74**	**0.46**	**27.74**	**47.13**	**4.25**
stdev	**6.07**	**0.39**	**117.03**	**5.30**	**1.22**	**15.51**	**0.20**	**7.62**	**11.58**	**1.91**

Table continued…

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Town Quay	41.27	1.55736	80.62	8.01	2.01	28.29	1.03	54.0949	53.5	4
Town Quay	31.10	0.45225	66.58	17.51	0.73	9.87	0.87	43.1568	40.6	2
Town Quay	35.47	1.00377	180.99	14.11	3.19	30.18	0.31	49.8593	40.6	2
Town Quay	28.33	0.73096	86.01	9.67	1.66	16.30	0.67	54.4226	49.9	3
Town Quay	43.10	1.1072	86.95	7.03	2.15	27.70	0.89	33.2243	35.4	6
Town Quay	27.17	1.18125	79.20	9.15	2.59	11.19	0.72	19.3528	45.7	3
Town Quay	40.53	1.12937	80.10	6.99	1.46	13.08	1.00	32.3642	44.7	4
Town Quay	36.77	0.76592	175.16	11.94	3.04	14.89	0.39	18.3256	38.0	5
average	**35.47**	**0.99**	**104.45**	**10.55**	**2.11**	**18.94**	**0.73**	**38.10**	**43.54**	**3.63**
stdev	**6.07**	**0.34**	**45.88**	**3.73**	**0.83**	**8.37**	**0.27**	**14.57**	**6.09**	**1.41**
Neal Point	33.60	1.62298	96.60	8.09	2.76	23.18	1.00	32.9471	42.0	3
Neal Point	26.30	1.11218	114.94	8.71	2.22	20.10	1.13	29.1079	24.6	2
Neal Point	25.87	1.21536	62.97	2.75	7.48	26.79	0.58	34.9971	50.7	6
Neal Point	40.07	1.89777	84.51	10.99	0.66	13.72	1.46	53.6727		1
Neal Point	36.60	1.58599	108.69	11.87	4.36	14.02	0.73	28.8290	46.7	2
Neal Point	26.63	1.58078	101.85	11.85	6.99	9.95	0.44	46.2471	59.4	3
Neal Point	41.43	1.53789	62.23	12.85	2.01	13.12	0.93	31.8858	35.1	3
Neal Point	25.03	1.16336	94.48		2.37	15.53	0.85	21.2940	13.0	4
average	**31.94**	**1.46**	**90.78**	**9.59**	**3.61**	**17.05**	**0.89**	**34.87**	**38.80**	**3.00**
stdev	**6.82**	**0.27**	**19.65**	**3.48**	**2.46**	**5.73**	**0.32**	**10.35**	**15.90**	**1.51**
Jupiter Point	25.90	1.87	141.38	24.97	1.91	49.90	0.46	31.3378	51.8	2
Jupiter Point	16.00	1.78	93.66	19.95	0.85	39.08	0.84	35.5907	59.0	2
Jupiter Point	27.48	1.70	105.09	18.31	2.07	41.31	0.79	35.7722	56.6	3
Jupiter Point	32.34	1.28	119.02	20.12	0.56	25.73	0.54	27.1390	32.5	4
Jupiter Point	1.60	147.79	23.14	0.63	18.76	0.61	34.4014	48.4	3	
Jupiter Point	25.45	1.34	191.23	39.61	1.86	42.53	0.37	34.1184	56.6	8
Jupiter Point	34.48	1.36	85.36	32.33	0.51	25.82	0.75	36.1469	58.1	2
Jupiter Point	25.90	1.38	148.20		0.56	35.75	0.52	28.3322	50.8	3
average	**26.79**	**1.54**	**128.97**	**25.49**	**1.12**	**34.86**	**0.61**	**32.85**	**51.75**	**3.38**
stdev	**5.92**	**0.23**	**34.99**	**7.78**	**0.69**	**10.48**	**0.17**	**3.51**	**8.63**	**2.00**

Table G Raw data for *C. maenas* sampled from the Fal & Helford SAC in 'spring'

Site	Heart rate	Ache	Frap	NR	Phag	Protein	MN
Gillian 1	64.73	1.26	6.28	0.83	7.54	60.10	X
	74.57	0.74	4.84	3.58	18.89	65.10	1
	78.00	2.38	5.80	1.53	12.01	72.35	1
	60.57	1.10	4.29	1.89	13.24	50.05	2
	91.03	1.34	6.01	0.55	13.28	62.90	1
	77.03	X	X	3.28	9.99	X	X
	70.60	4.00	24.74	10.30	53.90	19.05	2
	80.47	1.69	6.92	3.61	21.81	26.95	1
average	**74.63**	**1.79**	**8.41**	**3.20**	**18.83**	**50.93**	**1.33**
stdev	**9.50**	**1.10**	**7.25**	**3.11**	**14.89**	**20.33**	**0.52**
Helford 2	111.30	2.08	4.21	3.00	13.56	39.55	2
	109.60	1.02	7.36	3.69	16.10	45.55	1
	53.93	2.99	21.47	6.96	54.81	17.40	1
	72.40	X	X	1.23	8.27	X	X
	101.23	1.44	3.32	1.94	16.63	55.30	1
	78.07	1.46	4.26	0.73	17.10	60.65	2
	79.67	1.30	4.21	1.74	11.43	55.25	1
	72.67	1.56	3.39	2.75	8.65	71.25	5
average	**84.86**	**1.69**	**6.89**	**2.76**	**18.32**	**49.28**	**1.86**
stdev	**20.40**	**0.66**	**6.57**	**1.95**	**15.15**	**17.36**	**1.46**
Flushing 4	106.77	1.64	6.16	1.27	25.07	19.55	
	23.70	1.09	X	1.13	10.54	71.35	
	95.87	X	X	0.79	16.52	68.10	
	80.33	1.44	X	0.92	12.34	70.75	
	100.07	1.14	4.89	1.32	13.53	39.40	
	86.87	X	9.10	1.24	16.89	40.25	
	97.00	X	X	0.37	5.69	X	
	67.93	0.78	X	0.53	11.51	75.55	
average	**82.32**	**1.22**	**6.72**	**0.94**	**14.01**	**54.99**	
stdev	**26.67**	**0.33**	**2.16**	**0.36**	**5.70**	**21.71**	

Table continued…

Site	Heart rate	Ache	Frap	NR	Phag	Protein	MN
Weir Point 5	127.70	3.64	12.42	2.52	14.19	25.55	
	84.60	2.47	8.77	1.38	10.68	36.60	
	78.40	X	X	0.43	6.56	77.30	
		X	X	X	X	X	
	125.30	X	X	0.48	9.06	77.70	
	80.40	3.07	X	0.65	8.22	78.50	
	90.40	1.08	X	0.71	8.58	77.35	
	92.53	X	X	X	X	X	
average	**97.05**	**2.57**	**10.60**	**1.03**	**9.55**	**62.17**	
stdev	**20.75**	**1.10**	**2.58**	**0.81**	**2.63**	**24.34**	
King Harry Ferry 6		X	X	X	X	X	
	95.07	X	X	0.38	15.89	68.70	
	101.13	0.51	2.32	1.31	10.40	45.40	
	110.77	0.84	5.14	0.73	20.88	56.05	
	109.73	0.91	4.00	2.84	13.56	46.80	
	100.33	1.82	5.40	2.02	14.50	44.50	
		X	X	X	X	X	
		X	X	X	X	X	
average	**103.41**	**1.02**	**4.21**	**1.46**	**15.05**	**52.29**	
stdev	**6.68**	**0.56**	**1.40**	**0.99**	**3.84**	**10.27**	
Malpas 7	104.57	1.56	5.37	1.77	11.33	56.00	
	106.70	3.06	8.29	0.53	9.15	48.45	
	109.67	1.16	5.95	1.54	16.75	53.80	
		X	X	X	X	X	
	87.47	1.06	7.60	1.17	12.13	28.20	
	116.20	1.58	5.35	1.46	13.83	36.55	
	94.03	1.55	6.04	3.84	13.06	57.25	
	103.63	1.43	3.44	1.46	11.71	67.40	
average	**103.18**	**1.63**	**6.00**	**1.68**	**12.56**	**49.66**	
stdev	**9.63**	**0.66**	**1.59**	**1.03**	**2.36**	**13.31**	

Table H Raw data for *M. edulis* sampled from the Fal & Helford SAC in 'spring'

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Gillian 1	17.93	1.97	64.38	21.56	4.96	4.87	1.05	35.44	59.49	X
	22.90	2.86	81.88	19.28	4.02	11.17	0.77	23.05	31.65	X
	18.03	2.91	43.71	22.57	4.88	4.78	1.60	18.83	41.77	1
	17.77	1.93	79.14	12.19	6.53	10.41	1.10	26.12	48.35	2
	23.97	2.99	42.99	45.16	1.65	7.59	0.05	12.74	49.37	X
	4.63	3.44	4.83	2.53	5.44	9.01	0.83	23.32	62.03	0
	33.67	3.24	72.55	15.83	5.41	8.78	1.13	24.16	59.49	1
	16.50	0.98	91.56	14.98	4.94	8.72	0.89	20.59	39.24	1
average	**19.43**	**2.54**	**60.13**	**19.26**	**4.73**	**8.17**	**0.93**	**23.03**	**48.92**	**1.00**
stdev	**8.19**	**0.83**	**28.32**	**12.23**	**1.43**	**2.33**	**0.44**	**6.49**	**10.95**	**0.71**
Helford 2	19.87	2.42128	121.91	4.21	2.59	8.33	0.57	28.41	72.41	2
	39.73	2.87258	101.29	7.36	3.58	11.19	0.57	25.55	47.13	X
	17.23	3.62731	166.15	21.47	1.72	11.62	0.48	21.82	43.91	X
	45.97	1.13213	45.90	X	1.17	10.43	1.14	19.43	44.83	1
	33.83	2.59773	101.95	3.32	5.12	11.63	0.81	14.65	54.02	X
	21.40	3.41407	64.00	4.26	3.80	10.24	1.53			2
	27.10	3.32243	80.59	4.21	4.88	9.39	1.06	18.61	66.90	2
	37.57	3.62316	86.86	3.39	5.26	9.94	0.76	19.25	54.02	1
average	**30.34**	**2.88**	**96.08**	**6.89**	**3.52**	**10.35**	**0.86**	**21.10**	**54.75**	**1.60**
stdev	**10.48**	**0.84**	**36.86**	**6.57**	**1.56**	**1.14**	**0.36**	**4.61**	**11.07**	**0.55**
Maenporth 3	19.93	1.92801	95.93	12.31	0.50	8.12	0.17	22.42	34.88	X
	30.03	2.46712	X	X	1.82	10.92	1.04	22.02	62.79	2
	25.90	3.07671	34.06	5.65	0.64	9.67	0.53	22.69	27.91	1
	23.53	2.95263	64.45	X	5.29	9.30	1.04	25.40	36.28	3
	25.10	0.36977	X	X	7.26	12.77	0.69	12.90	44.19	7
	23.60	2.42705	64.07	5.63	6.38	9.29	1.14	12.46	34.88	4
	22.37	2.65527	52.06	21.62	0.50	7.09	0.34	12.55	50.23	X
	19.80	3.10978	196.65	12.89	5.60	11.71	0.42	22.97	65.12	2
average	**23.78**	**2.37**	**84.54**	**11.62**	**3.50**	**9.86**	**0.67**	**19.18**	**44.53**	**3.17**
stdev	**3.34**	**0.90**	**58.52**	**6.59**	**2.90**	**1.87**	**0.37**	**5.51**	**13.73**	**2.14**

Table continued…

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Flushing 4	31.47	1.88591	49.02	6.16	4.78	22.83	0.82	20.62	40.74	3
	26.43	0.80607	41.20	X	1.52	14.45	1.28	42.04	47.16	2
	20.70	1.0	86.45	X	1.41	18.00	0.87	34.17	45.68	3
	33.93	0.9	68.26	X	3.36	19.64	1.24	19.65	40.74	4
	13.00	1.1	66.44	4.89	1.50	13.02	1.12	18.60	45.68	3
	31.53	1.2	91.36	9.10	2.30	20.38	0.80	16.33	33.33	1
	28.60	1.1	44.97	X	2.05	11.97	0.60	14.86	27.41	3
	29.07	1.0	52.90	X	7.60	12.28	0.73	8.39	33.33	X
average	**26.84**	**1.13**	**62.58**	**6.72**	**3.07**	**16.57**	**0.93**	**21.83**	**39.26**	**2.71**
stdev	**6.88**	**0.33**	**18.86**	**2.16**	**2.17**	**4.17**	**0.25**	**10.94**	**7.17**	**0.95**
Weir Point 5	28.73	0.55424	X	X	2.54	25.19	0.54	41.33	44.11	2
	26.13	0.62351	105.84	X	4.07	26.77	0.64	26.37	28.77	X
	24.97	0.79898	53.21	49.11	0.97	34.37	0.76	20.90	19.45	X
	24.10	0.65606	87.80	44.79	2.70	46.36	0.42	20.60	46.85	3
		0.69727	70.96	X	1.76	30.78	0.61	43.29	24.93	2
	6.70	0.63241	86.21		0.44	23.76	0.36	19.86	23.29	X
	33.67	1.19899	X	X	2.31	21.33	1.08	19.72	9.59	1
	29.43	0.7595	29.46	27.72	6.10	6.03	0.28	40.05	49.59	1
average	**24.82**	**0.74**	**72.25**	**40.54**	**2.61**	**26.82**	**0.58**	**29.02**	**30.82**	**1.80**
stdev	**8.62**	**0.20**	**27.43**	**11.31**	**1.79**	**11.52**	**0.25**	**10.63**	**14.45**	**0.84**
King Harry Ferry 6	21.60	2.32456	126.97	19.26	0.57	66.45	0.66	14.86	29.83	X
	30.40	2.31991	147.92	23.00	4.31	47.56	0.30	21.56	37.95	2
	23.80	2.41576	112.65	18.57	4.25	26.92	0.45	26.63	36.99	5
	24.57	1.72289	304.67	21.71	3.09	28.15	0.28	13.06	18.85	1
		1.93381	108.75	18.01	0.71	36.75	0.39	14.21	47.49	3
	2.17	1.9068	116.96	16.10	5.38	25.74	0.60	21.95	37.95	2
	26.47	1.90647	311.00	37.91	3.27	22.49	0.12	25.08	30.79	1
	39.67	1.74252	119.49	14.84	3.04	20.12	0.47	25.29	14.08	1
average	**24.10**	**2.03**	**168.55**	**21.18**	**3.08**	**34.27**	**0.41**	**20.33**	**31.74**	**2.14**
stdev	**11.37**	**0.28**	**86.80**	**7.27**	**1.70**	**15.68**	**0.18**	**5.49**	**10.92**	**1.46**

Table continued…

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Malpas 7	26.93	1.26	184.40	19.31	2.37	51.64	0.57	26.05	25.06	3
	31.70	1.02	X	X	0.54	17.97	1.03	13.52	39.53	X
	25.47	0.91	77.33	14.42	3.97	35.75	0.93	23.49	25.06	X
	27.43	0.82	218.78	15.07	2.34	21.83	0.44	20.31	48.32	3
	20.97	1.29	150.80	23.09	1.89	22.19	0.52	25.69	48.32	1
	22.93	1.13	X	X	5.12	14.15	0.92	28.39	50.90	X
	23.67	1.18	71.20	5.94	4.35	17.06	1.26	18.75	40.57	1
	17.17	0.99	101.83	28.37	2.43	7.11	0.44	18.80	17.31	1
average	**24.53**	**1.07**	**134.06**	**17.70**	**2.88**	**23.46**	**0.76**	**21.87**	**36.89**	**1.80**
stdev	**4.42**	**0.17**	**60.33**	**7.77**	**1.49**	**14.01**	**0.31**	**4.91**	**12.77**	**1.10**

Table I Raw data for *C. maenas* sampled from the Fal & Helford SAC in 'autumn'

Site	Heart rate	Ache	Frap	NR	Phag	Protein
Gillian 1	77.0	2.54	3.26	1.89	19.51	54.20
	72.0	1.66	7.66	1.03	34.73	31.00
	97.6	1.97	1.65	1.16	3.31	45.20
	89.0	1.88	14.15	1.16	36.53	24.55
	77.2	2.88	5.30	1.78	33.88	47.20
	65.8	1.56	2.60	1.22	41.78	31.00
	72.3	2.25	2.55	1.28	5.24	48.60
	73.0	2.82	4.77	0.73	5.60	41.50
average	**77.98**	**2.20**	**5.24**	**1.28**	**22.57**	**40.41**
stdev	**10.35**	**0.51**	**4.08**	**0.38**	**16.07**	**10.39**
Helford 2	90.0	1.16	5.90	1.59	15.88	43.15
	73.4	2.18	9.79	1.22	25.89	34.25
	84.2	2.85	5.07	2.36	11.80	48.45
	101.1	2.23	3.50	1.60	20.83	46.10
	79.1	1.93	4.02	1.92	24.32	60.10
	111.2	6.17	2.15	1.00	37.39	42.05
	65.0	2.84	4.67	8.56	7.78	56.30
	78.0	1.88	2.78	1.03	9.73	47.10
average	**85.24**	**2.66**	**4.73**	**2.41**	**19.20**	**47.19**
stdev	**15.07**	**1.52**	**2.38**	**2.53**	**9.93**	**8.13**
Flushing 4	96.4	2.49	3.54	0.97	18.71	52.55
	93.1	1.71	5.73	0.68	28.53	51.80
	107.6	2.70	2.84			59.90
	92.8	X	X	1.53	5.21	X
	77.2	X	X			X
	121.3	1.41	5.93	0.25	42.30	42.90
	102.8	2.59	5.34	0.40	27.68	44.65
	87.7	1.78	4.07	1.88	51.93	60.95
average	**97.36**	**2.11**	**4.58**	**0.95**	**29.06**	**52.13**
stdev	**13.35**	**0.54**	**1.27**	**0.64**	**16.59**	**7.48**

Table continued…

Site	Heart rate	Ache	Frap	NR	Phag	Protein
Weir Point 5	126.2	3.21	3.45	1.73	14.23	57.05
	94.2	2.04	3.86	0.72	31.47	50.15
	87.6	1.72	5.98	1.59	22.72	39.00
	108.6	2.33	7.58	0.65	7.13	35.10
	88.0	2.42	9.42	1.22	6.05	23.35
	92.3	2.37	16.23	1.15	11.34	21.00
	99.8	2.17	5.24	0.87	52.22	41.80
	103.0	1.93	4.83	1.02	13.53	45.55
average	**99.97**	**2.27**	**7.07**	**1.12**	**19.84**	**39.13**
stdev	**12.87**	**0.45**	**4.18**	**0.39**	**15.53**	**12.45**
King Harry Ferry 6	105.7	2.06	3.33	0.81	13.10	65.05
	118.8	3.12	2.25	1.82	10.68	70.00
	92.6	3.00	1.11	2.35	15.47	62.55
	109.8	1.22	4.15	0.82	8.88	37.65
	76.3	1.36	2.60	1.42	12.64	61.05
	94.6	1.46	2.99	0.90	19.33	55.30
	87.9	6.01	8.71	1.16	13.52	18.60
	91.5	1.92	2.43	1.46	9.86	58.50
average	**97.15**	**2.52**	**3.45**	**1.34**	**12.94**	**53.59**
stdev	**13.51**	**1.58**	**2.30**	**0.54**	**3.36**	**17.08**
Malpas 7	101.4	3.17	1.59	1.37	8.65	65.40
	107.5	2.72	3.01	1.64	16.72	52.30
	124.8	3.01	2.04	0.87	11.82	67.30
	97.1	2.46	3.07	0.75	7.51	70.15
	86.6	2.82	4.22	1.64	15.96	64.85
	108.1	X	3.62	0.89	18.81	66.80
	76.1	2.13	9.31	0.84	15.71	41.25
	95.6	8.78	5.78	1.24	10.99	12.20
average	**99.65**	**3.59**	**4.08**	**1.15**	**13.27**	**55.03**
stdev	**14.72**	**2.32**	**2.48**	**0.37**	**4.10**	**19.83**

Table J Raw data for *M. edulis* sampled from the Fal & Helford SAC in 'autumn'

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Gillian 1	23.4	1.99	81.54	31.09	1.89	28.52	0.40	19.45	3.4	3
	30.3	1.82	51.93	9.25	2.81	19.23	0.75	22.56	26.6	2
	20.7	1.36	20.44	45.23	2.46	22.62	0.31	27.56	8.2	2
	22.8	1.79	98.95	6.39	3.10	33.52	0.45	33.17	29.0	8
	18.9	1.80	45.73	39.05	1.41	21.27	0.55	20.26	19.3	1
	16.7	1.63	X	X	1.32	19.05	0.74	19.60	19.3	0
	23.4	1.63	87.25	4.76	6.70	65.02	0.16	31.33	22.7	1
	23.5	0.17	X	X	3.19	36.94	0.48	17.42	21.7	x
average	**22.45**	**1.52**	**64.31**	**22.63**	**2.86**	**30.77**	**0.48**	**23.92**	**18.78**	**2.43**
stdev	**4.03**	**0.58**	**29.82**	**17.97**	**1.71**	**15.36**	**0.20**	**5.97**	**8.77**	**2.64**
Helford 2	22.5	1.35	124.68	32.29	2.18	21.37	0.65	32.46	21.7	1
	25.9	1.08	92.21	8.42	3.25	19.07	0.75	30.87	25.3	1
	35.7	1.81	165.76	21.66	2.36	14.63	0.92	32.25	21.7	0
	21.3	1.90	75.65	14.45	1.28	14.17	1.22	24.42	9.6	0
	23.7	1.16	63.27	13.86	1.45	10.56	1.32	22.68	10.5	2
	26.2	0.90	137.40	11.17	3.82	22.08	0.55	33.35	10.5	3
	25.7	1.73	104.33	14.77	3.82	25.12	0.52	40.90	26.2	0
	29.7	1.66	81.15	9.06	5.40	39.83	0.35	37.54	23.9	0
average	**26.34**	**1.45**	**105.55**	**15.71**	**2.95**	**20.85**	**0.78**	**31.81**	**18.68**	**0.88**
stdev	**4.56**	**0.38**	**34.77**	**7.88**	**1.39**	**9.04**	**0.34**	**6.06**	**7.18**	**1.13**
Maenporth 3	29.0	1.46	67.41	25.73	1.78	16.74	0.70	22.09	85.2	1
	29.6	1.31	71.78	19.90	1.27	17.43	0.88	21.77	32.9	1
	22.9	1.56	X	X	1.68	12.85	1.25	34.26	37.5	1
	32.9	1.52	49.67	14.41	1.34	15.34	0.93	20.86	28.2	2
	34.4	0.88	X	X	1.52	20.86	0.60	15.09	46.8	2
	20.8	1.53	95.75	48.39	3.47	19.15	0.61	11.73	34.3	0
	27.6	1.45	63.94	X	4.51	22.40	0.34	24.52	48.1	0
	29.0	1.36	X	X	X	X	X	19.71	30.6	x
average	**28.28**	**1.38**	**69.71**	**27.11**	**2.22**	**17.82**	**0.76**	**21.25**	**42.94**	**1.00**
stdev	**4.57**	**0.22**	**16.75**	**14.92**	**1.26**	**3.27**	**0.29**	**6.68**	**18.52**	**0.82**

Table continued…

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Flushing 4	30.3	1.01	36.58		3.09	25.39	0.43	42.01	28.5	2
	25.6	0.74	14.53		6.08	25.72	0.45	25.83	33.2	3
	29.8	1.02	X		0.85	20.05	0.47	29.63	38.8	x
	23.4	0.86	X		2.65	39.97	0.46	17.62	7.3	5
	38.5	0.61	X		5.36	22.77	0.68	18.43	9.6	1
	27.8	0.65	40.64		17.62	20.44	0.41	18.43	21.4	x
	28.2	0.69	27.79		1.74	30.25	0.53	16.12	24.7	1
	21.7	0.95	27.50		3.02	15.84	0.50	16.39	20.0	3
average	**28.15**	**0.82**	**29.41**		**5.05**	**25.05**	**0.49**	**23.06**	**22.94**	**2.50**
stdev	**5.12**	**0.16**	**10.06**		**5.36**	**7.43**	**0.09**	**9.06**	**10.84**	**1.52**
Weir Point 5	18.7	0.86	30.41		3.58	32.50	0.55	71.85	51.4	3
	30.3	0.89	63.77		1.05	47.40	0.29	31.40	28.6	4
	21.0	1.06	52.62		1.83	25.74	0.51	25.91	35.7	x
	28.9	0.98	56.99		3.30	20.04	0.70	62.36		1
	21.4	1.48	28.79		5.71	25.68	0.74	30.76	38.1	2
	22.5	1.44	52.73		4.53	32.73	0.48	25.06	37.1	4
	28.5	1.61	X		2.88	21.41	0.64	25.57	37.1	4
	26.7	1.54	32.75		4.93	19.37	0.86	34.26	42.9	2
average	**24.75**	**1.23**	**45.44**		**3.48**	**28.11**	**0.60**	**38.40**	**38.71**	**2.86**
stdev	**4.33**	**0.31**	**14.37**		**1.57**	**9.34**	**0.18**	**18.19**	**7.02**	**1.21**
King Harry Ferry 6	22.3	1.67	52.44		4.85	33.42	0.53	33.01	38.1	0
	37.5	1.48	26.83		2.60	24.62	0.54	35.70	38.1	1
	23.2	1.49	25.15		1.96	21.80	0.62	37.30	23.8	4
	24.1	1.63	28.24		3.71	12.65	0.60	36.70		2
	35.3	1.68	24.28		4.69	12.52	0.64	37.88	57.1	1
	21.5	1.49	45.48		4.08	34.41	0.56	28.30	38.1	3
	29.5	1.26	39.64		2.58	22.45	0.31	27.67	53.8	0
	20.1	1.45	38.90		2.45	14.93	0.61	37.63	38.1	1
average	**26.69**	**1.52**	**35.12**		**3.36**	**22.10**	**0.55**	**34.27**	**41.02**	**1.50**
stdev	**6.61**	**0.14**	**10.52**		**1.11**	**8.61**	**0.10**	**4.18**	**11.21**	**1.41**

Table continued…

Site	Heart rate	Feed rate	Ache	Frap	NR	Phag	Protein	MT	Tosc	MN
Malpas 7	25.9	1.07	24.35		2.33	17.45	1.05	36.41	50.0	1
	27.0	0.91	14.01		1.85	29.79	0.67	32.05		2
	25.7	0.86	18.55		3.35	18.44	0.96	38.68	52.4	1
	24.8	0.95	48.26		2.75	21.41	0.50	27.55	42.9	3
	23.8	1.13	38.80		3.28	21.08	0.72	30.48	52.4	1
	34.1	1.47	31.51		6.98	17.78	0.65	22.39	50.0	3
	24.5	0.65	33.54		3.91	14.05	0.76	20.03	31.0	1
	19.0	0.81	33.33		1.71	18.19	0.52	28.68	39.5	4
average	**25.59**	**0.98**	**30.29**		**3.27**	**19.77**	**0.73**	**29.53**	**45.44**	**2.00**
stdev	**4.19**	**0.25**	**11.05**		**1.68**	**4.65**	**0.19**	**6.37**	**8.06**	**1.20**

Table K PAH metabolites in *C. maenas* urine

		Naphthalene		Pyrenes		BaPs	
	Site	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Tamar	Whitsands			no samples			
	Torpoint	341.6	34.1	283.2	27.7	660.8	66.2
	Cove Head	593.7	147.8	345.2	79.4	1186.1	380.2
	Town Quay	419.3	5.1	247.9	30.9	688.8	107.7
	Neal Point	478.4	50.2	273.1	49.5	744.5	108.8
	Jupiter Point	488.9	5.4	396.6	10.3	908.8	122.1
Fal	Gillian	456.5	27.8	244.8	14.9	686.3	66.2
	Helford	611.8	64.3	358.3	9.1	860.9	12.2
	Flushing	671.0	145.2	432.3	89.9	1095.1	334.5
	Weir Point	448.5	43.8	279.6	34.9	639.2	91.6
	King Harry Ferry	686.8	72.8	549.0	32.7	1269.2	14.3
	Malpas	1236.6	48.7	1708.6	63.2	2704.3	133.8

Table L Microtox raw data

	Site	**% inhibition**	**St dev**
Tamar	Whitsands	-3.35	0.83
	Torpoint	8.64	1.27
	Cove Head	11.55	5.12
	Town Quay	3.56	0.63
	Neal Point	11.38	4.60
Fal	Gillian	18.61	0.91
	Helford	19.67	0.90
	Maenporth	0.14	1.01
	Flushing	5.13	0.98
	Weir Point	7.00	0.97
	King Harry Ferry	25.78	0.87
	Malpas	4.04	1.00

Table M Multivariate statistics for *M. edulis* from the Plymouth Sound & Estuaries SAC

Tamar Estuary	AChE				FRAP				Protein				Metallothionein				TOSC			
M. edulis	df	MS	F	P	df	MS	F	P	df	MS	F	P	df	MS	F	P	df	MS	F	P
Site	4	6028.867	4.390	0.010	4	198.386	4.740	0.010	4	0.041	0.780	NS	3	839.636	8.500	0.001	3	205.869		
Time	1	6146.158	4.470	0.050	1	689.544	16.460	0.001	1	0.690	13.220	0.001	1	1246.211	12.610	0.001	1	205.923		
Site x Time	4	1479.528	1.080	NS	4	15.367	0.370	NS	4	0.513	9.830	0.001	3	128.991	1.310	NS	3	553.554		
Residual	60	1374.018			60	41.890			60	0.052			56	98.793			56	76.875		
Cochran's value	C = 0.200 NS				0.2719 NS				0.3262 NS				0.1833 NS				0.2530 NS			
Transformations																				

Tamar Estuary	Micronuclei				Cellular Viability				Phagocytosis				Cardiac Rate				Clearance Rate			
M. edulis	df	MS	F	P	df	MS	F	P	df	MS	F	P	df	MS	F	P	df	MS	F	P
Site	4	19.959	5.990	0.001	4	3.763	1.020	NS	4	148.866	2.540	NS	4	77.382	1.470	NS	4	2.201	12.880	0.001
Time	1	1.114	0.330	NS	1	0.318	0.090	NS	1	496.503	8.490	0.010	1	59.709	1.130	NS	1	2.453	14.360	0.001
Site x Time	4	1.930	0.580	NS	4	9.231	2.500	NS	4	92.110	1.570	NS	4	106.418	2.020	NS	4	3.155	18.470	0.001
Residual	60	3.333			50	3.670			40	58.503			60	52.782			60	0.171		
Cochran's value	C = 0.200 NS				0.2719 NS				0.3262 NS				0.1833 NS				0.2530 NS			
Transformations																				

Table N Multivariate statistics for *C. maenas* from the Plymouth Sound & Estuaries SAC

Tamar Estuary	AChE				FRAP				Protein			
C. maenas	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
Site	3	0.230	3.130	0.050	3	0.878	4.240	0.050	3	266.750	0.930	NS
Time	1	1.812	23.930	0.001	1	1.597	7.720	0.010	1	0.865	0.000	NS
Site x Time	3	0.785	10.370	0.001	3	0.448	2.170	NS	3	94.897	0.330	NS
Residual	40	0.076			32	0.207			40	286.915		
Cochran's value	0.2897 NS				0.3506 NS				0.2649 NS			
Transformations												

Tamar Estuary	Cellular Viability				Phagocytosis				Cardiac Rate			
C. maenas	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
Site	3	7.764	3.670	0.050	3	0.829	3.410	0.050	3	854.959	4.910	0.010
Time	1	88.527	41.860	0.001	1	4.032	16.590	0.001	1	2.344	0.010	NS
Site x Time	3	5.201	2.460	NS	3	0.505	2.080	NS	3	98.123	0.560	NS
Residual	40	2.115			40	0.243			40	173.976		
Cochran's value	0.4974**				0.3086 NS				0.1989			
Transformations					Ln(x+1)							

Table O Multivariate statistics for *M. edulis* from the Fal & Helford SAC

Fal Estuary	AChE				Protein				Metallothionein				TOSC			
M. edulis	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
Site	6	4833.323	3.230	0.010	6	0.245	3.740	0.001	6	327.172	5.170	0.001	6	305.651	2.370	0.050
Time	1	40107.640	26.830	0.001	1	0.309	4.710	0.050	1	603.930	9.540	0.010	1	1774.803	13.770	0.001
Site x Time	6	8120.796	5.430	0.001	6	0.283	4.320	0.001	6	136.808	2.160	NS	6	1412.520	10.960	0.001
Residual	56	1494.821			84	0.066			84	63.289			84	128.891		
Cochran's value	0.3135*				0.1616 NS				0.4003**				0.2058 NS			
Transformations																

Fal Estuary	Micronuclei				Cellular Viability				Phagocytosis				Cardiac Rate				Clearance Rate			
M. edulis	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
Site	6	2.060	0.930	NS	6	2.698	0.590	NS	6	497.753	5.630	0.001	6	83.376	1.810	NS	6	4.233	18.430	0.001
Time	1	0.286	0.130	NS	1	0.091	0.020	NS	1	528.682	5.980	0.050	1	72.172	1.560	NS	1	6.754	29.410	0.001
Site x Time	6	4.619	2.090	NS	6	11.827	2.610	0.050	6	374.341	4.230	0.001	6	26.822	0.580	NS	6	1.439	6.260	0.001
Residual	42	2.214			84	4.540			84	88.396			84	46.117			84	0.230		
Cochran's value	0.2661 NS				0.4755**				0.2309 NS				0.2001 NS				0.2895**			
Transformations																				

Table P Multivariate statistics for *C. maenas* from the Fal & Helford SAC

Fal Estuary	AChE				FRAP				Protein			
C. maenas	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
Site	5	0.958	1.900	NS	5	28.309	2.850	0.050	5	143.427	0.690	NS
Time	1	5.333	10.570	0.010	1	103.056	10.360	0.010	1	37.763	0.180	NS
Site x Time	5	0.695	1.380	NS	5	4.975	0.500	NS	5	609.825	2.950	NS
Residual	36	0.504			24	9.944			48	206.829		
Cochran's value	0.1996 NS				0.7080**				0.2705 NS			
Transformations												

Fal Estuary	Cellular Viability				Phagocytosis				Cardiac Rate			
C. maenas	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
Site	5	3.188	3.610	0.010	5	83.652	1.890	NS	5	996.809	4.200	0.010
Time	1	1.568	1.770	NS	1	90.487	2.050	NS	1	75.466	0.320	NS
Site x Time	5	0.909	1.030	NS	5	55.354	1.250	NS	5	121.801	0.510	NS
Residual	48	0.884			48	44.223			48	237.179		
Cochran's value	0.5207				0.2410 NS				0.2190 NS			
Transformations					Arcsine							

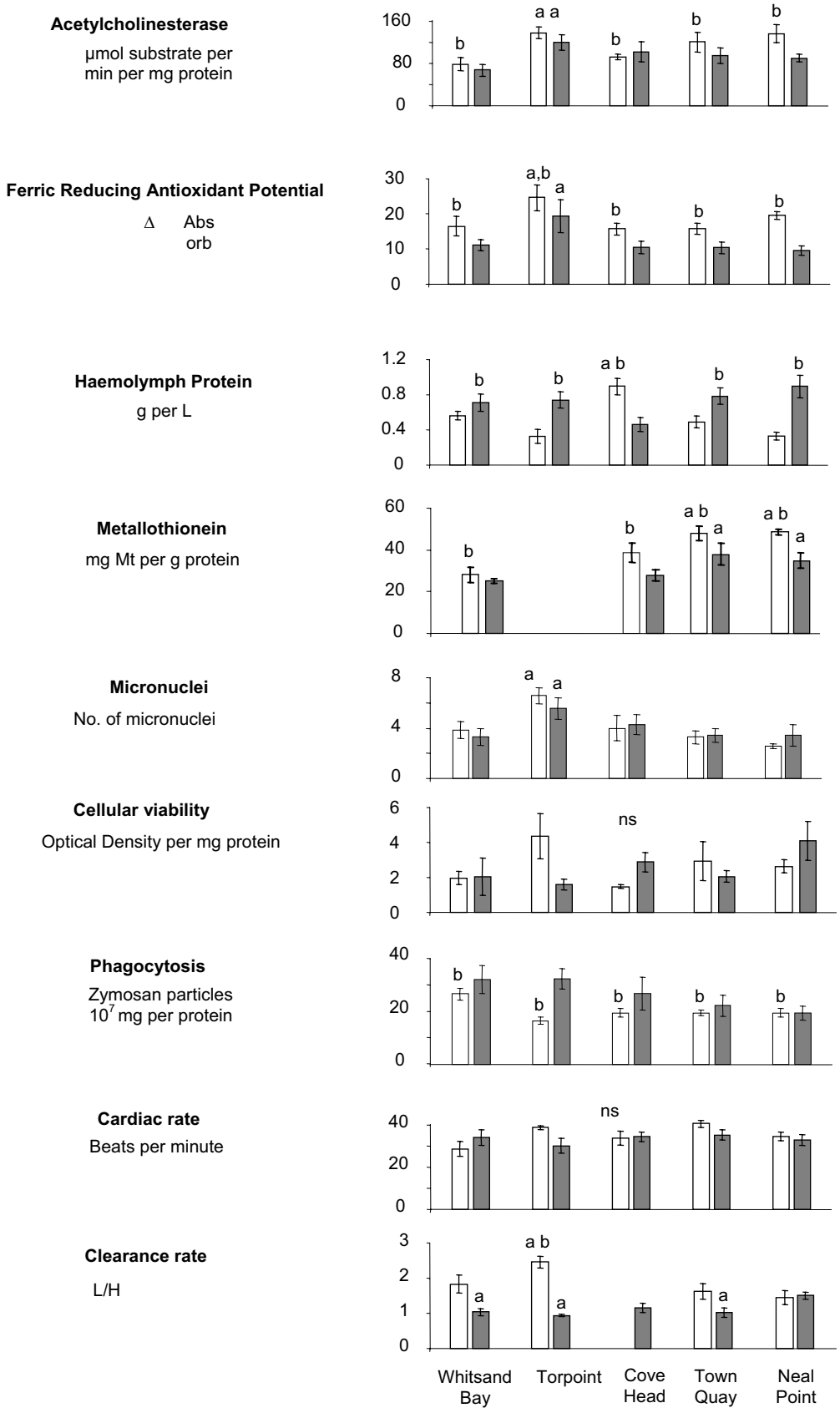

Figure G Biomarker results for Plymouth Sound & Estuaries SAC for *M. edulis*. Values expressed as mean ± standard error. Statistical significance is denoted by 'a' (site effect) and 'b' (time effect) and 'ns' no difference

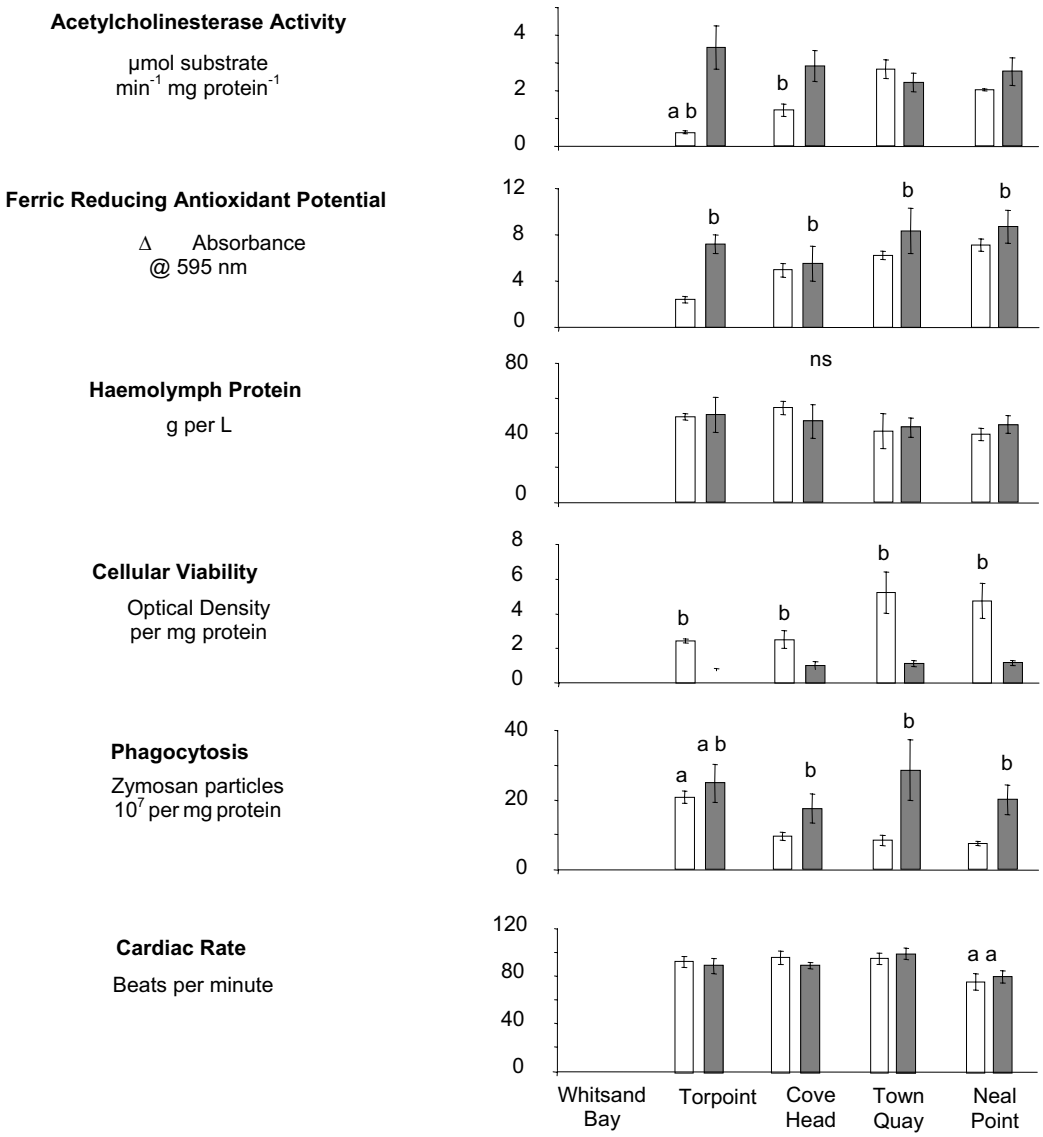

Figure H Biomarker results for Plymouth Sound & Estuaries SAC for *C. maenas*. Values expressed as mean ± standard error. Statistical significance is denoted by 'a' (site effect) and 'b' (time effect) and 'ns' no difference

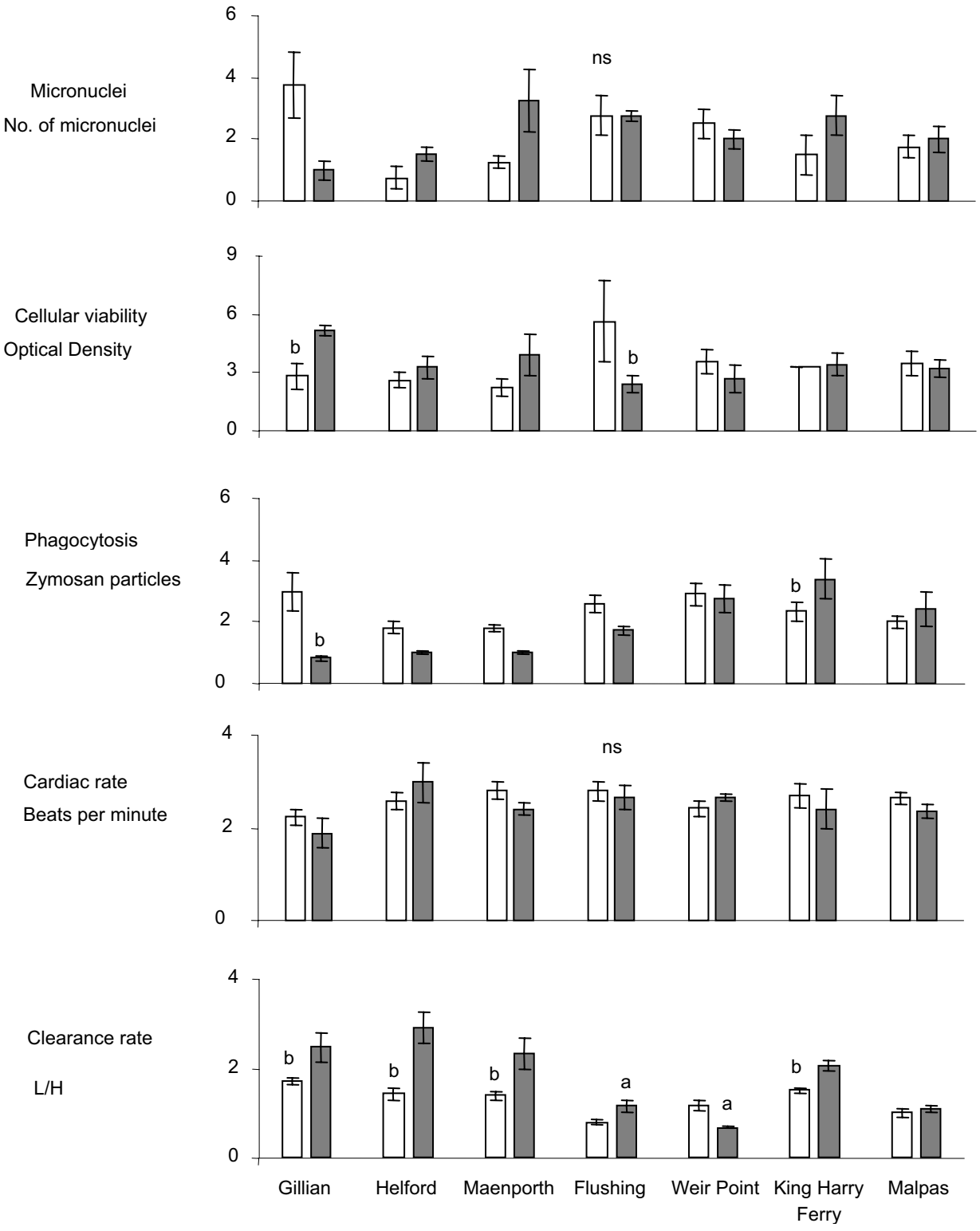

Figure continued…

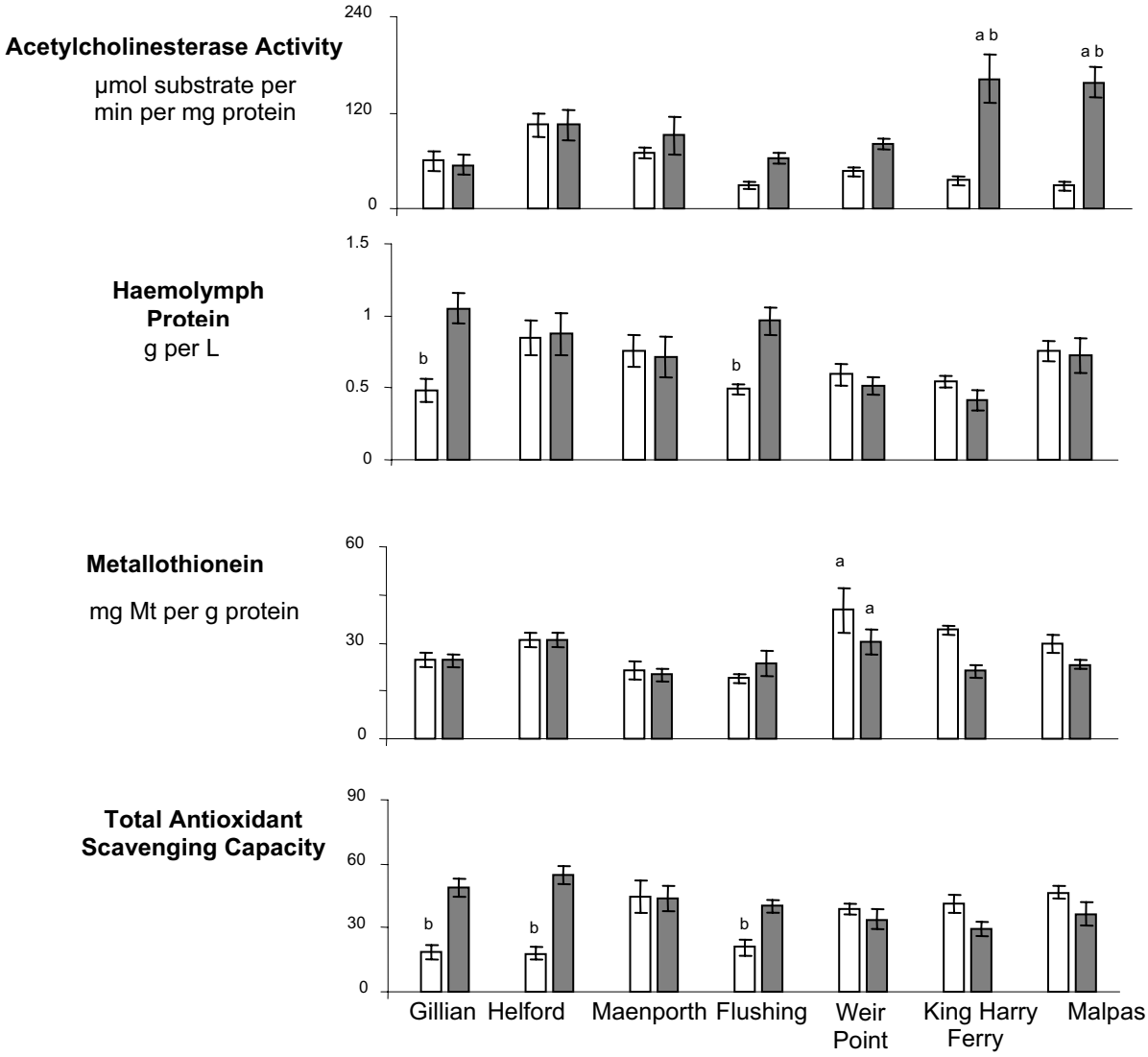

Figure I Biomarker results for Fal & Helford SAC for M. edulis. Values expressed as mean ± standard error. Statistical significance is denoted by 'a' (site effect) and 'b' (time effect) and 'ns' no difference

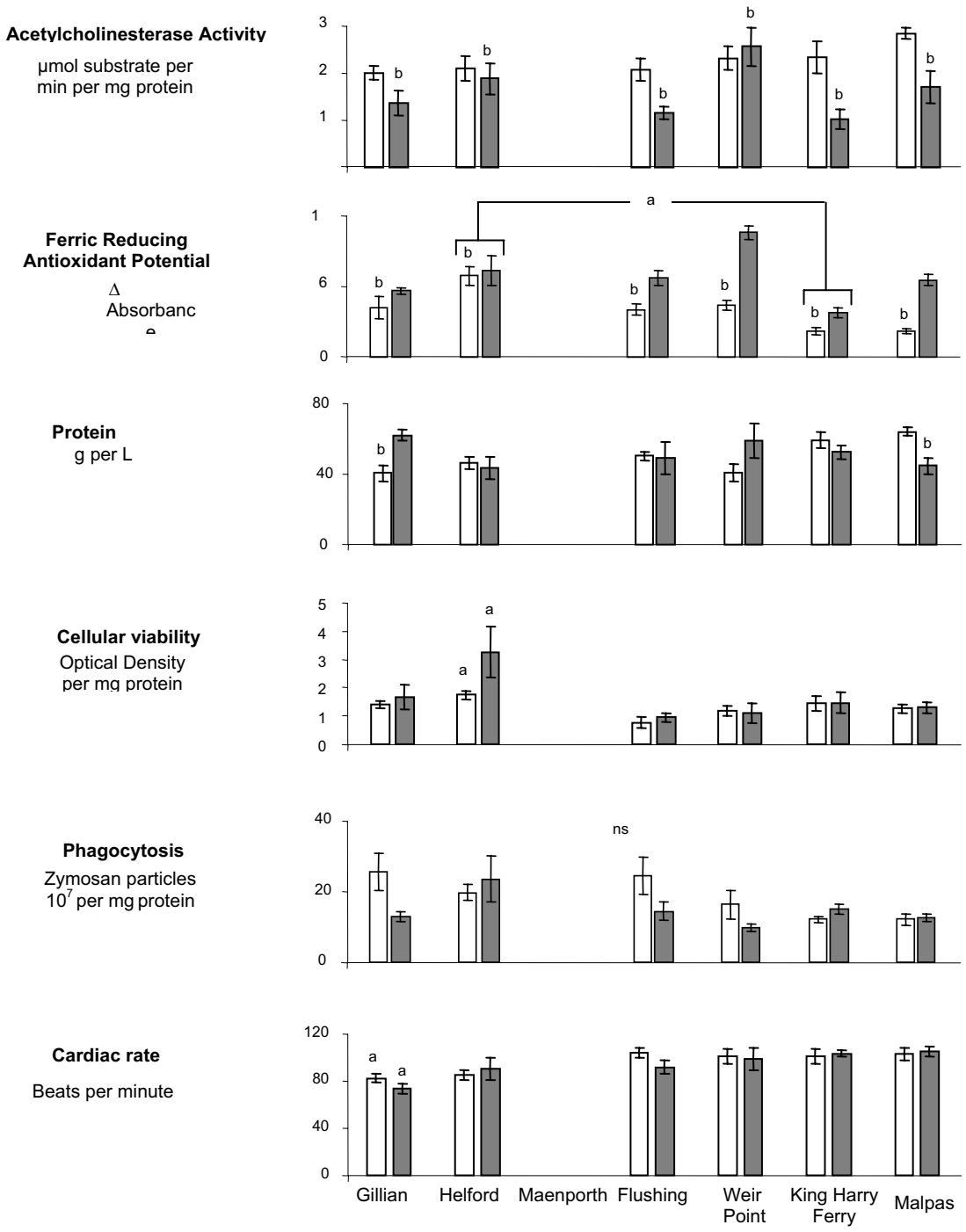

Figure J Biomarker results for Fal & Helford SAC for *C. maenas*. Values expressed as mean ± standard error. Statistical significance is denoted by 'a' (site effect) and 'b' (time effect) and 'ns' no difference

Appendix 5 - Favourable Condition Table for the Fal & Helford European marine site

Table Q Favourable Condition Table for the Fal and Helford European marine site

Feature	Sub-feature	Attribute	Measure	Target	Comments
Large shallow inlet and bay		Extent	Area (ha) of the large shallow inlet and bay, measured periodically (frequency to be determined).	No decrease in extent from an established baseline, subject to natural change.	Baseline established by aerial photography, with survey of existing landclaim. Losses since designation believed to be insignificant
		Water clarity	Average light attenuation measured periodically throughout the reporting cycle (frequency to be determined).	Average light attenuation should not deviate significantly from the established baseline, subject to natural change.	No baseline data on water clarity. Possible localised turbidity associated with Black Rock discharge, dredging activities. No evidence for condition assessment – presumed no changes
		Nutrient status - phytoplankton concentration	Average phytoplankton concentration in summer measured annually.	No significant increase in phytoplankton concentration from the established baseline, subject to natural change.	Evidence of eutrophication problems with toxic algal blooms in Carrick Roads and outer sections of Helford (Site characterisation report). Site put forward for Sensitive area status and nutrient reductions under AMP 4. Believed to be unfavourable
	Saltmarsh communities	Attributes and targets for this sub-feature are listed under the 'Atlantic Salt Meadows' interest feature covered in other sections of this table.			

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
	Intertidal mudflat and sandflat communities	Attributes and targets for this sub-feature are listed under the 'Intertidal mudflats and sandflats' interest feature covered in other sections of this table.			
Large shallow inlet and bay (cont.)	Rocky shore communities	Distribution of characteristic rocky shore communities	Distribution of intertidal rocky shore communities measured using littoral extent. Measured during summer, once during the reporting cycle.	No decrease in littoral extent of range of rocky shore communities from an established baseline value (intertidal biotope mapping survey 1998), subject to natural change.	Baseline established by biotope mapping of whole site. No repeat survey to demonstrate change, but no evidence of substantial change. Presumed favourable although substantial areas impacted by TBT and other contamination. Favourable
		Species composition of low-shore boulder communities	Presence and abundance of composite species from biotope MLR.Fse.Bo. Measured during summer, twice during reporting cycle.	Presence and abundance of composite species should not deviate significantly from the established baseline, subject to natural change.	No baseline data to demonstrate change TBT impacts so unfavourable
		Species composition of rockpool communities	Presence and abundance of composite species and percentage cover of *Sargassum muticum* (Japweed) from a representative series of rockpools, measured during summer twice during reporting cycle.	Presence and abundance of composite species should not deviate significantly from the established baseline, subject to natural change. Average percentage cover of *Sargassum* should not increase from the established baseline.	No baseline data to demonstrate change. Potentially favourable

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
	Subtidal sandbank communities	Attributes and targets for this sub-feature are listed under the 'Subtidal sandbanks' interest feature covered in other sections of this table.			
Large shallow inlet and bay (cont.)	Kelp forests communities	Algal species composition	Presence and abundance of composite of algal species from kelp zone. Measured during summer, twice during reporting cycle.	Presence and abundance of composite of algal species should not deviate significantly from an established baseline, subject to natural change.	Baseline data established by ASM surveys. No evidence of change
		Characteristic species - *Laminaria hyperborea & L. ochroleuca* population size	Relative proportions and density of each species in kelp forests at representative series of sites. Measure during summer, twice during reporting cycle.	Average ratio of *Laminaria hyperborea: L. ochroleuca* should not deviate significantly from an established baseline, subject to natural change. Average density of each species should not deviate significantly from an established baseline, subject to natural change.	Baseline data established by ASM surveys. No evidence of change
		Characteristic species - *Distomus variolosus* population size	Average abundance on kelp stipes (percentage of stipe length over which present and density of cover) measured twice during reporting cycle.	Average percentage cover should not deviate significantly from an established baseline, subject to natural change.	No baseline data? Check with ASM report but potentially favourable?

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Large shallow inlet and bay (cont.)	Subtidal rock and boulder communities	Species composition of characteristic biotopes MCR.ErSEun and ECR.AlcMas	Presence and abundance of composite species from biotopes MCR.ErSEun and ECR.AlcMas. Measured during summer, once during reporting cycle.	Presence and abundance of composite species should not deviate significantly from an established baseline, subject to natural change.	ASM report provides baseline – no probs Favourable
	Subtidal mud communities	Species composition of characteristic biotopes	Presence and occurrence of composite species. Measured during summer, once during reporting cycle	Presence and abundance of composite species should not deviate significantly from an established baseline, subject to natural change.	Baseline data from IECS report and sediment data from Site Characterisation report. No change since designation, but significant areas of site have sediment contaminants at above PEL, including heavy metals and TBT.Localised unfavourable condition
Subtidal sandbanks		Extent	Area (ha) of the subtidal sandbanks measured periodically (frequency to be determined).	No decrease in extent from an established baseline, subject to natural change.	Limited baseline data from Davies & Sotheran report (1996) but no repeat and based on Roxann survey, shown to be of limited value for monitoring. Area is unlikely to have changed, although maerl extraction (shown below) has impacted the site. Favourable.
		Sediment character	Particle size analysis (PSA). Parameters include percentage sand/silt/gravel, mean and median grain size, and sorting coefficient, used to characterise sediment type. Sediment character to be measured during summer once during reporting cycle.	Average PSA parameters should not deviate significantly from an established baseline, subject to natural change.	PSA data collected by IECS, and as part of AA for maerl extraction will form baseline. Evidence that maerl extraction causes change in PSA parameters and hence sediment character is changing. Believed to be unfavourable, although no direct evidence of actual change against the baseline Localised unfavourable.

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Subtidal sandbanks (cont.)		Topography	Depth distribution of sandbanks from selected sites, measured periodically (frequency to be determined).	Depth distribution should not deviate significantly from an established baseline, subject to natural change.	Limited baseline data from Roxann survey and maerl AA. Circumstantial evidence of loss of sediment in extraction areas, although some loss pre-dates designation. Localised unfavourable
		Water density	Average temperature/ salinity in the subtidal measured periodically throughout the reporting cycle (frequency to be determined).	Average temperature/ salinity should not deviate significantly from an established baseline, subject to natural change.	No data No perceived problems – remove from table?
	Eelgrass bed communities (*Zostera marina)*	Extent	Area (ha) of eelgrass beds measured during peak growth period twice during reporting cycle.	No decrease in extent from an established baseline, subject to natural change.	Baseline data provided by Coral Cay surveys and monitoring for Black Rock discharge. No evidence of change, presumed favourable
	Eelgrass bed communities (*Zostera marina)* (cont.)	Characteristic epiphytic species -density of *Zostera marina*	Average density, measured during peak growth period twice during reporting cycle.	Average density should not deviate significantly from an established baseline, subject to natural change.	Baseline data provided by monitoring of Black Rock discharge and Helford VMCA monitoring. Some evidence of heavy overgrowth of epiphytic algae in Fal beds. Locally unfavourable
		Characteristic species - epiphytic community	Presence and abundance of epiphytic species measured during summer twice during reporting cycle.	Presence and abundance of epiphytic species should not deviate significantly from the established baseline, subject to natural change.	Baseline data provided by monitoring of Black Rock discharge and Helford VMCA monitoring. Some evidence of heavy overgrowth of epiphytic algae in Fal beds. . Locally unfavourable

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Subtidal sandbanks (cont.)		Nutrient status - green algal mat	Extent across whole or parts of site, measured during peak growth period every 3 years during reporting cycle.	No increase in extent of green algal mats from an established baseline, subject to natural change.	Baseline data provided by monitoring of Black Rock discharge and Helford VMCA monitoring. Some evidence of heavy overgrowth of epiphytic algae in Fal beds. . Locally unfavourable
	Maerl bed communities	Extent	Area (ha) of maerl (live & dead maerl), measured once during reporting cycle.	No decrease in extent of maerl as whole, or of either dead or live maerl, from an established baseline, subject to natural change.	Concern over declines of live maerl, potentially through water quality issues (ASM data). Extraction of dead maerl has reduced extent and volume (appropriate assessment). Locally unfavourable declining
		Distribution of maerl bed communities	Distribution of maerl bed communities. Measured once per reporting cycle.	Distribution of maerl bed communities should not deviate significantly from an established baseline, subject to natural change.	Maerl extraction appropriate assessment shows changes in communities due to extraction still ongoing. Locally unfavourable declining
	Maerl bed communities (cont.)	Species composition of maerl bed communities	Presence and abundance of composite species of biotopes from maerl areas. Measured during summer, one during reporting cycle.	Presence and abundance of composite species should not deviate significantly from an established baseline, subject to natural change.	Maerl extraction appropriate assessment shows changes in communities due to extraction still ongoing. Locally unfavourable declining
		Nutrient status - green algal mats	Extent measured during peak growth period. Measured once during reporting cycle.	No increase in extent of algal mats from an established baseline, subject to natural change.	Baseline and monitoring data underway from Black Rock monitoring. Uncertain results give cause for concern?

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Subtidal sandbanks *(cont.)*	Gravel and sand communities	Species composition of characteristic biotopes	Presence and abundance of composite species. Measured during summer, once during reporting cycle.	Presence and abundance of composite species should not deviate significantly from an established baseline, subject to natural change.	Baseline data from IECS report. No repeat data to demonstrate change, but evidence of high sediment contaminants from Site Characterisation reports. Favourable since most seds contaminants are in mud areas.
	Mixed sediment communities	Species composition of characteristic biotopes	Frequency and occurrence of composite species. Measured during summer, once during reporting cycle.	Presence and abundance of composite species. Measured during summer, once during reporting cycle.	Baseline data from IECS report. No repeat data to demonstrate change, but evidence of high sediment contaminants from Site Characterisation reports. Favourable since most seds contaminants are in mud areas.
Intertidal mudflats and sandflats		Extent	Area of intertidal mudflats and sandflats measured periodically (frequency to be determined).	No decrease in extent from an established baseline, subject to natural change.	Baseline data from aerial photos and intertidal biotope mapping survey. No repeat survey, but no indications of significant losses.
		Sediment character	1. Particle size analysis. Parameters include percentage sand/silt/gravel, mean and median grain size, and sorting coefficient, used to characterise sediment type. Measured in summer once during reporting cycle.	Particle size parameters should not deviate significantly from an established baseline, subject to natural change.	No baseline data Favourable

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Intertidal mudflats and sandflats (cont.)			2. Sediment penetrability Degree of sinking.	Average measure should not deviate significantly from an baseline, subject to natural change.	No baseline data Favourable
		Topography	Tidal elevation and shore slope, measured periodically (frequency to be determined).	Shore profile measurements should not deviate significantly from an established baseline, subject to natural change.	No baseline data Favourable
		Nutrient status - green algal mats	Extent of green algal mats, measured during summer, annually throughout the summer.	Extent of green algal mats should not increase from an established baseline, subject to natural change.	No repeat data. Evidence of significant risk, potentially improved due to AMP 3 changes at Truro, but still risks from diffuse inputs. Potentially favourable, but not meeting management objectives.
	Sand and gravel communities	Extent and distribution of characteristic biotopes	Extent and distribution of biotopes. Measured during summer, once during reporting cycle.	Extent and distribution of the biotopes should not deviate significantly from an established baseline, subject to natural change.	Baseline data from intertidal biotope mapping. No repeat data, but no indications of change. Believed to be favourable.
	Muddy sand communities	Extent and distribution of characteristic biotopes	Extent and distribution of biotopes. Measured during summer, once during reporting cycle.	Extent and distribution of the biotopes should not deviate significantly from an established baseline, subject to natural change.	Baseline data from intertidal biotope mapping. No repeat data, but no indications of change. Believed to be favourable.

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Intertidal mudflats and sandflats (cont.)	Mud communities	Extent and distribution of characteristic biotopes	Extent and distribution of biotopes. Measured during summer, once during reporting cycle.	Extent and distribution of the biotopes should not deviate significantly from an established baseline, subject to natural change.	Baseline data from intertidal biotope mapping (although not at great detail for infaunal communities). No repeat data, but no indications of change. Believed to be favourable, although concerns over sediment contaminants at above PEL. Locally unfavourable on PEL data.
Atlantic salt meadows		Extent	Area (ha) of Atlantic salt meadows measured once during the reporting cycle.	No decrease in extent of Atlantic salt meadows from an established baseline, subject to natural change.	Baseline data on extent from aerial photos, NVC biotope surveys and established transects. No evidence of change, although known erosion loss in Fal Ruan as estuary re-adjusts to natural equilibrium. Presumed favourable.
		Creek patterns	Creek density and morphology measured periodically during reporting cycle (frequency to be determined).	No significant alteration of creek patterns from an established baseline, subject to natural change.	Baseline data from aerial photos. No repeat survey, but no evidence of significant anthropogenic change.
		Range and distribution of characteristic NVC saltmarsh communities.	Presence and distribution of characteristic saltmarsh communities measured once during reporting cycle.	Range and distribution of characteristic saltmarsh communities should not deviate significantly from the established baseline, subject to natural change.	Baseline data from aerial photos, NVC biotope surveys and established transects. No evidence of change.

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Atlantic salt meadows (cont.)		Vegetation structure	Range and distribution of varying heights of vegetation measured periodically (frequency to be determined).	Vegetation structure should not deviate significantly from an established baseline, subject to natural change.	Baseline data from aerial photos, NVC biotope surveys and established transects. No evidence of change.
Low, mid and upper saltmarsh and zonations between these are considered to be subfeatures of **Atlantic salt meadows**. These may exhibit considerable variation, and NVC communities have been assigned to each subfeature, according to The Saltmarsh Survey of Great Britain (Burd 1989). The presence of these on an individual site and their position within the saltmarsh zonation will need to be established by surveys which may also identify sub-communities typical of that site					
	Low Marsh and Low-Mid Marsh communities	Characterising species of: 1.Transitional low marsh vegetation with *Puccinellia maritima*, annual *Salicornia* species and *Sueda maritima* SM10.	Frequency and abundance of characterising species, particularly of: 1. *Puccinellia maritima*, annual *Salicornia* species and *Sueda maritima*. Measured once during reporting cycle	Frequency and abundance of characteristic species of low and low-mid marsh communities should not deviate significantly from an established baseline subject to natural change	Baseline data from aerial photos, NVC biotope surveys and established transects. No evidence of change.
		2. *Aster tripolium var. discoides* saltmarsh SM11.	2. *A. tripolium var. discoides*.		Baseline data from aerial photos, NVC biotope surveys and established transects. No evidence of change.
		3. *Puccinellia maritima* saltmarsh SM13	3. *P. maritima* with *Triglochin maritima*, *Plantago maritima, Armeria maritima*		Baseline data from aerial photos, NVC biotope surveys and established transects. No evidence of change.

Table continued…

Feature	Sub-feature	Attribute	Measure	Target	Comments
Atlantic salt meadows (cont.)	Mid and Mid-Upper marsh	Characterising species of: 1. *Juncus maritimus-Triglochin maritima* saltmarsh SM15.	Frequency and abundance of characterising species, particularly of: 1. *Juncus maritimus*	Frequency and abundance of characteristic species of low and low-mid marsh communities should not deviate significantly from an established baseline subject to natural change.	
		2 .*Festuca rubra* saltmarsh SM16	2. *Festuca rubra* and *Juncus gerardii*		Baseline data from aerial photos, NVC biotope surveys and established transects. No evidence of change.
		3. *Juncus maritimus* saltmarsh SM18	3. *Juncus maritimus*		Baseline data from aerial photos, NVC biotope surveys and established transects. No evidence of change.

NB - Many of the attributes will be able to be monitored at the same time or during the same survey. The frequency of sampling for many attributes may need to be greater during the first reporting cycle in order to characterise the site and establish the baseline.

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