Biological Indicators of Contamination in Auckland's Estuaries

An Initial Assessment

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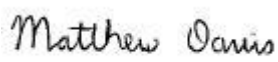

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Biological Indicators of Contamination in Auckland's Estuaries: An Initial Assessment

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Contents

1 Executive Summary

An ability to identify a specific contaminant or groups of contaminants that are potentially causing adverse effects in the aquatic environment could improve management practices nationally and internationally. The objectives of this initial study were:

- to conduct a literature review of the potential of biomarkers for assessing the adverse effects of metals in estuarine and coastal environments;
- to obtain quantitative data on biochemical changes and cellular damage in shellfish and fish sampled along a contaminant gradient that covered eight estuaries in the Auckland region, and
- to provide recommendations on the possible incorporation of biological assessment tools in existing contaminant monitoring programmes.

The literature review explains how the application of biological effects tools can be used in regulatory environmental monitoring programmes and which tools are available to assess the biological effects of metal exposure in marine and estuarine sediments. The biological effects tools identified were:

- metallothionein (MT) induction;
- δ-aminolevulinic acid dehydratase (ALA-D) induction;
- lysosomal stability;
- oxidative stress biomarkers;
- DNA strand breaks and micronuclei (MN) assay.

These biological effect tools showed they were fit for the purpose of an environmental monitoring programme, some being more specific to either metal-related sediments or more general environmental stressors. The most appropriate tool depends on the aims and objectives of the monitoring required.

This study then focused on using four selected gene expression tools (for metal-specific effects and other general stressor effects) and the micronuclei assay tool (for metal-related and general stress effects) to identify a specific contaminant or groups of contaminants that were potentially causing adverse effects in the aquatic environment.

Sediments, cockles (*Austrovenus stutchburyi*) and yellowbelly flounder (*Rhombosolea leporina*) were collected from eight estuaries in the Auckland region. Sediments were analysed for metal concentrations. Fish liver tissues were analysed for metallothionein (MT) induction, aldehyde dehydrogenase expression, glutathione-S-transferase expression and Cu/Zn superoxide dismutase expression using quantitative real-time polymerase chain reaction (PCR) analysis. The micronuclei assay was performed on gill tissues from fish and shellfish.

Results of the micronuclei assay showed some linear relationship between copper and zinc in sediments and micronuclei numbers in tissue of flounder caught in the upper Waitemata Harbour but not in the other harbours that were sampled. There was, however, induction of micronuclei in tissues at all sites suggesting biochemical and cellular damage is occurring. This suggests that there is sublethal stress occurring but it is unclear if this is occurring to some individuals and not others and it is also unclear what is driving this response in winter. In summer, Reed & Lyons (2009) found a linear relationship between micronuclei numbers in cockles and total zinc concentrations in sediments collected in the inner Waitemata Harbour during 2008, but this relationship was not observed in cockles in this winter study.

Results of the gene expression analysis showed increased levels of metallothionein in yellowbelly flounder caught in Cox's Bay, Meola and Shoal Bay compared to lower levels in Te Matuku Bay, Tamaki and Pahurehure. Interestingly, the sites that appeared to be more impacted were geographically close and are recognised by Auckland Council as being sites which may show occasional adverse effects due to contaminants.

Low levels of aldehyde dehydrogenase expression were also observed in flounder caught in the Tamaki Estuary (suggesting lower levels of PAHs at this site), however, expression was notably higher at Shoal, Cox's Bay and Whau than at Tamaki, suggesting higher levels of polycyclic aromatic hydrocarbon (PAH) contamination than previously expected.

Recommendations resulting from this study for consideration are to:

- Undertake additional study of the use of biological indicators to assess the initial findings in this study.
- Characterise the use of yellowbelly flounder as a species to use in biological effects monitoring and to confirm observed effects in flounder with bioavailable metals.
- Investigate why results from this study show zinc and copper are the main metals influencing the biological responses observed (plus PAHs at some sites) when the Benthic Health Model shows zinc and lead are the main metals influencing benthic species distributions.
- Assess the use of oysters and or mussels as sentinel species for monitoring the effects of chemicals in the inner estuaries.
- Explore the design of a monitoring programme that integrates chemical and biological tools for use in the Auckland region.

2 Introduction

2.1 Background

One of the key issues facing environmental managers and resource users of the marine environment is a restricted ability to identify and verify the adverse environmental effects of contaminants beyond coarse-scale changes, and to attribute changes to specific contaminants. The inability to clearly answer questions of contaminant effects at finer scales means that risk management is not always robust. Consenting processes are therefore based on potentially incomplete information and money spent on environmental management (including that spent on restoration/remedial activities) is not always targeted efficiently. There can also be a gradual reduction in ecosystem functioning and productivity, with inshore biological resources potentially compromised before contaminant impacts are observed using traditional methods. An ability to identify a specific contaminant, or groups of contaminants, that are potentially causing adverse effects in the aquatic environment could improve management practices nationally and internationally.

In order to address these gaps in knowledge, this initial study investigates the use of a range of biochemical techniques using international best practice. The cellular and molecular responses of New Zealand species to urban contaminants were examined by assessing the use of a number of environmental biomarkers. The ultimate goal of the study was to identify a set of biological markers that could be used to provide an early warning of environmental impact (i.e., adverse effects) and the possible use this information to improve the regulation of discharges by regulatory authorities.

This study focuses on using biomarkers to test the hypothesis that exposure of New Zealand shellfish and fish to low-level contamination in New Zealand's urban estuaries causes biological effects which can be measured through altered gene expression and cellular damage.

2.1.1 Aim and objectives

This initial study aims to use biological effects markers to detect early signs of stress at the cellular and molecular level for a New Zealand native estuarine shellfish and fish species. These biomarkers could subsequently be used to evaluate the causal relationship between contaminant exposure and biological changes in estuarine marine species. The study focuses on measuring physiological parameters capable of indicating sublethal stress at the cellular and organism level and detecting deteriorating health in benthic dwelling fauna. The objectives of this study were to:

- Conduct a literature review of the potential of biomarkers (or biological effects tools) for assessing the impact of metals in estuarine and coastal environments.
- Obtain quantitative data on biochemical changes and cellular damage in shellfish and fish sampled along a contaminant gradient that covered eight estuaries in the Auckland region.
- Provide recommendations on the possible implementation of biological assessment tools for contaminant monitoring.

3 Literature Review

3.1 Introduction

Contaminants (e.g., heavy metals, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs)) are important stressors in many urbanised estuaries and coastal areas. However, the presence of a chemical contaminant, in itself, does not necessarily indicate that a detrimental effect will occur. Chemicals tend to be deposited in sediments and sediment quality guidelines (SQGs) are routinely used to assess contamination in marine and estuarine sediments.

Hübner and co-workers recently reviewed SQGs for the assessment of metal contamination and critically evaluated 15 sets of commonly used SQGs, including values developed for use in Australia/New Zealand, Canada, Japan, USA and Europe (Hübner *et al.*, 2009). Their review highlighted the potential problems encountered by environmental managers when applying SQGs developed in one region or country with another due to differences in sediment type, exposure duration, geochemical parameters, contaminant bioavailability, species sensitivity and other factors. Other limitations of SQGs are that they only cover well known and thoroughly tested chemicals, and they provide little, if any data, on possible antagonistic and synergistic effects of chemical mixtures.

A recent pilot study that quantified levels of chemicals of emerging concern in Auckland's aquatic sediment highlighted several sites with elevated levels of metal contamination (Table 1, Stewart *et al.*, 2009). A generalised assessment of the ecotoxicological significance of these values can be gained by a comparison with the Threshold Effects Levels (TELs) and Probable Effects Levels (PELs) developed for use by the National Oceanographic and Atmospheric Administration (NOAA) in North America (Buchman, 2008). These two values provide three separate ranges (based on Hübner *et al.*, 2009):

- <TEL (adverse effects rarely observed);
- >TEL<PEL (adverse effects occasionally observed); and
- >PEL (adverse effects frequently observed).

These ranges are shown in Table 1 with the Australia and New Zealand interim sediment quality guidelines ISQG-low and –high values (ANZECC, 2000). PELs were exceeded for one or more metals at the Milford marina (copper (Cu), zinc (Zn)), Westhaven (Cu), Hobson (Cu, lead (Pb), Zn), Shoal Bay (Zn) and Puketutu (Zn) sites.

Table 1

Concentration of bioavailable metals in estuarine sediments collected from Auckland Harbour areas, and colour coded based on NOAA SQGs (Buchman, 2008). (Table adapted from Stewart *et al.*, 2009).

Sample code	Auckland site	Extractable metals <63µm mg/kg dry weight		
		Copper (Cu)	Lead (Pb)	Zinc (Zn)
133/1	Cox's Bay	38	110	270
133/2	Cox's Bay	39	94	250
133/3	Meola	39	82	230
133/4	Meola	40	84	240
133/5	Motions	39	69	220

Sample code	Auckland site	Extractable metals <63µm mg/kg dry weight		
		Copper (Cu)	Lead (Pb)	Zinc (Zn)
133/6	Motions	39	68	210
133/7	Milford Marina	120	82	**770**
133/8	Milford Marina	120	92	**1000**
133/9	Westhaven Marina	170	62	190
133/10	Westhaven Marina	170	63	190
133/11	Hobson Bay	110	**230**	**630**
133/12	Hobson Bay	120	**240**	**640**
133/13	Shoal Bay	36	83	340
133/14	Shoal Bay	37	84	350
133/15	Halfmoon Bay	67	31	140
133/16	Halfmoon Bay	64	32	140
133/17	Pakuranga	37	46	260
133/18	Pakuranga	39	47	270
133/19	Whau	36	77	270
133/20	Whau	35	76	260
133/21	Taihiki	7.7	17	81
133/22	Taihiki	7.4	17	78
133/23	Mahurangi	24	11	70
133/24	Mahurangi	25	11	70
133/26	Puketutu	80	57	290
133/27	Puketutu	80	58	300

Metal	ANZECC (2000) guideline		
	Low(mg/kg dw)		High (mg/kg dw)
Zinc	200		**410**
Copper	65		**270**
Lead	50		**220**
	NOAA (2008) guideline		
	TEL (mg/kg dw)		PEL (mg/kg dw)
Zinc	124		271
Copper	18.7		108
Lead	30		112
	<TEL	>TEL <PEL	>PEL
Key colour code for sediment guidelines in relation to metal values			

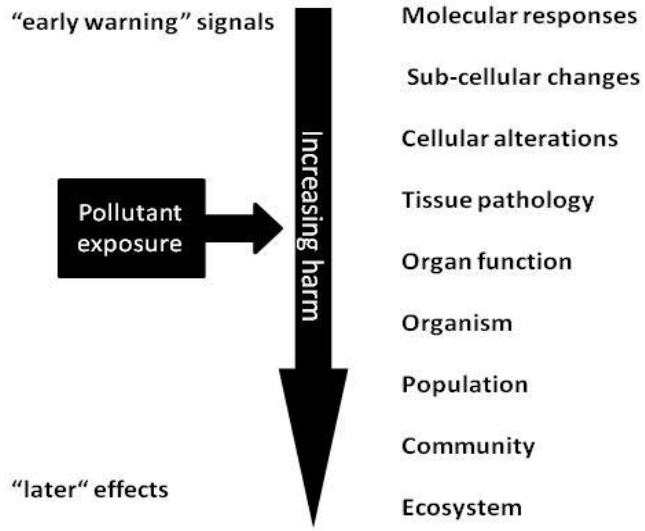
It should not be assumed that exceeding SQGs necessarily means adverse effects will inevitably occur and examples are available where they have been breached without a direct toxic effect on resident biota being detected (Hübner *et al.*, 2009). Conversely, biological effects have also been observed where SQG's have not been exceeded (Kelly 2007). While appropriately developed SQGs are useful indicators of potential effects, it is recommended that they shouldn't be used alone (Hübner *et al.*, 2009). It is generally recognised that contaminant effects should, at the very least, integrate chemical with biological data (Thain *et al.*, 2008).

Many strategies and approaches have been advocated to assess ecosystem health in the scientific literature (EEA, 2001; IOC, 2002; Hewitt *et al.*, 2005; Jorgensen *et al.,* 2005; Thain *et al.*, 2008). Biological effects indicators (also referred to as biomarkers) are frequently used in environmental monitoring programmes because they provide a link between contaminants and ecological responses. A biological effect may be defined as the response of an organism, a population, or a community, to changes in its environment. These changes can be either man-made or natural. The usefulness of any biological-effect method will depend on how well it is able to separate anthropogenic stressors from the influence of natural variability. The output should be a measure of the well-being or health of some ecosystem component.

Vital aspects determining health relate to questions including: are organisms, populations, or communities viable; can they reproduce; do individuals or populations fulfil their potential for growth under relevant natural ecosystem conditions; do they behave normally, and are they physiologically competent? The early warning signs of deteriorating ecosystem health can be more subtle and include the homeostatic responses of an organism or measures of detrimental genetic, biochemical, or physiological changes that may be Indicative of higher-level effects. The sequence of response to contaminant stress within a biological system is displayed in Figure 1 (modified from Bayne *et al.*, 1985). The potential sensitivity of physiological and cellular indicators has triggered a large field of research to establish markers that provide an early warning of adverse biological effects from contaminant exposure.

Figure 1

Schematic representation of the sequential order of response to contaminant stress within a biological system. Adapted from Bayne *et al.* (1985).



Physiological, molecular and cellular markers have most commonly been used to identify the effects of contaminants (e.g. at point source discharges, non-point diffuse inputs or after accidental spills) and to assess the health status of marine habitats. It is important that cause-and-effect relationships between

the presence of contaminants and biological-effect responses be clearly established if they are to be used to inform management and direct environmental policy.

A good example of cause-and-effect relationships is tributyltin (TBT)-induced imposex in a species of marine snail (dogwhelk, *Nucella lapillus*). Imposex is the imposition of male sexual characteristics on a female and it has been found to be the most sensitive indicator of TBT exposure in marine species. Severe cases of imposex can lead to sterility in females and the collapse of the local population. Once the link between TBT exposure in the marine environment and population effects had been established in the 1980's (Gibbs *et al.*, 1987), management actions were taken to reduce TBT use. International policies through the International Maritime Organisation (IMO) were introduced, which resulted in a decrease in the prevalence and severity of imposex (Birchenough *et al.*, 2002).

The link between a specific contaminant and a biological-effects response is unfortunately not always so clear-cut or expressed in so readily identifiable traits as TBT-induced imposex. Many biological effects are also not specific to an individual contaminant. In such situations, physiological and cellular markers can potentially provide an early measure of response within exposed organisms prior to the observation of population level effects.

More than 100,000 known man-made chemicals are released into the environment but analytical techniques are currently only able to measure a fraction of those chemicals. Therefore robust biological-effect tools are needed to measure responses to contaminants at the individual, population and community level. These tools also need to be capable of integrating the synergistic and antagonistic effects of contaminants in a complex chemical mixture, which is often found in the environment. Minimum criteria for biological effects tools used in monitoring programmes have recently been proposed (Van der Oost *et al.*, 2003; Lyons, *et al.*, 2010) and are as follows:

- The assay to quantify the biological effect should be reliable (with quality assurance (QA) programmes in place), relatively cheap and easy to perform.
- The biological effect response should be sensitive to pollutant exposure and/or effects in order to serve as an early warning parameter.
- Baseline data of the biological effect should be well defined in order to distinguish between natural variability (noise) and contaminant-induced stress (signal).
- The impacts of confounding factors to the biological effect response should be well established.
- The underlying mechanism of the relationships between biological effect response and pollutant exposure (dosage and time) should be established.
- The toxicological significance of the biological effect, e.g. the relationships between the response and the (long-term) impact to the organism, should be established and, where possible, assessment criteria developed.

3.2 The application of biological effects in regulatory environmental monitoring programmes

There are a vast number of scientific studies detailing the use of biological effects tools in a biomonitoring context (for a detailed review, see Van der Oost *et al.*, 2003). However, only limited data is available on the application of biological effects tools in an environmental management and regulatory context.

In North America, the US Geological Survey undertakes a comprehensive biological effects monitoring programme known as the Biomonitoring of Environmental Status and Trends (BEST) programme (http://biology.usgs.gov/status_trends/) (Bauch *et al.*, 2005). This programme assesses water quality in large US river basins, including the Rio Grande, Colorado and Mississippi, and uses a combination of animal health assessments (e.g. histopathology) and toxicological status (e.g. biological effects tools of contaminant exposure).

Within Europe, two large biomonitoring programmes exist. The Joint Assessment and Monitoring Programme (JAMP) has been developed within the framework of the Convention for the Protection of the Marine Environment of the North East Atlantic (the OSPAR Convention, www.ospar.org/) and the Program for the Assessment and Control of Pollution in the Mediterranean Region (MEDPOL) under the United Nations Environment programme (www.unep.org/). Both of these programmes use a combination of chemical and biological effects tools to assess the concentrations, trends and effects of specific contaminants in the marine environment (e.g. Polycyclic Aromatic Hydrocarbons (PAHs), tributyltin and heavy metals).

The JAMP and MEDPOL programmes provide a large repository of contaminant-related biological effects data, and it is highly likely that these will be used to support the new European Union Marine Strategy Framework Directive (MSFD), which is a wide-ranging framework directive with the overall objective of achieving or maintaining Good Environmental Status (GES) in Europe's seas by 2020 (MSFD, 2008). Eleven high-level qualitative descriptors of GES have been defined by the MSFD and these will be the key policy drivers shaping the future requirements for marine monitoring across Europe (Lyons *et al.*, 2010). One of these, GES Descriptor 8 entitled "Concentrations of contaminants are at levels not giving rise to pollution effects", will wherever possible rely on the approaches developed under MEDPOL and JAMP. One of the clear advantages of using contaminant-related biological effects tools is that they indicate links between chemical exposure and ecological endpoints. They can also detect the impacts of substances (or combinations of substances) that may not be analysed for as part of routine chemical monitoring programmes (Van der Oost *et al.*, 2003; Thain *et al.*, 2008).

3.3 Tools to assess the biological effects of metal exposure in marine and estuarine sediments

Various international organizations (e.g. Oslo and Paris Commissions (OSPAR), as part of their Joint Assessment and Monitoring Programme) have issued guidelines for general biological effects monitoring and also for contaminant-specific biological effects monitoring (ICES, 2006; ICES, 2009). Within these guidelines, strategies have been recommended for the monitoring of metal-specific biological effects incorporating a "cascade" of stages that include the measurement of **metallothionein** (MT) and **δ-amino levulinic acid dehydratase** (ALA-D). Other "general" early warning techniques, including **lysosomal stability**, a range of **antioxidant enzymes,** the **comet assay** and **micronucleus assay** have also been suggested as potential biochemical, sub-cellular and cellular biomarkers of metal-mediated effects.

This review summarises the use of a selection of biological effects techniques for assessing the impacts of metals in research and monitoring programmes. In particular, it addresses their practicalities in a field-monitoring context, ecological relevance and their application specifically to the investigation of metal-mediated biological effects.

3.3.1 Metallothionein (MT)

Metallothioneins (MT) are low molecular weight metal-binding proteins that are present in vertebrates and some invertebrates. They are known to play a vital role in the homeostatic control of the essential trace elements zinc and copper. They also act as part of the detoxification mechanism for other metallic pollutants, especially cadmium, silver and mercury. MT synthesis above species specific baseline levels has been used in a wide number of marine species as a biomarker for exposure to metal contamination (George and Olsson, 1994; George *et al.*, 2004; Faria *et al.*, 2010; Oliveira *et al.*, 2010).

3.3.1.1 Methods available for measuring MT induction

There are a number of techniques in the literature that have been used to detect MT induction in aquatic species. The most routinely used assays exploit some of the characteristics of the MT protein, including its metal content, the number of sulphydryl groups, its size and/or heat-stability. A number of immunochemical assays using MT-specific antisera have also been established (Roesijadi, 1992; Hogstrand *et al.*, 1989; Hylland, 1999). More recently with the advent of sensitive molecular technology, MT gene expression (mRNA) has been quantified in aquatic species using a method called quantitative PCR which measures the level of transcription of the MT gene (qPCR; Tom *et al.*, 2004; Sheader *et al.*, 2006).

3.3.1.2 Ecological relevance and field use: MT induction

The use of MT induction as a biological marker of metal exposure has been trialled in a wide number of invertebrate and vertebrate aquatic species (>75; Roesijadi, 1992). This wide inter-species application of MT analysis means that it can be used in many different geographical areas and habitats. Laboratory studies have demonstrated that MT levels in fish increase in both a dose responsive (e.g. George and Young, 1986; Hogstrand and Haux, 1991) and/or a time responsive (Beyer *et al.*, 1997) manner after exposure to heavy metals.

Studies have detected correlations between hepatic MT levels and waterborne concentrations of metals (zinc, copper and cadmium in rainbow trout (*Onchorhyncus mykiss*) in a Canadian river study (Roch and McCarter, 1984). Similar relationships have been detected between MT and hepatic cadmium concentrations in perch (*Perca fluviatilis*) from a contaminated river in Sweden (Olsson and Haux, 1986). Klaverkamp *et al.* (1996) measured MT in various tissues in whitefish (*Coregonus clupeaformis*) and pike (*Esox lucius*) from a metal contaminated lake in Canada. They showed some correlation with metal concentrations in various tissues (including liver) but a confusing picture with respect to links with metal concentrations in sediments. Studies conducted in estuarine environments have detected increasing levels of hepatic MT induction in the European flounder (*Platichthys flesus*), at several sites along the Forth Estuary (Sulaiman *et al.*, 1991). While high levels of variability were detected in each sample, there was a general trend of MT levels corresponding with hepatic metal concentrations (copper, cadmium, zinc and lead).

When undertaking studies in aquatic species it is vitally important to take into account other endogenous and exogenous factors that may also influence MT induction. Seasonal studies conducted on cod (*Gadus morhua*) demonstrated that hepatic MT levels varied through the year in both male and female fish. This was attributed to the demand for zinc in gonad development and sexual maturation (Ruus *et al.*, 2003).

Similar studies in the marine flatfish dab (*Limanda limanda*) have also identified seasonality in hepatic MT induction linked to both sexual maturation and water temperature (Lacorn *et al.*, 2001).

The extensive scientific information base detailing the use of MT induction following metal exposure means this biological effects tool is useful for environmental managers undertaking *in situ* risk assessments of metals toxicity. The ICES Working Group for the Biological Effects of Contaminants (WGBEC) has recommended its application in monitoring fish for national programmes (ICES, 2006). Efforts are now underway in Europe to develop background response levels and assessment criteria for MT biological effect tools (ICES, 2009). These tools are species specific, having been developed by analysing international datasets for Atlantic cod and the flatfish dab and plaice (*Pleuronectes platessa*) (Ruus *et al.*, 2003). It is apparent that MT induction is not always a reliable indicator of the presence of metal contamination on its own, but in combination with other data its induction can be used to support findings on metal bioavailability and accumulation in the tissues of exposed organisms.

As detailed above, various factors can influence MT induction and it can only be reliably used if the seasonal dynamics of MT induction are well understood in the bioindicator species of choice. Furthermore, as with many other biomarkers, the data provided by MT are of most benefit if the test is deployed as part of a suite of biological effects assays and chemical analyses (specifically heavy metals), so that the results can be interpreted with consideration to other relevant factors.

3.3.2 δ-aminolevulinic acid dehydratase (ALA-D)

The enzyme δ-aminolevulinic acid dehydratase (ALA-D) catalyses a step in the synthesis pathway of heme molecules in fish and other vertebrate. Heme is then incorporated into macromolecules such as haemoglobin and cytochromes. A significant and prolonged inhibition in the activity of ALA-D could therefore result in anaemia since it is one of the rate limiting enzymes in heme (and hence haemoglobin) production. Environmental managers are interested in using ALA-D as a biological marker for metal exposure because certain metals (in particular lead) are known to inhibit ALA-D at very low exposure levels (Hodson *et al.*, 1984; Haux and Förlin, 1988).

3.3.2.1 Methods available for measuring ALA-D induction

At present no universally adopted assay for ALA-D inhibition exists, with most studies using relatively simple spectrophotometric procedures. This involves the target tissue being homogenised (or haemolysed for blood cells) and used as raw material for the source of ALA-D. This material is incubated with ALA as the substrate, and the activity is based on the rate of synthesis of the product, porphobilinogen (PBG). The product is reacted with a colour producing reagent, and the absorbance recorded spectro-photometrically. The activity is finally expressed as a factor of weight of tissue, volume of blood/tissue, milligrams (mg) of protein or milligrams (mg) of haemoglobin (in blood). At present no standard reference material is available and the majority of laboratories using the technique produce their own.

3.3.2.2 Ecological relevance and field use: ALA-D induction

The ALA-D assay has been applied to aquatic species from a wide range of environments. As blood is frequently used as the target tissue, there is the opportunity for non-destructive sampling. In cases where enough blood cannot be extracted, or specific tissue excision is impractical, ALA-D has successfully been used to monitor lead poisoning in whole body homogenates (Burden *et al.*, 1998). The assay is relatively easy and inexpensive to perform, and in general could be performed in the field given access to a spectrophotometer. Moreover the enzyme is relatively stable during storage and studies have

demonstrated that blood samples could be held on ice for up to 24 hours without loss of activity and freezing would maintain activity for prolonged periods (Hodson *et al.,* 1984).

The use of ALA-D as a biological effects tool for metal exposure has mainly been restricted to the freshwater environment, in species including carp *Cyprinus carpio* and *Carassius auratus langsdorfii* (Nakagawa *et al.*, 1995; Nakagawa *et al.*, 1998) and rainbow trout (Addison *et al.*, 1990; Sordyl and Osterland, 1990). It is also important to note that lead has low bioavailability at the pH found in seawater. Similar to MT induction ALA-D inhibition is also known to be influenced by a wide range of factors including seasonality, size and age. Therefore, species specific traits need to be fully understood before using ALA-D in a bio-monitoring programme.

There have been a number of studies undertaken using ALA-D for marine and estuarine species. For example, between 1997 and 2001 the Norwegian national monitoring programme used ALA-D activity in red blood cells of Atlantic cod, flounder and dab (Ruus *et al.*, 2003). In this programme, ALA-D was generally found to be inhibited in the blood of fish from sites with known anthropogenic impacts (Ruus *et al.*, 2003). Of note, the data also indicated that there could be lead toxicity in individual fish with hepatic lead concentrations below the limit of detection for routine chemical analysis. A similar study investigated ALA-D in Atlantic cod and flounder collected from southern Norway (Holth, 2004). In this study the researchers observed clear inhibition of the ALA-D enzyme in both species sampled in the inner Oslofjord compared to individuals collected from the outer Oslofjord, where the fish were deemed to have baseline values of enzyme activity.

In general, ALA-D is regarded as being specific for lead, although there may be some degree of interference in the presence of other metals. While the specificity of ALA-D inhibition means that the assay can be very targeted and useful for certain circumstances, it also means that these applications are potentially limited to those locations impacted by lead contamination.

Due to the low solubility of lead in sea water, the application of ALA-D inhibition assays to survey marine waters maybe of limited value. For example, while ALA-D inhibition has been detected at 5 µg/L in rainbow trout (Haux *et al.*, 1986) the actual concentrations of lead in marine waters in the UK do not exceed 0.2 µg/L (CEFAS, 1998). ALA-D does, however, remain a very useful tool in the investigation of areas where lead contamination, particularly associated with sediments, is known or suspected to be an issue. As with other enzyme markers, it is best used as part of a suite of other assays and in species whose physiology is well understood.

3.3.3 Lysosomal stability

Lysosomes are cell vesicles containing enzymes that are involved in the breakdown of proteins, polysachorides and lipids. If lysosomes break open, these enzymes are released and can destroy the cell. Lysosomal stability is affected by environmental stressors in many different taxa. It is therefore a potentially useful biological effects marker of individual health status (Moore, 2006a, 2006b).

Lysosomes are known to accumulate many metals and organic xenobiotics. For example metals including copper, cadmium and mercury have been shown to induce lysosomal destabilisation in mussels (Viarengo *et al.*, 1981, 1985a, 1985b). There are now numerous studies suggesting that various forms of chemical and physical stress can damage or destabilise the lysosomal membrane, thereby leading to the leakage of lysosomal enzymes into the cytosol of the cell. Recent studies have shown that lysosomal autophagy (a process whereby a cells own components are degraded in order to clear damaged or superfluous proteins) provides a second line of defence against oxidative stress (Cuervo, 2004; Moore *et al.*, 2006b). An organism's ability to up-regulate this process is a significant factor contributing to its ability to tolerate stressful and polluted environments.

3.3.3.1 Methods available for measuring lysosomal stability

There are two main methods for measuring lysosomal stability and comparative studies have shown that the two techniques correlate well (Lowe *et al.*, 1995). The first, a cyto-chemical method termed lysosomal latency, is most commonly used in fish tissue (Kohler *et al.*, 1992). The technique requires the immediate removal of the target tissue (usually liver) from the animal followed by preservation at -70°C. The frozen sample is then processed in a cryostat and ultra thin sections produced for analysis. Lysosomal membrane stability is assessed by measuring the time of acid labilisation required to fully destabilise the membrane, with various marker enzymes used to assess membrane stability. The point of full membrane destabilisation is detected at the point when maximal lysosome staining occurs (using an appropriate dye). This denotes penetration of the membrane by the substrate and the faster this occurs the more damaged the membrane is considered to be.

The second and more widely used technique is the neutral red retention assay (NRR) (Lowe *et al.*, 1992; Lowe and Pipe, 1994). This technique is conducted in freshly collected tissue (e.g. bivalve haemocytes) and assesses the release of a dye from the lysosomes. Briefly, the assay works on the basis that lysosomal contents are acidic in nature and therefore weak bases such as neutral red are rapidly taken up into them. Using a microscope, the point at which the neutral red dye "leaks out" of the lysosome into the cytosol of the cell is determined. The more rapid the leakage the more damaged the lysosomal membrane is considered to be.

3.3.3.2 Ecological relevance and field use: lysosomal stability

Lysosomal integrity has been directly correlated with physiological scope for growth (SFG) and has also been shown to correlate with larval viability in oysters (*Crassostrea spp*) (Moore *et al.*, 2006a; Ringwood *et al.*, 2004). In addition, lysosomal stability was also directly correlated with diversity of macro-benthic organisms in an investigation in Langesund Fjord in Norway (Moore *et al.*, 2006a). Health status thresholds for NRR and cytochemical methods for lysosomal stability have been determined from data based on numerous studies (e.g. Cajaraville *et al.*, 2000; Moore *et al.*, 2006a). For the cytochemical method, animals are considered to be healthy if the lysosomal stability is $\geq$20 minutes; stressed but compensating if <20 but $\geq$10 minutes and severely stressed and probably exhibiting pathology if <10 minutes (ICES, 2009).

Similarly for the NRR method, animals are considered to be healthy if NRR is $\geq$120 minutes; stressed but compensating if <120 but $\geq$50 minutes and severely stressed and probably exhibiting pathology if <50 minutes (ICES, 2009). It should be noted that as with the other biological effects tools discussed, lysosomal stability can be influenced by non-contaminant stressors. Preliminary studies should always be performed before the assay is used in a new sentinel species or tissue type. For example, between tissues there can be large differences in the size and number of lysosomes depending on the cells particular function.

In this respect the lysosomes of molluscan digestive gland tissue are a particularly useful target tissue, whereas other tissues like fish hepatocytes have relatively few and smaller lysosomes under normal conditions making their visual assessment more difficult (Kohler, 1991). The choice species and tissue is therefore not straight-forward and must be selected after consideration of the aims of the monitoring programme. This in-depth investigation has been carried out for certain species and lysosomal stability in fish and *Mytilus* spp. has been recommended by ICES at the national/international level as a measure of cellular damage (ICES, 2009).

The measurement of lysosomal stability has the potential for use as a valuable biomonitoring measure of contaminant exposure. The technique responds to a wide array of contaminants of which metals are a significant group. As a result of this non-specificity, its use for the monitoring of metal pollution is

limited, but worth consideration in areas where metals are potential contaminants of concern. With this in mind lysosomal assays should be viewed as a general indicator of health (ICES, 2009). Furthermore, as with other assays using biological systems that can be affected by non-contaminant stressors, and that interact significantly with other biological systems, they are best used as part of a battery of biological effects tools only after careful consideration of the test species and likely external influences.

3.3.4 Oxidative stress

The presence of elevated intra-cellular concentrations of reactive oxygen species (ROS), including the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) in tissues, can have severe effects on cellular health. These effects can include changes in redox balance, general cellular and skeletal damage, DNA damage and lipid peroxidation (Livingstone *et al.*, 1992). Elevated concentrations of ROS have been observed to occur in response to contaminant exposure and consequently assays for measuring ROS and associated enzymes (produced as a natural response to neutralise the presence of ROS) have been suggested as potentially useful monitoring tools (Van der Oost *et al.*, 2003; Valavanidis *et al.*, 2006). Several biomarkers have been proposed including:

- superoxide dismutase (SOD) which converts O_2- to H_2O_2;
- catalase which converts H_2O_2 to water:
- glutathione peroxidase (GPX) which also converts H_2O_2 to water;
- glutathione reductase (GR) which maintains cellular reduced glutathione (an important antioxidant); and malonedialdehyde which is a measure of lipid peroxidation.

3.3.4.1 **Methods available for measuring oxidative stress**

Various methods exist for the measurement of oxidative stress biomarkers, including both relatively simple spectrophotometric assays and molecular methods using qPCR (Van der Oost *et al.*, 2003; Valavanidis *et al.*, 2006). The methods usually rely on samples being collected in the field and cryogenically stored for analysis at a later date.

3.3.4.2 **Ecological relevance and field use: oxidative stress**

Superoxide dismutase (SOD) has been measured in a variety of fish species including the dab (Livingstone *et al.*, 1992), European flounder (Sheader *et al.*, 2006), and in molluscs including mussels (*Mytilus* spp) (Sole *et al.*, 1995). Elevated levels have been detected following laboratory exposures to cadmium (Sheader *et al.*, 2006). Catalase (CAT) has been widely investigated across a broad range of species including dab (Livingstone *et al.*, 1992) and mussels (Regoli and Principato, 1995). The activity of glutathione reductase (GR) and glutathione peroxidase (GPx) are also measurable in most organisms. For example, Doyotte *et al.* (1997) used caged bivalves (*Unio tumidis*) to investigate the effects of a cokery discharge.

Lipid peroxidation (LP) has been shown to increase in dab after exposure to sediments known to be contaminated with a range of pollutants including metals (Livingstone *et al.*, 1993). However, a recurring theme in the literature reviewed is the variability in response of biological effects markers to oxidative stress. Laboratory data confirm that the systems triggered by oxidative stress respond to contaminant exposure (including metals), but field data routinely provide ambiguous results. This is thought to be due to these oxidative stress markers being highly influenced by a range of factors other than contaminant exposure. Several studies have demonstrated that gender, age and seasonality can all influence oxidative

stress responses (Sole *et al.*, 1995; Machala *et al.*, 1997; McFarland *et al.,* 1999; Zielinski and Portner, 2000).

It is suggested that while these biological effects tools are flexible and straight-forward to perform, their suitability for metal-specific monitoring purposes is questionable. This is because they are influenced by a range of other non-contaminant related factors. Furthermore, several other classes of chemicals have been shown to induce responses in antioxidant enzymes (e.g. PAH and PCBs), so their specificity to metals is very poor. Nevertheless, as with the other biological effects tools the measurement of oxidative stress has its place as part of a battery of techniques for monitoring environmental quality. The ICES Working Group on Biological Effects of Contaminants (WGBEC) concluded there was sufficient data to recommend the application of oxidative stress techniques in fish and mussels (ICES, 2009).

3.3.5 DNA strand breaks

The analysis of modified or damaged DNA has been shown to be a promising method for assessing exposure to genotoxic contaminants in aquatic environments. In general, the methods developed are sensitive to a range of contaminant concentrations and are applicable to a wide range of species. The method also has the ability to detect and quantify exposure to genotoxins without a detailed knowledge of the contaminants present.

The Single Cell Gel Electrophoresis (SCGE) or comet assay was first applied to ecotoxicology over 15 years ago, and has since become one of the most widely used tests for detecting DNA strand breaks in aquatic animals (see Figure 2) (Fenzilli *et al.*, 2009). The comet assay has many advantages over other methods commonly used to assess genotoxic exposure, including:

- genotoxic damage can be detected in most eukaryotic cell types at the single cell level;
- only a small number of cells are required;
- it is a rapid and sensitive technique; and
- due to the nature of DNA strand break formation, it provides an early warning response of genotoxic exposure.

3.3.5.1 **Methods available for measuring DNA strand breaks: comet assay**

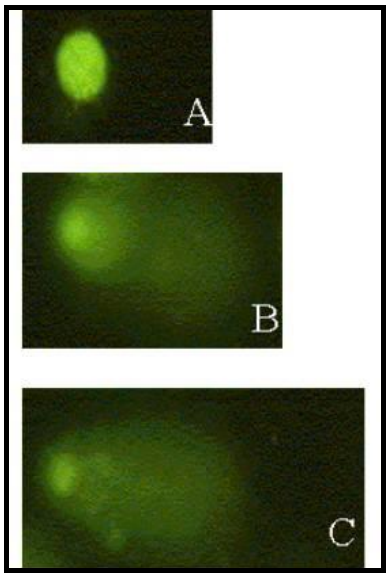
The majority of aquatic studies published to date have used circulating blood cells (either haemocytes or erythrocytes) as target cells for comet assay analysis. This is likely to be due to the practical advantage of processing tissues from a ready-made supply of nucleated cells in suspension. Solid tissues like gill or fish hepatocytes require dissociation prior to analysis, with the potential for introducing damage through enzymatic or mechanical processes. Studies have also demonstrated that different cell types respond with different sensitivities to contaminant exposure.

When comparing cells types it has been observed that circulating cells are less sensitive than hepatocytes or gill cells (Jha, 2008; Fenzilli *et al.*, 2009). In addition to the variation in response depending on cell type, it is also apparent that a range of comet assay protocols (differing in terms of agarose concentrations, lysing and electrophoresis parameters) have been used in studies with aquatic organisms (Jha, 2008; Fenzilli *et al.*, 2009). Therefore, effort is required to establish standardised protocols for the main species and cell types commonly used in environmental studies. The production of standard protocols, or the initiation of inter laboratory ring testing workshops focused on aquatic species are essential if the comet assay is to develop further as an environmental monitoring tool. The level of damage can be expressed in

several different ways including the per cent of DNA in the tail of the comet in relative to the head, or tail length (Fenzilli *et al.*, 2009).

Figure 2

Level of DNA damage in a cell using the comet assay: A is a normal cell; B is a cell with low-level DNA damage and C is a cell with a long tail and a high-level of DNA damage.



3.3.5.2 **Ecological relevance and field use: comet assay**

Marine invertebrates have been widely used as sentinel species in environmental monitoring programs. This is mainly due to their often relatively sessile nature, ability to bioaccumulate contaminants and general ease of capture. For example, Hartl *et al.* (2004) used the clam (*Tapes semidecussatus*) as an indicator species for the presence of potentially genotoxic substances in estuarine environments. The investigation demonstrated an increase in DNA damage in haemocytes, gill and digestive gland cells of animals exposed to contaminated sediments (Hartl *et al.,* 2004). Furthermore, the blue mussel (*Mytilus spp.*) has also been extensively deployed as a sentinel organism to assess the genotoxic effects of crude oil spills (for review see Martínez-Gómez *et al.*, 2010).

There are a limited number of comet assay studies utilising marine fish species in comparison to those using freshwater species (for detailed review see Jha, 2008; Fenzilli *et al.*, 2009). This is mainly due to the logistical problems associated with collecting fish at sea (e.g. need for a research vessel) and technical problems inherent with the assay, including the difficulty of performing reproducible electrophoresis at sea (e.g. dealing with adverse weather conditions). To date those studies undertaken have mainly focused on flatfish and bottom-feeding species, which due to their close association with sediment bound contaminants are widely used in marine monitoring programmes (ICES, 2009).

No formal quality assurance programmes are currently run within the marine monitoring community. However, a series of comet assay workshops have taken place with the aim of drafting a common regulatory strategy for industrial genotoxicology screening (Burlinson *et al.*, 2007). Final guidelines were drafted after the 4th International Workgroup on Genotoxicity testing. Results of the in-vivo comet assay workgroup provide a useful starting point for developing quality assurance programmes specifically focused on protocols employed in marine species. These include consideration of:

- cell isolation processes;
- cryopreservation processes;

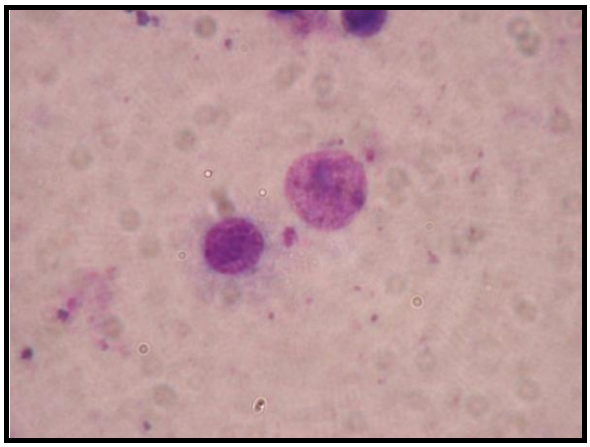
- concurrent measures of cytotoxicity, and
- image analysis and scoring methods.

3.3.6 Comet assay micronuclei (MN)

Micronuclei are acentric fragments or whole chromosomes that are not incorporated into daughter nuclei at anaphase (for review, see Chaudhary *et al.*, 2006). A micronucleus (MN) arises due to either spindle apparatus malfunction or chromosomal fragmentation. The lagging or whole/fragmented chromosome then forms a secondary small nucleus during the telophase stage of the cell cycle (see Figure 3). Scoring MN during interphase provides a measure of genotoxicity. Due to the fact that MN may arise from fragmented or whole chromosomes, their measurement can indicate exposure to either clastogenic (causing breaks in chromosomes) or aneupoidy (abnormal number of chromosomes) inducing contaminants.

Figure 3

Micronuclei in gill tissue from green lipped mussel (Reed, 2006).



3.3.6.1 Comet assay micronuclei (MN) methods available for measuring micronuclei

Detecting micronuclei is a relatively simple and inexpensive procedure, only requiring some basic preparation, involving smearing tissue (usually blood) samples onto a slide, staining the material and viewing under a microscope. When selecting a test species, consideration must be given to its karyotype. For example, many fish species are characterised by having an elevated number of small chromosomes (Udroiu, 2006).

In certain cases micronuclei formed after exposure to clastogenic contaminants will be very small and hard to detect under light microscopy. This can be addressed to a certain extent by using fluorescent staining. After selecting a suitable species researchers should also ensure that other factors including age, sex, temperature and diet are similar between sample groups. If conducting transplantation studies consideration needs to be given to cellular turn-over rate of the tissue being examined to ensure that sufficient cells have gone through cell division. For example, if using blood the times of erythropoiesis should be known prior to sampling. After sampling, slides should be coded and 'blindly' scored. The level of MN in a sample are expressed as a percentage relative to normal cells.

3.3.6.2 Ecological relevance and field use: micronucleus assay

Hooftman and de Raat (1982) were the first to successfully apply the MN assay to aquatic species when they demonstrated the induction of micronuclei in erythrocytes of the eastern mudminnow (*Umbra*

pygmaea), following waterborne exposure to the known mutagen ethyl methanosulphate (EMS). Since these initial experiments, other studies have validated the detection of micronuclei as a suitable biomarker of genotoxicity in a wide range of both vertebrate and invertebrate species (for review see Chaudhary *et al.*, 2006; Udroiu, 2006).

The application of the MN assay to blood samples is particularly attractive due to the ease of sampling and the number of cells present. However, recent studies have demonstrated that the frequencies of MN may differ between tissues and species (Barŝiené *et al.*, 2005). The frequencies of micronucleated erythrocytes sampled from peripheral blood and cephalic kidney differed and the authors and proposed the hypothesis that species such as cod and turbot may use their spleen to remove micronuclei from the peripheral. This phenomenon is recognised in mammalian biology and has led to the Organization for Economic Cooperation & Economic Development (OECD) statement "any appropriate mammalian species may be used provided it is a species in which the spleen does not remove micronucleated erythrocytes" (OECD, 1997).

Similar to the comet assay (and the other non-specific biological effects techniques), the MN assay detects genetic damage caused by a wide range of environmental contaminants. It therefore cannot be considered to be solely a metal contaminant specific biological effects marker.

3.4 Conclusion

Each of the biological effects techniques discussed above is known to show some form of a dose response to metal contaminant exposure. In most cases (except near point source discharges) the dissolved metal concentrations in marine waters are unlikely to reach high enough levels to result in detectable biological effects. However in urbanised estuaries, metal concentrations in sediments, or bioconcentrated or bioaccumulated biota, could produce levels which are high enough to induce detectable changes in one or all of the methods assessed in this report.

The biological effects techniques reviewed vary in their specificity to metals. MT induction is regarded as specific for metals (primarily cadmium, copper, mercury and zinc) and ALA-D is regarded as being specific to lead. Lysosomal and oxidative stress, along with genetic damage as detected using the MN and comet assay, are indicators of general stress caused by a range of anthropogenic chemicals, not specifically metals.

All of the biological effects techniques discussed are known to be affected by a range of physical and/or seasonal influences that can confuse the picture when trying to correlate observed effects with environmental metal concentrations. It is therefore essential that environmental managers use biological effects tools that have been extensively characterised in the selected sentinel species. No single biological effects tool offers particularly useful monitoring or risk assessment data when applied in isolation. Where possible they should be used in conjunction with a battery of other techniques (e.g. chemical analysis and SQGs) to enable a more complete picture of the health of a population to be established. This weight of evidence approach can effectively determine when adverse environmental effects occur.

Internationally recognised expert groups are currently developing assessment criteria for many of the metal-specific and general biological effects tools (Thain *et al.*, 2008; Lyons *et al.*, 2010). If used in an integrated chemical and biological monitoring programme, biological effects tools offer managers a promising toolbox to aid environmental risk assessment.

With respect to the application of biological effect tools it is important to realise that different strategies are required depending on the purpose of the environmental monitoring programme. These have been outlined by Van der Oost *et al.* (2003). Steps include first carrying out cost-effective measurements in a

stepwise approach, then obtaining insights into the cause of observed effects in the field, followed by studying trends in time or spatial variation, or using biological effect responses as signals of negative effects on the ecosystem. The characteristics and specific research needs for the application of biological effects tools to perform screening, diagnosis, trend monitoring (both in temporal and spatial) or risk assessment are outlined in Table 2.

Table 2

Recommendations for the implementation of biological effects tools in environmental monitoring (modified from Van der Oost *et al.*, 2003).

Requirement	Purpose	Use of biological effects tools	Specific needs for further implementation of this concept
Investigative and operational monitoring	Cost-effective use of biomarkers as an initial screening step. Early-warning marker system of the biological effects of contaminants. To signal the possible cause for observed adverse effects in populations of a certain species.	Simple biological effects measurements in an integrated approach (together with chemical analyses and bioassays).	Sensitive biological effects tools with specificity for certain types of contaminants or certain types of effect mechanism (for example see lists contaminant specific biomarkers recommended by ICES (for a complete list see Appendix 1).
Trend monitoring and assessment of spatial variation	Study contaminant-related effect responses over time and across geographical areas. To compare changes in contaminant-related responses over time with quality objectives (e.g. to check environmental improvement after remedial action).	Spatial and temporal sampling and biological effects monitoring.	Knowledge of the influence of confounding factors is required for each biomarker before they are deployed. Biomarkers require the development of background documents and the development of internationally accepted assessment criteria (e.g. those proposed by ICES, 2009).

Requirement	Purpose	Use of biological effects tools	Specific needs for further implementation of this concept
Site-specific risk assessment	Study of contaminant bio-availability and related risks at polluted sites (hotspots). Ideally signalling effects at higher levels of organisation.	Biological effects tools acting as indicators of contaminant exposure. With biological effects tools acting as an early warning signal of adverse effects on the individual, population or community level.	Biological effects techniques must be applicable in local species or *in situ* bioassays, using internationally established techniques (for comprehensive review, see Van der Oost *et al.*, 2003).

4 Methods

4.1 Introduction

initial study aims to use biological effects markers to detect early signs of stress at the cellular and molecular level for a New Zealand native estuarine shellfish and fish species. The objective was to measure cellular damage in shellfish and fish sampled along a contaminant gradient that covered eight estuaries in the Auckland region. Sites were selected based on their expected contamination levels and a control site.

4.2 Sampling

Sediments, cockles (*Austrovenus stutchburyi*) and yellowbelly flounder (*Rhombosolea leporina*) were collected from eight estuaries in the Auckland region. The majority of sites were located in the Waitemata Harbour due to the higher number of discharges from stormwater, industries and other inputs into these estuaries (Figure 4). One estuary in each of the Manukau and Kaipara Harbours was also sampled (Figure 5). Exact locations of each sampling site for flounder, cockles and sediments are shown in Tables 3 and 4, respectively. All samples were collected between 23 August and 10 September 2010.

4.2.1 Sediment collection and analysis

Surficial sediments (0-2 cm) were collected from a 2 m^2 area adjacent to the cockles sampled using a plastic scoop previously cleaned using 10 per cent hydrochloric acid solution. Sediments were placed in plastic bags and sent to Hill laboratories (Hamilton) for the analysis of total metals (< 500 µm particle size) and extractable metals (<63 µm particle size) for zinc (Zn), copper (Cu) and lead (Pb).

4.2.2 Cockle tissue collection and preparation

A total of 20 adult cockles were collected at each site based on their size (~2 cm). The cockles were kept in a 10 L bucket of seawater during transportation to the laboratory. The length and weight of five adult cockles were measured and a 0.2 cm^2 area of gill tissue was dissected and immediately placed in 1 ml Hanks Balanced Salt Solution (HBSS) solution for 20 minutes at room temperature. All cockles were processed within four hours of collection (i.e., after transportation of samples to the laboratory).

4.2.3 Flounder tissue collection and preparation

Flounder were caught by a commercial fisherman. Only a few large female flounder (~30 cm) were typically caught due to the time of year. The length, weight and sex of each flounder collected was measured and a 0.5 cm^2 area of gill tissue was dissected and immediately placed in 1.5 ml HBSS for approximately three hours at room temperature (i.e., after transportation of samples to the laboratory). These gill samples were taken to NorthTec (Whangarei) for the micronuclei assay analysis.

A 0.2 cm^2 sample of the liver tissue was dissected and immediately placed into 1 ml RNALater® and kept refrigerated at 4ºC. These were sent to Cefas, UK, for gene expression analysis unrefrigerated.

Figure 4

Location of sampling sites in (A) Waitemata Harbour and (B) Waiheke Island.

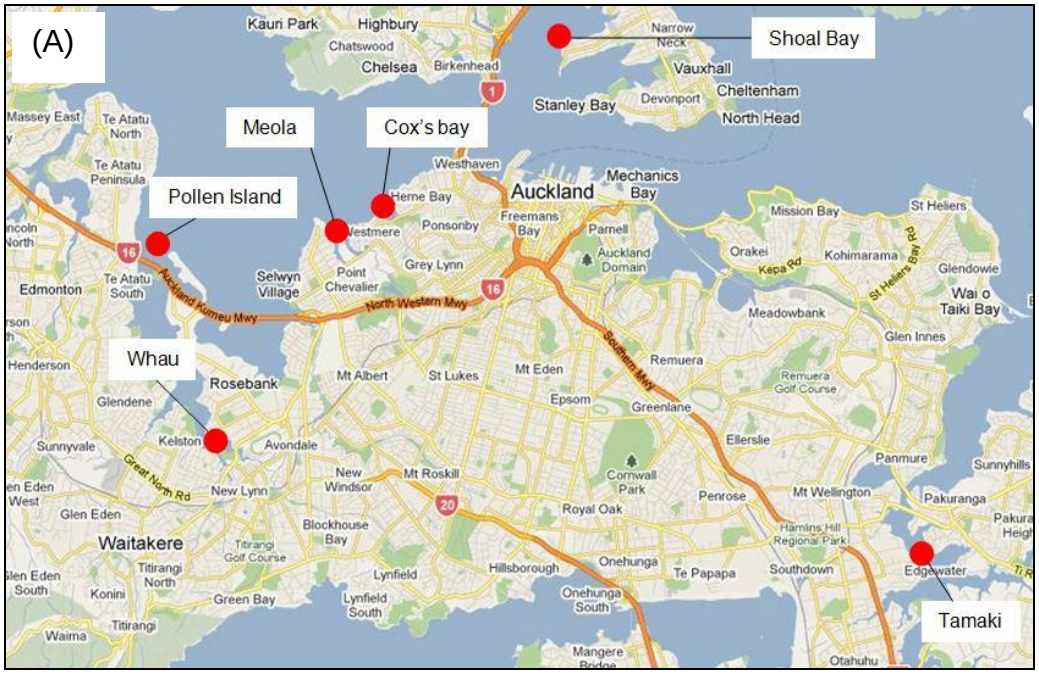


Figure 5

Location of sampling sites in the (A) Manukau Harbour and (B) Kaipara Harbour.

Table 3

Location of flounder sampling sites in Auckland's estuaries in 2010.

Site	Date	Sample ID	GPS	Comment
Waitemata Harbour				
Te Matuku Bay (Waiheke Island)	8/9/2010	H1F – H2F	Net 1 36.831821 S 175.108981 E Net 2 36.829279 S 175.103145 E	Only 2 fish caught. Good condition.
Meola	23/8/2010	M1F - M5F	36.853527 S 174.712315 E	4 out of 5 fish with fin damage
Cox's Bay	23/8/2010	C1F – C5F	36.848341 S 174.72240 E	2 out of 5 fish with fin damage
Shoal Bay	23/8/2010	S1F – S5F	36.810315 S 174.767418 E	2 out of 5 fish with fin damage and 1 fish with growth on upper side
Whau	24/8/2010	W1F – W5F	36.887859 S 174.666653 E	3 out of 5 fish with fin damage and 1 fish had severe ulcerations
Tamaki *	24/8/2010	T1F – T6F	Net 1 near SH1 36.946325 S 174.858634 E Net 2 at Ti Rakau Dr. 36.926172 S 174.882023 E	1 out of 6 fish with fin damage
Manukau Harbour				
Pahurehure Inlet	31/8/2010	P1F – P5F	Net 1 Opposite Weymouth 37.0646 S 174.874433 E Net 2 Upper Inlet 37.056133 S 174.906783 E	1 out of 5 fish with fin damage
Kaipara Harbour				
Shelly Beach	30/8/2010	K1F – K6F	Net 1 36.608879 S 174.359465 E Net 2 36.611945 S 174.366524 E Net 3 36.596166 S 174.365988 E	6 out of 6 fish were in good condition.

* Special permits were obtained from the Ministry of Fisheries to sample in this estuary.

Table 4

Location of cockle and sediment sampling sites in Auckland's estuaries in 2010.

Site	Date	Sample Id	GPS	Comment
Waitemata Harbour				
Te Matuku Bay, Waiheke Island*	8/9/2010	H1C – H5C	36.836783 S 175.133083 E	Large size, excellent shell condition & colour.
Meola	10/9/2010	M1C - M5C	36.855222 S 174.707645 E	Small size, shells in poor condition, not very numerous.
Cox's Bay	10/9/2010	C1C – C5C	36.85045 S 174.72485 E	Numerous, poor condition, medium size.
Shoal Bay	10/9/2010	S1C – S5C	36.812991 S 174.771412 E	Small size, poor condition, not very numerous.
Pollen Island (Motu Manawa)*	23/8/2010	P1C - P5C	36.858779 S 174.659381 E	Small size, shell in good condition.
Tamaki (inner)	23/8/2010	T1C - T5C	36.906666 S 174.867024 E	Large size, shell damage by borers.
Manukau Harbour				
Pahurehure Inlet	31/8/2010	W1C – W5C	37.0646 S 174.874433 E	Large size, good condition.
Kaipara Harbour				
Shelly Beach (flats north west)	30/8/2010	K1C - K5C	36.571114 S 174.379318 E	Small size, shells in good condition, not very numerous.

* Permits were obtained by Department of Conservation to sample in marine reserves.

4.3 Micronuclei assay of cockle and flounder tissue

The 0.5 cm^2 area of gill tissue of the cockles and flounders were transferred into 1 ml dispase:HBSS solution pre-heated to 37°C. The gill tissues were incubated at 37°C for 20 minutes and an aliquot of 1 ml of dispase:HBSS solution was discarded from each sample. Then 1 ml of HBSS (at room temperature) was added. Each sample was filtered using a 100 µm mesh nylon filter to remove and separate the cells and tissues. The remaining samples were centrifuged at 2,000 RPM for five minutes and the supernatant removed. A 800 µl quantity of prefix solution was added to the suspended cell pellet, homogenised, and left for 20 minutes at room temperature. The samples were centrifuged at 2,000 RPM for five minutes and the supernatant removed.

A 750 µl quantity of a fixative solution was added and homogenised. A further 750 µl quantity of fix solution was added and homogenised. The samples were centrifuged at 2,000 RPM for five minutes. This last step was repeated. A 300 µl of cells was added to each microscope slide and one slide was prepared for each cockle and flounder. Slides were left to dry at room temperature. Once dry, slides were placed into 5 per cent Giemsa solution for 10 minutes. Slides were washed in deionised water, dried and covered with a microscope cover slip. Each slide was analysed under a 100x magnification microscope and 500 cells counted to assess the number of micronuclei.

4.4 Gene expression in flounder tissue

4.4.1 RNA extraction

Fish livers were dissected from the flounder and fixed overnight in RNAlater® before being stored at -80°C until further use. RNA extraction was carried with GenElute Mammalian Total RNA Miniprep Kit (Sigma, Poole, UK). Roughly 20 mg of liver was ground with a pellet pestle in 500 µl of lysis solution before being extracted according to manufacturer's protocol, including the optional DNaseI step. RNA was quantified using nanodrop and quality checked on a semi-denaturing agarose gel. Reverse transcription was performed with 1 µg total RNA, oligo dT and random hexamer primer mix and SSII reverse transcriptase (Invitrogen) according to manufacturers protocol. A 1:10 dilution of this product was then used for qPCR expression analysis.

4.4.2 Real-time quantitative PCR

Promega GoTaq SYBR Green readymix (Promega, UK) was used with primers at 0.2 mM (see Table 5) and 2 µl dilute cDNA (equating to 5 ng total RNA) as template in a 20 L reaction. The cycling conditions were 95 °C for two minutes, then 40 cycles of 95°C for 15 seconds, 60°C for one minute with fluorescence being recorded during each 60°C step. Each reaction was carried out in duplicate in an Applied Biosystems StepOnePlus real-time PCR system and normalised to levels of a beta-actin housekeeping-gene.

4.4.3 PCR data analysis

Quantitative PCR data were analysed in MS Excel by standard relative quantification method. Gene expression was normalised back to the previously characterised house-keeping gene beta actin using the $2^{-\Delta\Delta Ct}$ equation and calibrated to reference fish (R2).

4.4.4 Reference material

The expression on biomarker genes in yellowbelly flounder were compared against reference material from yellowbelly founder tissues sampled from highly contaminated areas in the North Sea and around UK. This reference material has demonstrated a link between contaminant signal and expression of biomarker genes. The New Zealand and UK species are very similar and have been cited in international literature as being >80% match for biomarker genes (CEFAS pers. com.).

Table 5

Primers used during qPCR analysis of gene expression.

Gene	Acc No.	Primers (5'-3')		Reference
Aldehyde dehydrogenase	DV568325	F-	GGGAGAAGATTGCAAAGCTG	Falciani 2008
		R-	GAGCAGGAGCAGACTTCCAC	
Metallothionein	AJ291833	F-	CTGCGAATGCTCCAAGACT	Pers comms
		R-	AGCCGAATGGGCAGCATGG	
Glutathione-S-Transferase	AJ310428	F-	CCTTCGACAAAAAGGAGCAC	Pers comms
		R-	TTTCCCTGGGACTTGAACTG	
Copper/Zinc superoxide dismutase	AJ291980	F-	TGGAGACAACACAAACGGG	Williams 2003
		R-	CATTGAGGGTGAGCATCTTG	
Beta-actin	AF135499	F-	GACCAACTGGGATGACATGG	Falciani 2008
		R-	GCGTACAGGGACAGCACAGC	

5 Results and Discussion

5.1 Sediment metal concentrations

Bioavailable metal concentrations (<63 µm particle size) measured in Auckland's estuarine sediments are shown in Table 6 and total metal concentrations (<500 µm particle size) are shown in Table 7. PELs were not exceeded at any site surveyed. TELs were exceeded for one or more bioavailable metals at the Shoal Bay (Pb), Meola (Pb), Pollen Island (Cu, Zn, Pb), Tamaki (Zn, Cu, Pb) and Cox's Bay (Zn, Cu, Pb) sites. Concentrations that are in bold also exceed the ANZECC ISQG-Low guidelines. As stated in Section 3.1, it should not be assumed that exceeding SQGs necessarily means adverse effects will inevitably occur (Hübner *et al.*, 2009). It is generally recognised that contaminant effects should integrate chemical with biological data.

Table 6

Concentration of bioavailable metals (<63 µm mg/kg dw) in estuarine sediments collected from the Auckland region and colour coded based on NOAA SQGs (Buchman, 2008).

Sample code	Auckland site	Zinc (Zn)	Copper (Cu)	Lead (Pb)
826681.22	Shelly Beach	36	4.6	6.5
826681.24	Te Matuku Bay	45	4.2	13.5
826681.20	Pahurehure Inlet	59	5.4	11.4
826681.21	Shoal Bay	94	16.8	32
826681.17	Meola	117	18.3	37
826681.23	Pollen Island	143	21	37
826681.18	Tamaki	195	26	35
826681.19	Cox's Bay	**240**	40	**85**

* TEL is Threshold Effects Level and PEL is Probable Effects Level (Hübner *et al.*, 2009). Concentration in **bold** exceed the ANZECC ISQG-Low guidelines (ANZECC 2000). Concentrations are highlighted green if below the TEL (adverse effects rarely observed), yellow if above the TEL but below the PEL (adverse effects occasionally observed) and red if the PEL is exceeded (adverse effects frequently observed).

The results obtained were similar to bioavailable metal concentrations in estuarine sediments collected from Auckland Harbour by Stewart *et al.* (2009) except at Shoal Bay and Meola. All bioavailable metal concentrations in Shoal Bay sediments were lower and zinc did not exceed the PEL guideline in this study. In Meola sediments, all bioavailable metal concentrations were lower in this study.

Total metal concentrations in the <500 µm particle size exceeded TELs for one or more metals at two sites, Tamaki (Zn) and Cox's Bay (Zn, Pb). Total metal concentrations did not exceed PELs or the ANZECC ISQG-Low guidelines.

Based on the metal results in this study and the SQGs, the following would be expected:

(1) rare adverse effects at Te Matuku Bay, Shelly Beach and Pahurehure Inlet; and

(2) occasional adverse effects at Shoal Bay, Meola, Pollen Island, Tamaki and Cox's Bay.

The latter sites are all located in the Waitemata Harbour where there are known inputs of contaminants from stormwater and industrial discharges. The former sites are in the Hauraki Gulf, Kaipara Harbour and Manukau Harbour which tend to have less contaminant inputs from urban areas.

Table 7

Concentration of total metals (<500 µm mg/kg dw) in estuarine sediments collected from the Auckland region and colour coded based on NOAA (Buchman, 2008) SQGs.

Sample number	Auckland site	Zinc (Zn)	Copper (Cu)	Lead (Pb)
826681.16	Te Matuku Bay	21	2.4	5.2
826681.14	Shelly Beach	28	3	3.9
826681.12	Pahurehure Inlet	38	3.7	6.4
826681.13	Shoal Bay	66	9	17.3
826681.15	Pollen Island	78	7.6	18.6
826681.9	Meola	93	13	27
826681.10	Tamaki	144	13	19.2
826681.11	Cox's Bay	145	16.9	36

* TEL is Threshold Effects Level and PEL is Probable Effects Level (Hübner *et al.*, 2009). Concentrations are highlighted green if below the TEL (adverse effects rarely observed), yellow if above the TEL but below the PEL (adverse effects occasionally observed) and red if the PEL is exceeded (adverse effects frequently observed).

5.2 Micronuclei in shellfish

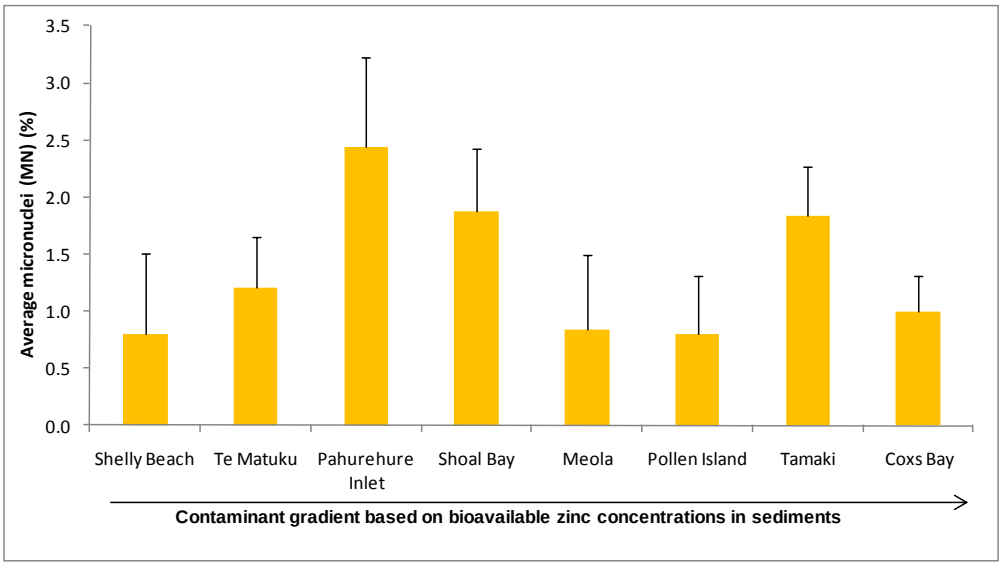
Average micronuclei (± standard deviation) counted in gill tissue of cockles (*Austrovenus stutchburyi*) and yellowbelly flounder (*Rhombosolea leporina*) are shown in Figures 5 and 6, respectively. The length and weight of each adult cockle and each flounder are shown in Tables E and F (in Appendix 1).

Micronuclei were lowest in cockles at the Pollen Island, Shelly Beach and Meola sites and highest at the Pahurehure Inlet, Tamaki and Shoal Bay sites. Pahurehure Inlet cockles had highest micronuclei measured at any of the sites. Highest micronuclei in cockles in the Waitemata Harbour sites were Tamaki Estuary>Shoal Bay>Te Matuku>Cox's Bay >Pollen Island>Meola.

The induction of micronuclei in cockle gill tissue suggests that cellular damage is occurring at all sites but there was no clear relationship between the percentage of micronuclei in cockles and the increasing contaminant gradient for bioavailable concentration of zinc (Figure 5). A linear relationship was previously detected (R^2 = 0.91) between micronuclei in cockles and total zinc concentrations in sediments at Cox's Bay>Hobson> Motions>Tamaki >Pollen Island in 2008 (Reed & Lyons, 2009) (see Figure 9). Average micronuclei were four times higher in 2010 than at the same sites sampled in 2008. Further investigation of seasonal differences in cockle response and contaminants detected in sediments would aid the use of the micronuclei assay for monitoring purposes.

Figure 5

Average percent micronuclei per 500 cells (±standard deviation) in gill tissue of cockles (*Austrovenus stutchburyi*) collected from the Auckland region, August 2010.

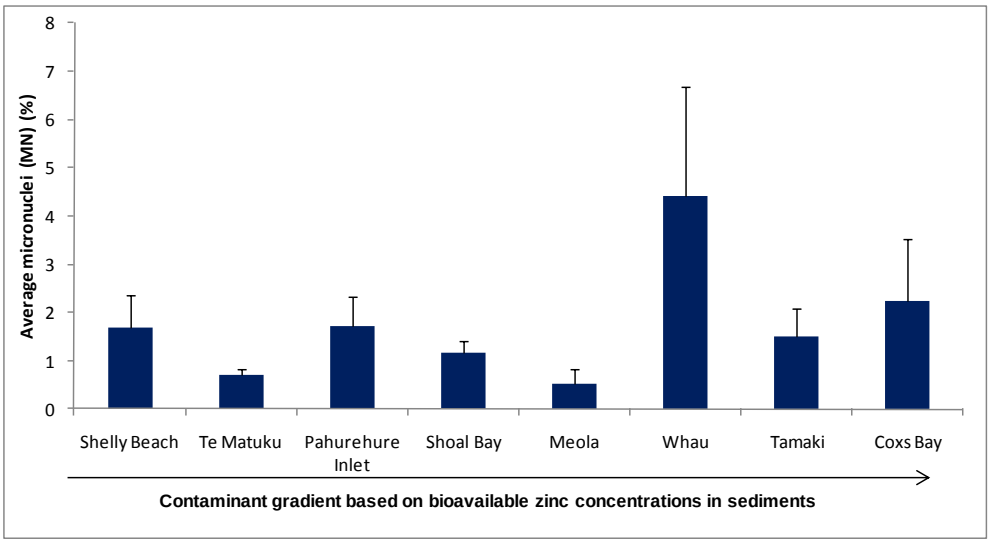


5.3 Micronuclei in fish

Micronuclei in yellowbelly flounder gill tissue were lowest at the Te Matuku Bay and Meola sites and highest in the Whau Estuary and Cox's Bay sites. In the Whau Estuary, micronuclei in the flounder were markedly higher than all other sites but were highly variable between fish. These fish were collected in the upper reaches of the Whau some distance from Pollen Island where the cockles were collected. One of the flounder caught in the Whau Estuary had severe ulcerations on its underside as shown in Figure 7. High concentrations of bioavailable zinc, copper and lead (see Table 1) were measured in the upper Whau sediments by Stewart *et al.* (2009). There was no clear relationship between the percentage of micronuclei in yellowbelly flounder and the increasing contaminant gradient for bioavailable concentration of metals. Figure 6 shows the percentage of micronuclei in flounder as zinc concentrations increase in sediments.

Figure 6

Average percent micronuclei per 500 cells micronuclei (±standard deviation) in gill tissue of yellowbelly flounder (*Rhombosolea leporina*) collected from the Auckland region, August 2010.



5.3.1 A comparison of micronuclei in shellfish and fish

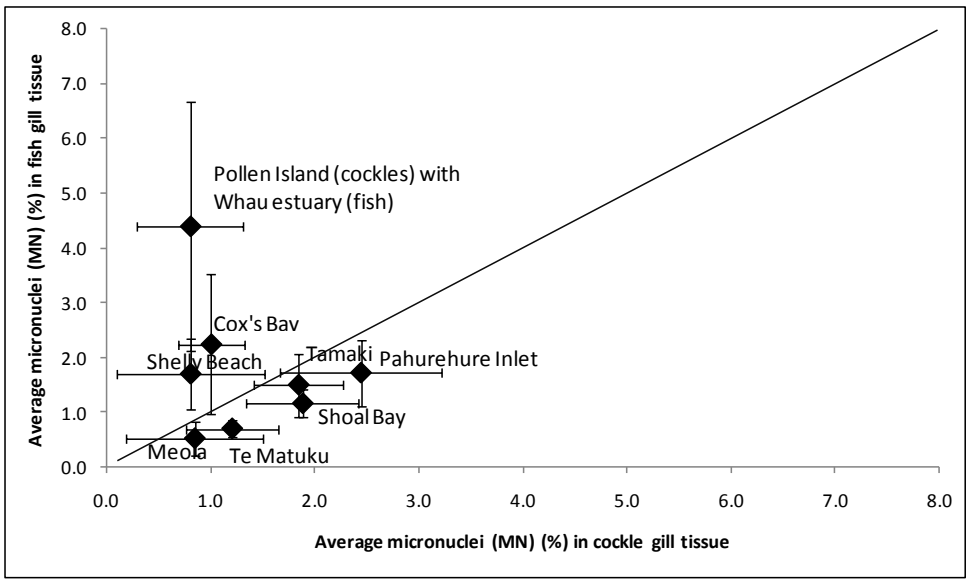
A comparison of the average micronuclei (±standard deviation) in gill tissue of cockles and the yellowbelly flounder was undertaken to determine whether there were similar responses occurring at each site (Figure 8). Three sites had a higher percentage of micronuclei in flounder than cockles and these were in the Whau Estuary, Cox's Bay and Shelly Beach. The Whau Estuary and Pollen Island sites were the most different because the Whau Estuary had higher metal concentrations in sediments than Pollen Island and therefore higher genetic damage in comparison. More micronuclei were measured in cockles than in flounder at the Pahurehure Inlet, Shoal Bay, Te Matuku Bay, Meola and Tamaki Estuary sites. Overall, there was no clear relationship between the percentage of micronuclei in cockles and yellowbelly flounder at these sites.

Figure 7

An example of a yellowbelly flounder caught in the upper reaches of the Whau Estuary on 24 August 2010. There is a large ulcer on the underside of this fish.

Figure 8

Comparison of average micronuclei in gill tissue of cockles and yellowbelly flounder collected from the Auckland region, August 2010. Note: the line is not a regression line it is simply a visual divide between cockle and flounder gill tissue data.



5.3.2 Micronuclei and metal concentrations in sediments

In this study, sites were ranked according to the total percentage of micronuclei measured at each site (Table 8). Sites were colour coded based on their ecological condition using ARC's benthic health model described in Anderson *et al.*, (2006). Based on output from the benthic health model, Green indicates a healthy ecosystem and red indicates a degraded ecosystem. Earlier in Figure 1 (Section 3.1) ecosystem and community responses were described as "later effects" of exposure to pollutant(s) as increasing harm

is realised (Bayne *et al.*, 1985). Cellular alteration responses are expected to be an earlier sign of harm by pollutant(s) and could therefore be used as an "early warning" signal by resource managers prior to an ecosystem response being realised. Results in Table 8 suggest that this hypothesis is not proven in this case. For example, Meola has the lowest number of cellular alterations but is classified by Anderson *et al.* (2006) as being a degraded ecosystem.

Table 8

Total percentage of micronuclei in cockle and yellowbelly flounder collected from the Auckland region, August 2010, and colour coded based on Anderson *et al.* (2006) ecological condition (in which a benthic health rating is used where green indicates a healthy ecosystem and red indicates a degraded ecosystem).

Auckland site	Cockle micronuclei	Flounder micronuclei	Total micronuclei at each site
Whau/Pollen Island#	0.8	4.4	-
Pahurehure Inlet	2.4	1.7	4.1
Tamaki	1.8	1.5	3.3
Cox's Bay	1.0	2.2	3.2
Shoal Bay	1.9	1.2	3.1
Shelly Beach*	0.8	1.7	2.5
Te Matuku Bay*	1.2	0.7	1.9
Meola	0.8	0.5	1.3

* No colour = sites not included in Anderson *et al.* (2006) study.

Summed totals not used due to distance between sites.

A comparison of bioavailable metals in sediments and the average percentage of micronuclei in gill tissues of cockles and fish are shown in Figure 9 and 10, respectively. Adverse effects were expected at the Whau, Cox's Bay, Tamaki, Shoal Bay, Meola and Pollen Island sites on occasion based on their >TEL<PEL bioavailable metal concentrations in sediments.

Micronuclei from gill tissues in cockles show no clear relationship with sediment zinc, copper or lead concentrations (Figure 9). Sites with known contamination issues, for example Cox's bay, Meola and Pollen Island had similar or lower percentages of MN than more healthy sites like Te Matuku and Shelly Beach.

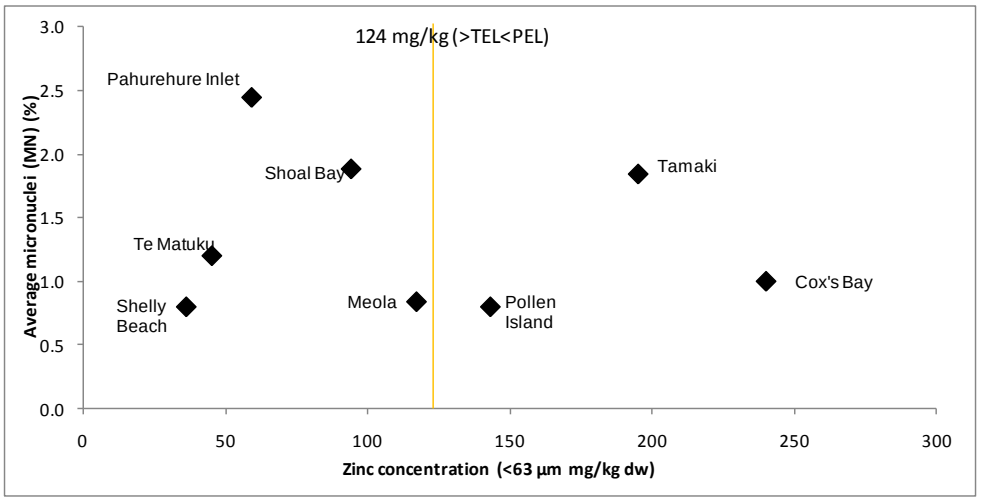
Some relationship between MN and contaminant concentrations was more evident in yellowbelly flounder however, particularly in response to copper and zinc sediment concentrations (Figure 10). The relationship may not be linear however, with lower levels of contaminants resulting in more variation in MN which may be indicative of threshold physiological stress points.

The relationship between the number of micronuclei in yellowbelly flounder and the concentrations of bioavailable zinc and copper becomes more evident for the inner Waitemata Harbour sites (Figure 11). A strong linear relationship (R^2 = 0.97) was found for bioavailable copper and, to a lesser extent, for bioavailable zinc (R^2 = 0.71) in sediments. There was no clear relationship with lead which is in contrast to results from the benthic health model which attributed their ecological condition data to zinc and lead concentrations in the sediments (Anderson *et al.*, 2006). Further investigation is required to clarify this relationship.

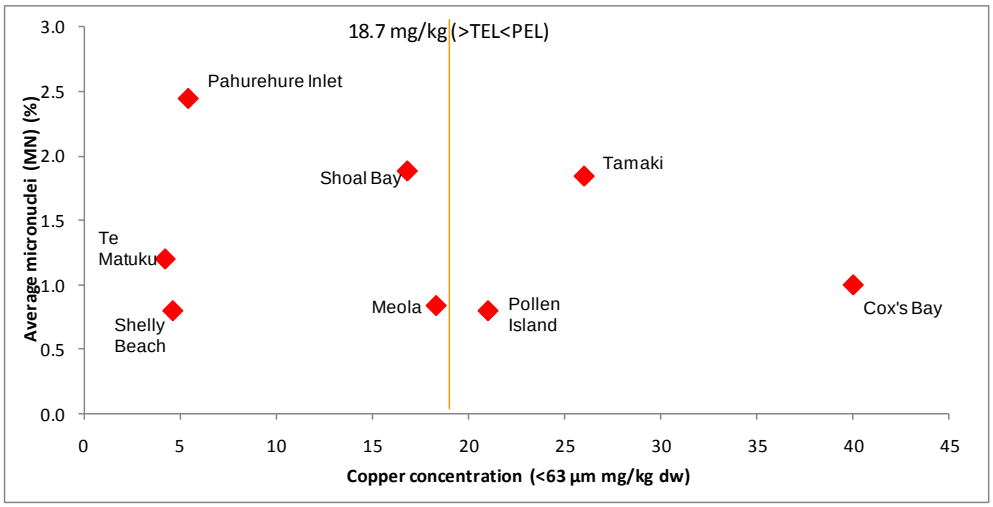
Figure 9

Comparison of average percent micronuclei per 500 cells in gill tissue of cockles and bioavailable metal concentrations in sediments collected from the Auckland region: (A) zinc, (B) copper, (C) lead.

(A)



(B)



(C)

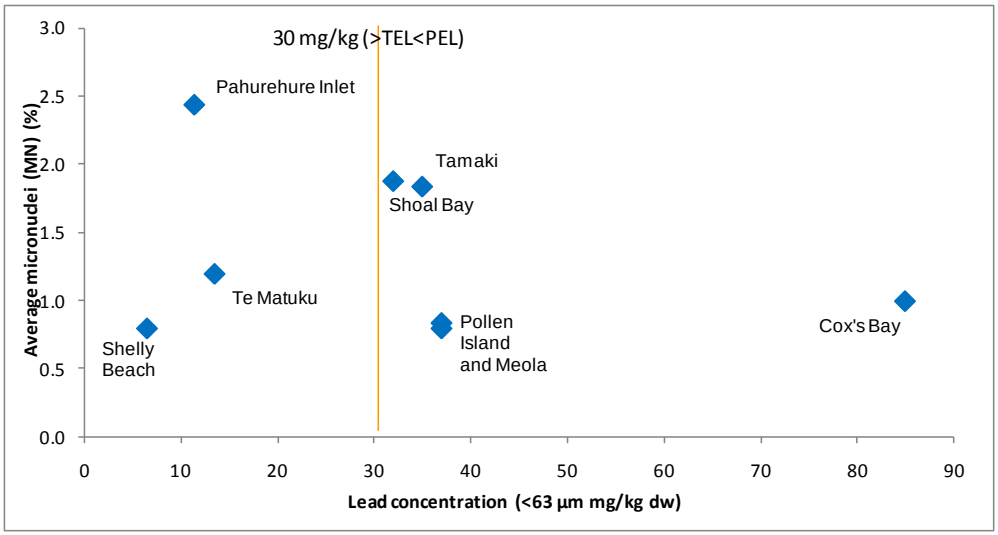
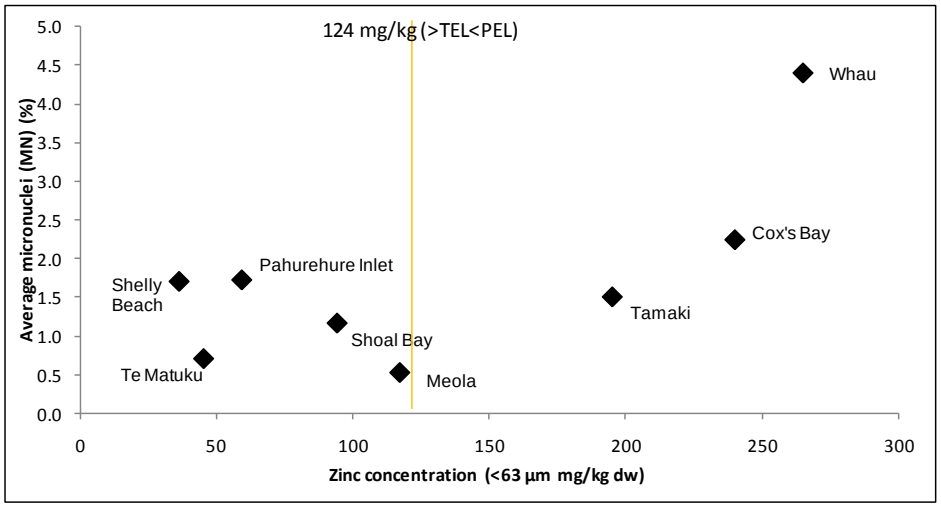


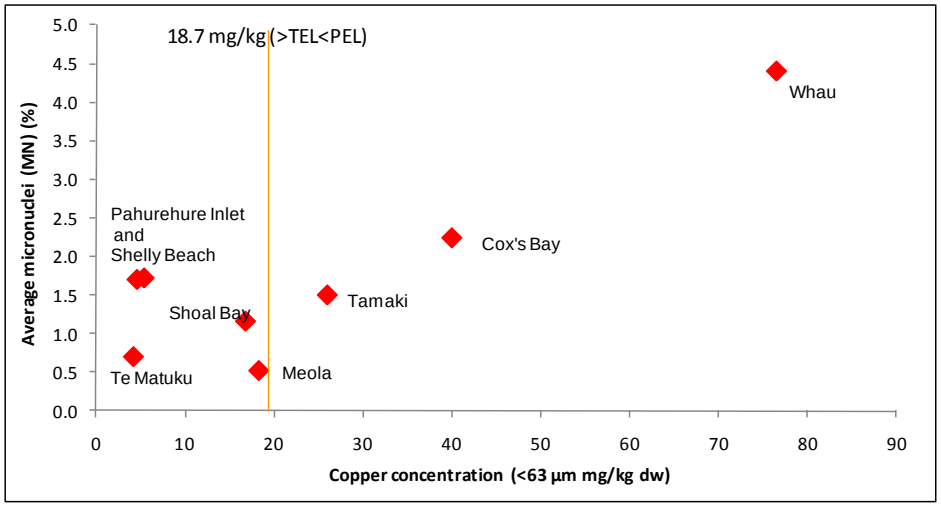
Figure 10

Comparison of average micronuclei in gill tissue of yellowbelly flounder and bioavailable metal concentrations in sediments collected from the Auckland region: (A) zinc, (B) copper, (C) lead. (Note concentrations in the Whau estuary are from Stewart *et al.*, 2009).

(A)



(B)



(C)

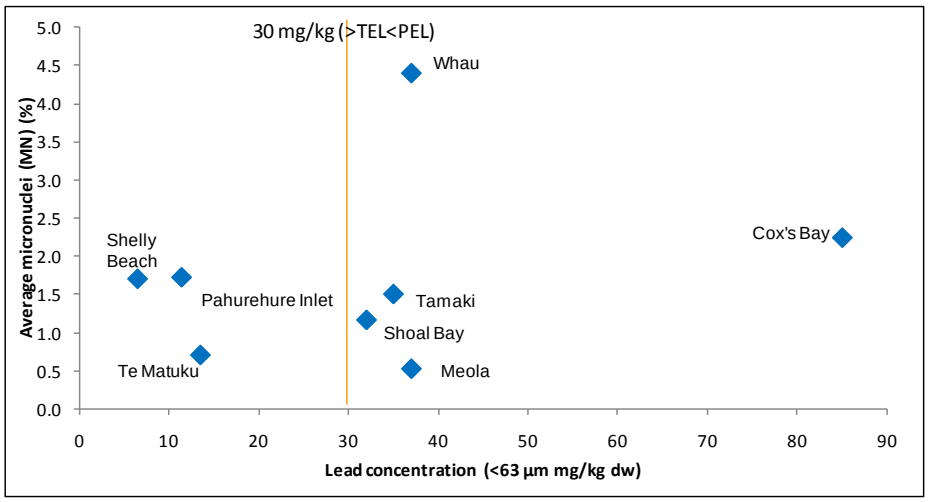
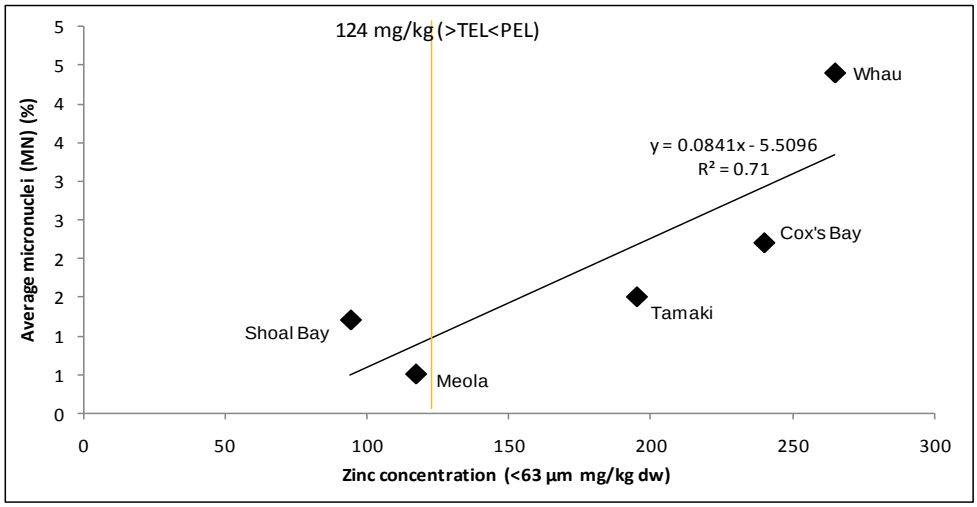


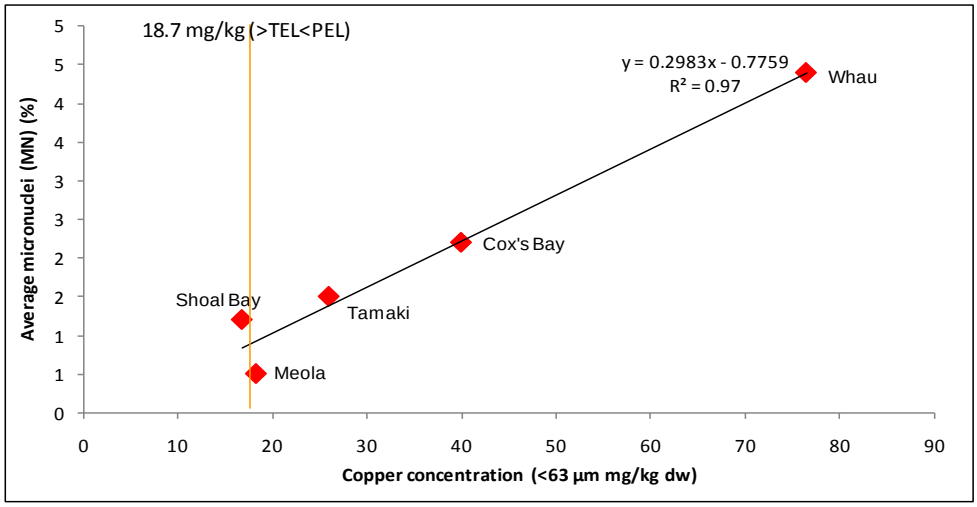
Figure 11

Comparison of micronuclei in gill tissue of flounder and bioavailable metal concentrations in sediments from the inner Waitemata Harbour in winter 2010: (A) zinc, (B) copper, (C) lead. (Note concentrations in the Whau estuary are from Stewart *et al.*, 2009).

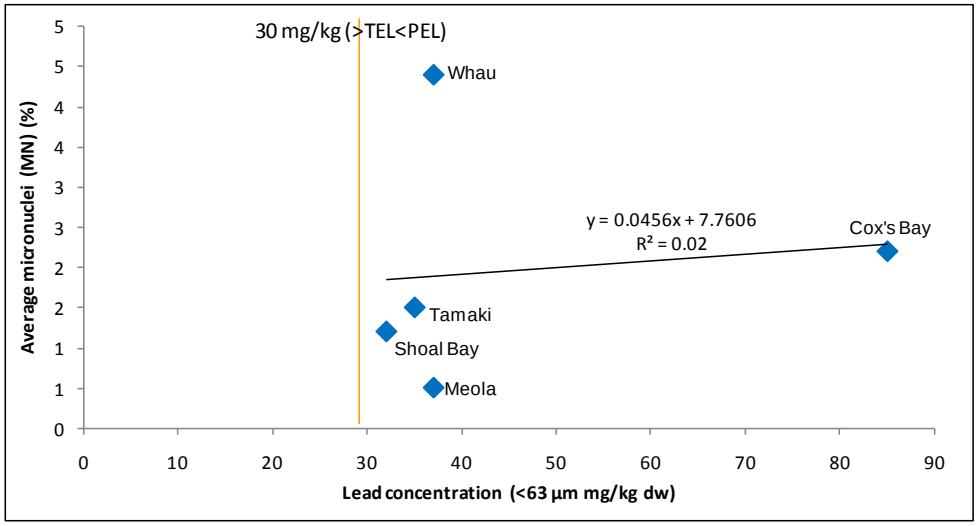
(A)



(B)



(C)



5.4 Gene expression in flounder

Expression of four known biomarker genes was measured in hepatic tissue of female yellowbelly flounder from eight sites known to differ in levels of bioavailable contaminants. The four genes selected for quantitative real-time PCR analysis were metallothionein, aldehyde dehydrogenase, glutathione-S-transferase and Cu/Zn superoxide dismutase. In general, Te Matuku appeared to be the healthiest ecosystem with low levels of metallothionein, glutathione-S-transferase and Cu/Zn superoxide dismutase expressed relative to other sites. The results for each gene expression assay are discussed in greater detail in the following sections.

5.4.1 Metallothionein

Metallothionein, a low molecular weight protein, is a known scavenger of heavy metals including copper, cadmium and lead and is overexpressed in fish exposed to these contaminants *in vitro* and *in situ* (George *et al.*, 2004). In this situation, where levels of metal bioavailability are known, metallothionein is the most indicative of the biomarker genes.

In this investigation, yellowbelly flounder had increased levels of metallothionein at Cox's Bay, Meola and Shoal Bay while lowest levels were observed at Te Matuku, Tamaki and Pahurehure. Interestingly the sites that appeared to be more impacted were geographically close and were all suggested earlier in this report as being sites which may show occasional adverse effects due to contamination. However, an absolute correlation between metal concentration and gene expression is not directly evident. The changes observed between fish are in the range of those observed by Sheader *et al* (2006) when European flounder were exposed directly to cadmium through intraperitonial injection, suggesting the results here represent genuine biological responses. However, it should be noted that expression of this gene can be induced through other forms of oxidative stress (Andrews, 2000) which may confound results in environmental situations; this suggests a need to combine a study of this gene alongside others in a weight-of-evidence approach to monitoring.

5.4.2 Glutathione-S-transferase

Glutathione-S-transferase enzymes are directly involved in detoxification of many xenobiotic compounds and can be overexpressed when the animal is under heavy anthropogenic or natural pressures (Williams *et al.*, 2003). In this study little difference between the majority of sites was observed, with only Te Matuku showing a significantly lower expression.

5.4.3 Aldehyde dehydrogenase

Aldehyde dehydrogenase is overexpressed in European flounder exposed to benzo(a)pyrene (Winzer *et al.*, 2002) and is a known biomarker of PAH exposure in contaminated estuaries (Williams *et al.*, 2003). Here low levels of aldehyde dehydrogenase expression in the Tamaki estuary were observed, suggesting lower levels of PAHs. However, expression was notably higher at Shoal Bay, Cox's Bay and the Whau estuary than at Tamaki suggesting higher levels of PAH contamination and a possible need to clarify the root cause of this difference. Aldehyde dehydrogenase is directly involved in the detoxification of aldehyde groups and, therefore indirectly in PAH detoxification (Glatt *et al.*, 2008). However, more direct markers of PAH exposure, such as the cytochrome P450 CYP1A exist and may be useful to use alongside aldehyde dehydrogenase in the future. CYP1A activity is the most utilised biomarker of PAH exposure, as it is directly involved in the metabolism of PAHs (Jacob, 1996) and is over-expressed in response to aromatic

hydrocarbon receptor ligands. Development of a suite of markers for PAH exposure, such as CYP1A, UGT1B (Leaver *et al.*, 2007) and aldehyde dehydrogenase to be used alongside each other could in future be used to provide convincing evidence of exposure to this form of contaminant. On this occasion there is interesting but not conclusive evidence to suggest differential exposure to xenobiotics between estuaries.

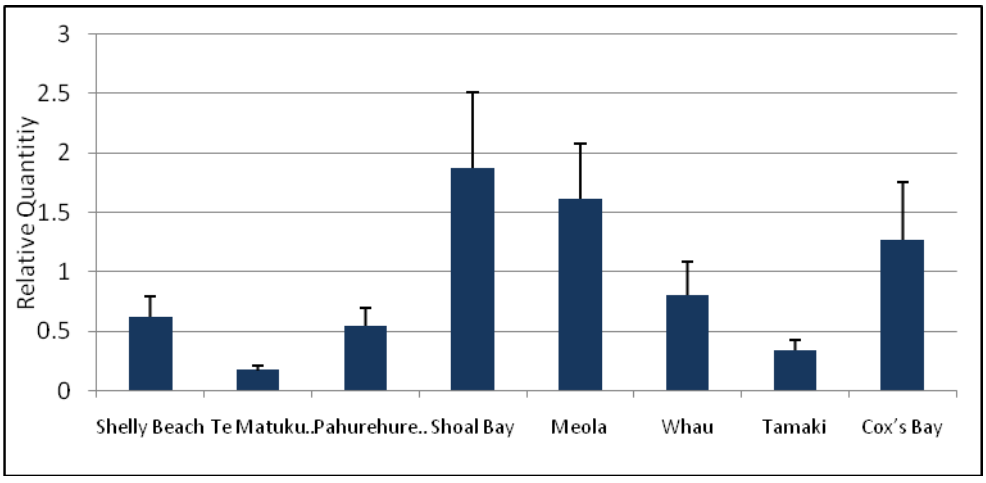
5.4.4 Cu/Zn superoxide dismutase

Cu/Zn superoxide dismutase is a protein involved in the scavenging of superoxides in the cytosol (which should not be confused with a Cu/Zn scavenger) and is commonly found to be over-expressed in European flounder sampled from heavily impacted estuaries such as the River Tyne in North East England (Williams *et al.*, 2003). Here notably lower expression was observed at Te Matuku Bay compared with other sites. Tamaki showed lower levels than Meola but other sites showed little difference. Used as part of a weight of evidence approach this would suggest that Te Matuku Bay is the cleanest site in this study. Used alone, the data is not convincing as increased expression of this gene can be caused by either contaminant- or disease-related pressures.

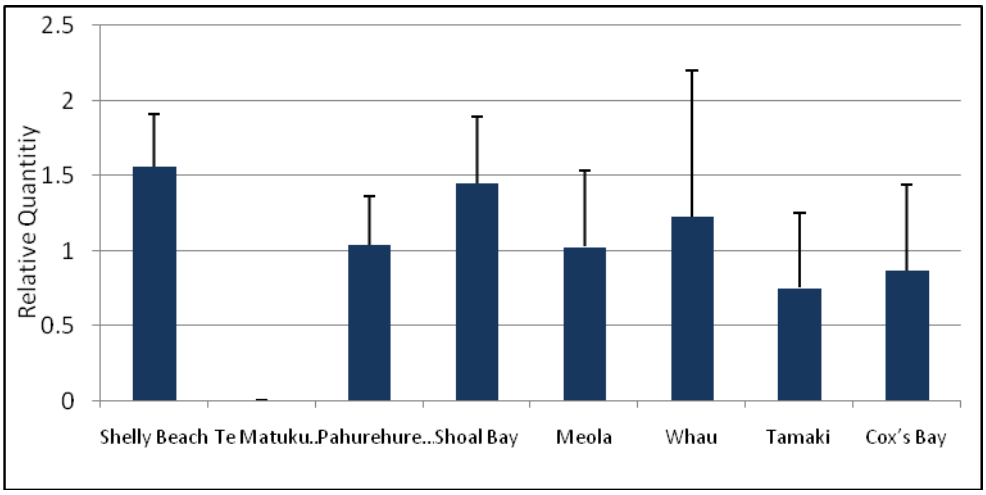
Figure 12

Expression of biomarker genes in yellowbelly flounder relative to a reference sample. Error bars represent standard error. (A) Metallothionein; (B) Glutathione-S-Transferase; (C) Aldehyde dehydrogenase; and (D) Cu/Zn Superoxide dismutase. Sites are arranged in order of zinc concentration from low to high.

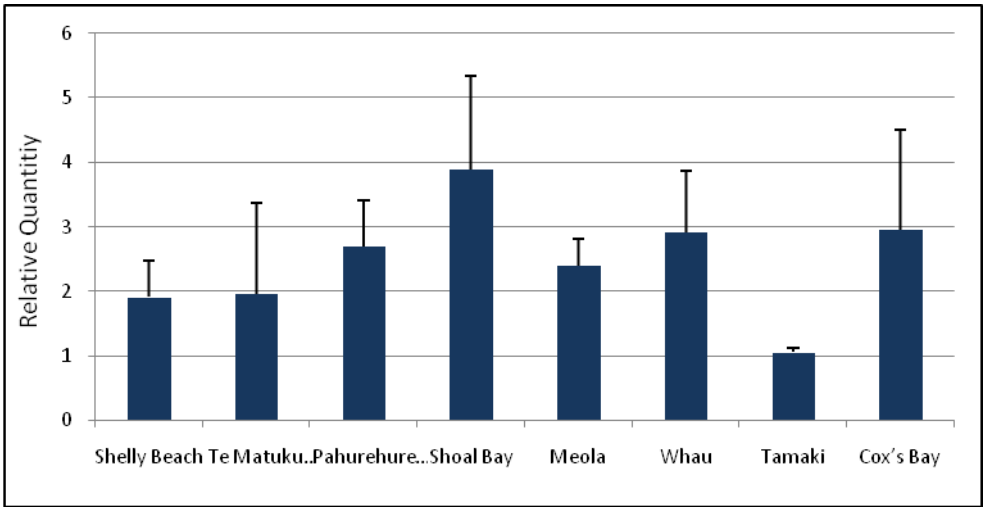
(A) – Metallothionein



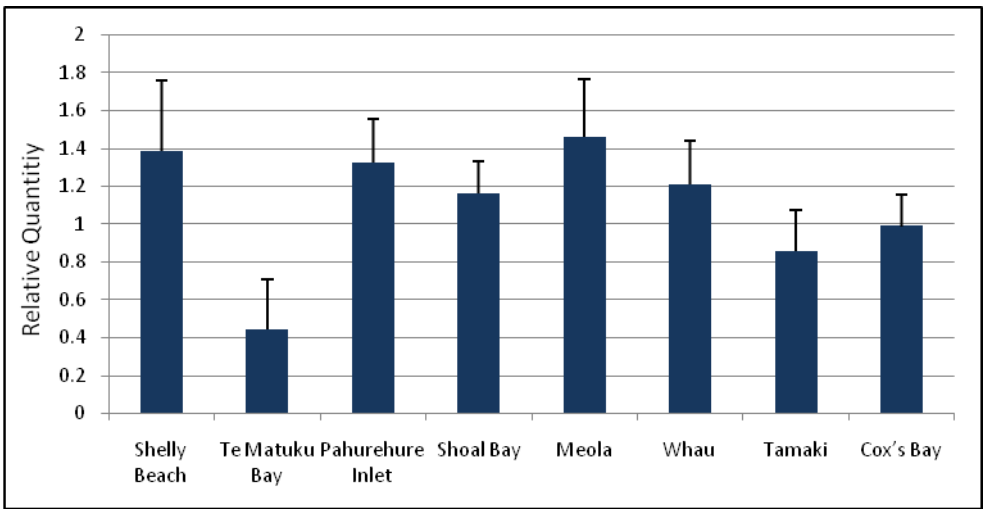
(B) – Glutathione-S-Transferase



(C) – Aldehyde dehydrogenase



(D) – Cu/Zn Superoxide dismutase



6 Conclusion

This study used two biological effects markers (the micronuclei assay and gene expression) to detect early signs of stress at the cellular and biochemical level for a New Zealand native estuarine shellfish (cockle) and fish (yellowbelly flounder) species.

Results of the micronuclei assay showed a potential causal relationship between total copper and zinc in sediments (<63 µm) and micronuclei in gill tissue of flounder caught in the upper Waitemata Harbour. There was no clear relationship along the contaminant gradient of the other estuary sites sampled in this study. There was, however, induction of micronuclei in both cockle and flounder gill tissues at all sites suggesting biochemical and cellular damage is occurring but this was highly variable within and between sites. This suggests that there is sublethal stress occurring but it is unclear if this is occurring to some individuals and not others and it is also unclear what is driving this response in winter. In summer 2008/09, Reed & Lyons (2009) found a linear relationship between micronuclei numbers in cockles and total zinc concentrations in sediments collected in the inner Waitemata Harbour during 2008, but this relationship was not observed in this winter 2010 study.

Results of the gene expression analysis were able to distinguish the cleanest of the site at Te Matuku Bay from the more impacted sites at Cox's Bay, Meola and Shoal Bay. Other sites fall in between and cannot be totally distinguished from each other, however, data suggests that the site at Tamaki is perhaps less impacted by contaminants than other data suggest. Metallothionein induction demonstrated this relationship most clearly but the three other genes used in this study added weight to this evidence.

The genes used in this study were selected to detect the effects of several types of contaminants but it should be noted that with a small amount of further development this list of genes can be increased to include many others such as CYP1A, a direct marker of PAH exposure; UGT1B, a glycosyltransferase involved in elimination of large numbers of xenobiotics in vertebrates (Leaver *et al.*, 2007) and vitellogenin, a marker of endocrine disruption in male fish (Scott and Hylland, 2002). The gene expression data also demonstrated the difficulties associated with cellular tools, in that results can vary depending on the indicator and species used. This highlights that careful interpretation of effects data is required (utilising baseline data of the species being studied) and illustrates the importance of using a weight of evidence approach.

The literature review (Section 3) provided a list of potential biomarkers (or biological effects tools) for assessing the impact of metals in estuarine and coastal environments and identified several tools suitable for use in New Zealand. Before using any of these biomarkers, Van der Oost *et al.* (2003) has recommended that they should, as a minimum:

- Reliably quantify the biological effect (with quality assurance (QA) programmes in place), so that they are relatively cheap and easy to perform.
- The biological effect response should be sensitive to pollutant exposure and/or effects in order to serve as an early warning parameter.
- Baseline data of the biological effect should be well defined in order to distinguish between natural variability (noise) and contaminant-induced stress (signal).
- The impacts of confounding factors to the biological effect response should be well established.
- The underlying mechanism of the relationships between biological effect response and pollutant exposure (dosage and time) should be established.

- The toxicological significance of the biological effect, e.g. the relationships between its response and the (long-term) impact to the organism, should be established and, where possible, assessment criteria developed.

This is a good approach to developing a suite of biomarkers to assess the concentrations, trends and effects of specific contaminants in the estuarine, coastal and marine environments. One advantage of this approach over other approaches is that contaminant-related biological effects tools link chemical exposure and ecological endpoints and can detect the impact of chemicals (or group of chemicals) that may not be analysed as part of routine monitoring programmes.

7 Recommendations

Early warning signs of adverse biological effects from contaminant exposure can assist resource managers by identifying chemicals or groups of chemicals causing increasing harm to biological systems before whole ecosystems are affected. Biological tools that may be useful to resource managers in the Auckland region include:

1. Measuring gene expressions in fish to identify effects of metals, PAH contamination, oestrogenic compounds or substances that mimic the action of female hormones and others suitable marker genes (George *et al.*, 2004; Williams *et al.*, 2008; Oliveira *et al.*, 2010).
2. Measure the induction of micronuclei as a "general" early warning technique for a wide range of metal-mediated biological effects (Chaudhary *et al.*, 2006; Udroiu, 2006).
3. Measure lysosomal stability in fish and mussels to assess cellular damage from pollutants (including metal and organic chemicals) and/or physical stress (ICES, 2009).
4. Measure ALA-D induction in fish as a specific biomarker for lead contamination (Holth, 2004).

The biological tools above require further evaluation once the aims of the future monitoring programme in the Auckland region are established. Table 2 in Section 3.1 recommends establishing the requirement and purpose of the monitoring before selecting one or more biological tools to meet that purpose.

In light of the results of this study, several recommendations are suggested:

1. Undertake additional study of the biological indicators to assess initial findings in this study.
2. Characterise the use of cockles and yellowbelly flounder as species to use in biological effects monitoring (and assess ambient biomarker levels and the relationship between biomarkers and chemicals in the environment).
3. Confirm observed effects in yellowbelly flounder (e.g. by assessing variability between individuals at sites in the upper Waitemata Harbour and obtaining more data from highly contaminated sites) with bioavailable metals and PAHs in sediments.
4. Investigate why results from this study and those from the benthic health model show different contaminants (e.g. PAHs at some sites) are influencing the biological responses observed in the two studies.
5. Assess the use of oysters and or mussels as sentinel species (or caged *in situ* assessments of hotspots) for monitoring the effects of chemicals in muddy inner estuaries as opposed to cockles in the more sandy outer estuaries. Flounder samples could be successfully used in these environments. In addition, other estuarine fish species could be considered as a sentinel species for estuarine monitoring and other examples exist including the use of species of mullet (Boglione *et al.,* 2006; Oliveira *et al.*, 2010).
6. Explore the design of a monitoring programme that integrates chemical and biological tools for the Auckland region. This could include the assessment of a suitable species, selection of the most appropriate biological tool and the development of criteria to assess results of monitoring over large geographical areas and different timescales.

8 Acknowledgements

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10 Appendix 1

Tables of recommended and promising biological effects tools as proposed by the ICES Working Group for the Biological Effects of Contaminants (ICES, 2007).

10.1 Table A

Recommended techniques for biological monitoring programmes at the national or international level – methods for fish (Adapted from ICES WGBEC 2007) B: BEQUALM; Q: QUASIMEME; O: Other (EU projects BEEP, UNEP MEDPOL).

Method	Organism	Inter-calibration	Issues addressed	Biological significance
Bulky DNA Adduct formation	Fish	B	PAHs; other synthetic organics, e.g., nitro-organics, amino triazine pesticides (triazines).	Measures genotoxic effects. Possible predictor of pathology through mechanistic links. Sensitive indicator of past and present exposure.
AChE inhibition	Fish	O	Organophosphates and carbamates or similar molecules.	Measures exposure.
Metallothionein induction	Fish	O	Measures induction of metallothionein protein by certain metals (e.g., zinc, copper and cadmium).	Measures exposure and disturbance of copper and zinc metabolism.
EROD or P4501A induction	Fish	B	Measures induction of enzymes which metabolize planar organic contaminants (e.g., PAHs, planar PCBs, dioxins).	Possible predictor of pathology through mechanistic links. Sensitive indicator of past and present exposure.
ALA-D inhibition	Fish	B	Lead.	Index of exposure.
PAH bile metabolites	Fish	Q	PAHs.	Measures exposure to and metabolism of PAHs.
Lysosomal stability	Fish	B	Not contaminant specific but responds to a wide variety of xenobiotic contaminants and metals.	Measures cellular damage and is a good predictor of pathology. Provides a link between exposure and pathological endpoints. Possibly, a tool for immune-suppression studies in white blood cells.
Externally visible diseases	*Limanda limanda, Platichthys flesus,Gadus morhua*	B	Responds to a wide variety of environmental contaminants and non-specific stressors.	Integrative response; measures general fish health; elevated prevalence may indicate exposure to contaminants.
Macroscopic liver neoplasms	*Limanda limanda, Platichthys flesus*	B	Effects of carcinogenic substances.	Indicative of contaminant associated liver carcinogenesis.

Method	Organism	Inter-calibration	Issues addressed	Biological significance
Liver histopathology	*Limanda limanda, Platichthys flesus*	B	Effects of carcinogenic and non-carcinogenic contaminants.	Indicative of nonspecific and specific contaminant effects at cellular or tissue level.
Vitellogenin induction	Male and juvenile fish	B	Oestrogenic substances.	Measures feminization of male fish and reproductive impairment.
Intersex	Male flounder		Oestrogenic substances.	Measures feminization of male fish and reproductive impairment.
Reproductive success in *Zoarces viviparus*	*Zoarces viviparous*			Measures reproductive output and survival of eggs and fry in relation to contaminants. Restricted to period when young are carried by female viviparous fish.

10.2 Table B

Recommended techniques for biological monitoring programmes at the national or international level - methods for invertebrates (Adapted from ICES WGBEC 2007) B: BEQUALM; Q: QUASIMEME; O: Other (EU projects BEEP, UNEP MEDPOL).

Method	Organism	Inter-calibration	Issues addressed	Biological significance
AChE inhibition	Molluscs and crustaceans	O	Organophosphates and carbamates or similar molecules Possibly algal toxins.	Measures exposure to a wide range of compounds and a marker of stress.
Metallothionein induction	*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.
Lysosomal stability (including NRR)	*Mytilus spp.* Oyster	O/B	Not contaminant specific, But responds to a wide variety of xenobiotic contaminants and metals.	Measures 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.
Scope for growth	Bivalve molluscs, e.g., *Mytilus spp.* and oysters	O	Responds to a wide variety of contaminants	Integrative response, a sensitive sub-lethal measure of energy available for growth.
Imposex	Neogastropod	Q	Specific to	Reproductive interference.

Method	Organism	Inter-calibration	Issues addressed	Biological significance
	Mollusks (*Nucella lapillus,Buccinum undatum, Hinia reticulata, Neptunea antiqua*)		organotins.	Estuarine and coastal littoral waters (*Nucella*) and offshore waters (*Buccinum*).
Intersex	*Littorina littorea*	B	Specific to reproductive effects of organotins.	Reproductive interference in coastal (littoral) waters.
Induction/inhibition of Multidrug/multi xenobiotic resistance (MDR/MXR).	*Mytilus edulis*		Multiple contaminants (organics and metals).	Adaptation/inhibition in response to xenobiotic stress.
Histopathology	Blue mussels		Not contaminant specific.	General responses.
Embryo aberrations in field-collected amphipod crustaceans	Amphipods		Contaminant specific.	Measures frequency of different types of lethal embryo aberrations; allows for separating effects of contaminants and environmental climate variables.

10.3 Table C

Recommended techniques for biological monitoring programmes at the national- or international-level – bioassays and methods for specific matrices.

Method	Organism	Inter-calibration	Issues addressed	Biological significance
Benthic community analysis	Macro-, meio-, and epibenthos	B	Responds to a wide variety of contaminants, particularly those resulting in organic enrichment.	Ecosystem level (retrospective). Particularly useful for point sources. Most appropriate for deployment when other monitoring methods indicate that a problem may exist.
Whole sediment bioassays	*Corophium Arenicola, Ampelisca brevicornis*	B	Not contaminant specific, will respond to a wide range of environmental contaminants in sediments.	Acute/lethal and acute/sub-lethal toxicity only at present. May enable retrospective interpretation of community changes.
Bioassays of sediment pore	Bivalve embryo *Acartia*		Will respond to a wide range of	Acute and sublethal toxicity, Including genotoxicity, etc.

Method	Organism	Inter-calibration	Issues addressed	Biological significance
waters, sea water elutriates, sea water samples			environmental contaminants, Useful for dredge spoils, sediments liable to re suspension.	Toxicity of hydrophobic contaminants might be underestimated in pore water assays.
CALUX	Reporter gene assay		Ah receptor active compounds.	Predictor of dioxin like toxicity.
YES	Reporter gene assay (yeast)		Oestrogen receptor-active compounds.	Potential endocrine disruption.
YAS	Reporter gene assay (yeast)		Androgen receptor-active compounds.	Potential endocrine disruption.

10.4 Table D

Promising biological effects monitoring methods that require further research before they can be recommended for monitoring (both fish, and invertebrates).

Method	Organism	Issues addressed	Biological significance
Pre-neoplastic and neoplastic liver lesions by NADPH-producing enzymes	Fish	PAHs, other synthetic organics, e.g., nitro-organics, amino triazine pesticides (triazines).	Diagnosis of pathological changes and enzymatic markers of carcinogenesis associated with exposure to genotoxic and nongenotoxic carcinogens.
DNA strand breaks including Comet assay	Fish and mussel cells	Not contaminant specific, will respond to a wide range of environmental contaminants.	Measures genotoxic effects, but is also extremely sensitive to other environmental parameters.
BaP Hydroxylase –like enzymes	Invertebrates	Induced enzyme response to PAHs, planar PCBs, dioxins and/or furans.	Measures exposure to organic contaminants.
Induction/inhibition of Multidrug/multixenobiotic resistance (MDR/MXR)	Fish and invertebrates other than *Mytilus spp*	Multiple contaminants (organics and metals).	Adaptation/inhibition in response to xenobiotic stress.
Glutathion-Stransferase (GST)	Fish and molluscs	Predominantly organic xenobiotics.	Measures exposure and the capacity of the major group of

Method	Organism	Issues addressed	Biological significance
			phase II enzymes. Considered most promising for isoenzyme-specific measurements.
Oxidative stress	Fish, invertebrates	Not contaminant specific, will respond to a wide range of environmental contaminants.	Measures the presence of free radicals.
Immunocompetence	Fish, invertebrates	Not contaminant specific, will respond to a wide range of environmental contaminants.	Measures factors that influence susceptibility to disease.
On-line monitoring	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.
Abnormalities in wild fish embryos and larvae	Fish, including demersal and pelagic species	Not linked unequivocally to contaminants.	Measures frequency of probably lethal abnormalities in fish larvae. Mutagenic, teratogenic.
Bulky DNA adduct formation	Invertebrates	PAHs, other synthetic organics.	Measures genotoxic effects.
Gene arrays	Fish, mussels	Various (stress and contaminant specific).	Combined responses from various biomarkers (potentially whole genome).
Histopathology	Invertebrates (other than mussels)	Not contaminant specific.	General response.
Spiggin	3-spined stickleback	Androgens.	Measures environmental androgens.
Micronuclei	Fish and bivalve molluscs	Not contaminant specific.	Exposure to aneugenic and clastogenic contaminants.
Peroxisomal proliferation (enzyme assays)	Fish and invertebrates	Contaminant specific.	Potential alterations in lipid metabolism, non-genotoxic carcinogenesis.
Alkylphenol- bile metabolites	Fish (cod)	Alkyl phenols.	Measures exposure to and metabolism of alkylated phenols.
Cellular Energy Allocation	Invertebrates and small fish	Wide range of stressors.	Changes in metabolic turnover and specific allocations will be linked to effects at higher levels of ecological organization.

10.5 Table E

Measurements of cockles sampled in Auckland estuaries in 2010.

Site	Sample ID	Length (cm)	Weigh (g)	Micronuclei (MN) (*n*)	Average MN	Std dev
Waitemata Harbour						
Te Matuku Waiheke Island	H1C	2.57	6.66	5		
	H2C	2.53	5.97	10		
	H3C	2.57	6.17	5	6	2
	H4C	2.47	5.88	5		
	H5C	2.40	5.10	5		
Meola	M1C	2.16	4.38	2		
	M2C	1.95	2.93	6		
	M3C	1.95	3.50	1	4	3
	M4C	1.87	2.68	3		
	M5C	1.87	3.37	9		
Cox's Bay	C1C	2.21	4.35	4		
	C2C	2.14	3.39	3		
	C3C	2.06	3.52	7	5	2
	C4C	2.01	3.41	5		
	C5C	2.04	3.90	6		
Shoal Bay	S1C	1.89	2.82	12		
	S2C	1.73	2.09	5		
	S3C	1.70	1.88	10	9	3
	S4C	1.78	2.08	11		
	S5C	1.72	2.02	9		
Pollen Island, outer Whau estuary	P1C	2.67	7.87	2		
	P2C	2.65	7.39	5		
	P3C	2.51	6.26	8	4	3
	P4C	2.57	6.41	2		
	P5C	2.53	6.75	3		
Tamaki	T1C	3.02	10.71	7		
	T2C	3.04	10.31	7		
	T3C	2.73	7.22	12	9	2
	T4C	2.95	11.24	10		
	T5C	3.00	8.26	10		
Manukau Harbour						
Pahurehure Inlet	W1C	2.29	4.16	11		
	W2C	2.12	4.92	11		
	W3C	2.25	4.09	9	12	4
	W4C	2.31	4.18	11		
	W5C	2.27	3.70	19		
Kaipara Harbour						
Shelly Beach	K1C	2.34	4.80	2		
	K2C	2.34	4.36	3		
	K3C	2.32	4.29	4	4	4
	K4C	2.18	3.09	1		
	K5C	2.27	3.88	10		

10.6 Table F

Measurements of flounder sampled in Auckland estuaries in 2010.

Site	Sample ID	Sex	Length (mm)	Weight (g)	Micronuclei (MN) (*n*)	Average MN	Std dev
Waitemata Harbour							
Te Matuku	H1F	F	345	486	4	4	1
Waiheke Island	H2F	F	365	514	3		
Meola	M1F	F	335	465	2		
	M2F	F	346	485	2		
	M3F	F	343	505	3	3	2
	M4F	F	285	305	5		
	M5F	F	395	760	1		
Cox's Bay	C1F	F	349	610	20		
	C2F	F	342	485	11		
	C3F	F	336	480	14	11	6
	C4F	F	335	480	8		
	C5F	F	343	465	3		
Shoal Bay	S1F	F	350	535	5		
	S2F	F	300	315	6		
	S3F	F	311	370	8	6	1
	S4F	M	264	250	5		
	S5F	F	276	305	5		
Whau	W1F	F	356	531	28		
	W2F	F	360	518	39		
	W3F	F	345	487	16	22	11
	W4F	F	349	463	16		
	W5F	F	324	439	11		
Tamaki	T1F	F	340	419	13		
	T2F	F	395	739	7		
	T3F	F	334	467	5	8	3
	T4F	F	300	306	6		
	T5F	F	345	462	8		
	T6F	F	377	493	6		
Manukau Harbour							
Pahurehure Inlet	P1F	F	356	521	8		
	P2F	F	340	574	7		
	P3F	F	393	660	13	9	3
	P4F	F	340	427	10		
	P5F	F	324	333	5		
Kaipara Harbour							
Shelly Beach	K1F	F	377	576	9		
	K2F	F	288	309	12		
	K3F	F	312	367	10	9	3
	K4F	F	350	472	11		
	K5F	F	328	382	5		
	K6F	M	285	259	4		