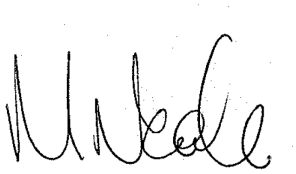
Auckland
Regional Council
TE RAUHĪTANGA TAIAO

Intergenerational Responses of Aquatic Biota to Low Level Contamination in Urban Streams

December 2009 TR2009/085

Auckland Regional Council
Technical Report No.085 December 2009
ISSN 1179-0504 (Print)
ISSN 1179-0512 (Online)
ISBN 978-1-877528-97-2

Reviewed by:



Name: Dr Martin Neale

Position: Project Leader - Freshwater

Organisation: Auckland Regional Council

Date: 25 June 2009

Approved for ARC Publication by:

Name: Matthew Davis

Position: Group Manager, Partnerships and Community Programmes

Organisation: Auckland Regional Council

Date: 15 December 2009

Recommended Citation:

Phillips, N., Croker, G., Albert, A. and Rijkenberg, M. (2009). Intergenerational responses of aquatic biota to low level contamination in urban streams. Prepared by NIWA for Auckland Regional Council. Auckland Regional Council Technical Publication 2009/085.

Intergenerational Responses of Aquatic Biota to Low Level Contamination in Urban Streams

Ngaire Phillips
Glenys Croker
Anathea Albert
Micha Rijkenberg

Prepared for
Auckland Regional Council

NIWA Client Report: HAM2009-048
May 2009

NIWA Project: ARC08222

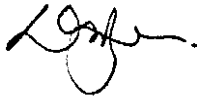
National Institute of Water and Atmospheric Research Ltd
Gate 10, Silverdale Road, Hamilton
PO Box 11115, Hamilton, New Zealand
Phone +64-7-856 7026, Fax +64-7-856 0151
www.niwa.co.nz

Contents

Reviewed by:

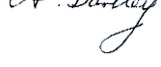
Dr Chris Hickey

Approved for release by:



Dr David Roper

Formatting checked



1 Executive Summary

There are many examples of rapid genetic changes or small-scale evolutionary processes (micro-evolution) resulting from exposure to contaminants. These changes result in an increase in contaminant-tolerant genetic variants, with loss of more sensitive forms. Given the importance of genetic diversity as one of the components of biodiversity, a method suitable for detecting such effects of contamination within the confines of a monitoring programme is required.

This project aimed to provide a tool for detecting the effects of chronic, low-level contamination in streams on multiple generations of aquatic biota, using changes in genetic structure as a marker. We have combined field and laboratory experiments ranging from acute (4 days) to chronic (38 days) exposure periods. We have examined effects on both adult and juvenile populations produced during the experiments and have assessed both physiological and reproductive measures of fitness. Using the technique of allozyme electrophoresis, we have also examined changes in genetic structure between adults used and juveniles produced during the experiments. This has allowed us to determine whether or not stormwater contamination can induce selection of specific genetic variants over one generation. We have chosen as our "model species" the native freshwater bivalve *Sphaerium novaezelandiae*. It is an ideal "model" for developing this tool, as its filtering activities mean it is exposed to both sediment and water column contamination. It also has a relatively rapid life-cycle and produces live young, starting at around 3 months of age.

We have found that pre-exposure to natural streamwaters contaminated with stormwater does not affect adult mortality but may have some effect on fecundity, at least for those animals exposed to Oakley Creek stream water. Importantly, we found a significant reduction (14.6%) in the frequency of individuals with one genetic variant (PGM 44) when we compared the genetic composition of adult and juvenile populations used for and produced during the pre-exposure experiment.

When the test organisms were subsequently exposed to low levels of zinc in solution for 38 days, we observed significant differences in both adult mortality and fecundity. There was also some suggestion of an influence of pre-exposure to contamination on the susceptibility of organisms to further contamination, with pre-exposure to Oakley Creek stream water reducing both overall survival and rate of mortality. We observed differences in the genetic composition of juvenile populations produced during the chronic exposure, with significantly lower (22.4%) frequencies of individuals with PGM 44 in the contaminated population. In addition, genetic variants PGM 22 and PGM 33 were increased in frequency in contaminated populations (8.8% and 11.5% respectively), although these results were variable and therefore not statistically significant. These results are also supported by the finding of a significant reduction in fecundity when considering the overall exposure regime (pre-exposure plus chronic exposure). This supports our original hypothesis that increased susceptibility to additional stressors is likely with organisms pre-exposed to stormwater contamination.

The implications of these results include changes in genetic diversity and associated species and functional diversity, as well as subsequent alterations to ecosystem

functioning. While we have used a "model" aquatic organism, we would expect that other species within stream communities would display a similar response, although the response times will vary depending on the life cycle of each species.

Collectively these results provide strong evidence for selection effects of stormwater resulting in heritable shifts in genetic composition. The characterisation of the extent of genetic variability associated with contaminant exposure provides the first step towards incorporating micro-evolutionary changes into an ecological risk assessment framework. This study has demonstrated a sensitive and relatively simple method for assessing the chronic effects and genetic responses to stormwater contamination on aquatic communities. We have identified significant chronic effects of stormwater contamination on aquatic organisms using an experimental design which could be applied over only a few months. Development of a monitoring programme based around this method would be a desirable next step and would complement existing ecologically-based stormwater monitoring programmes.

2 Introduction

2.1 Background to research

In urban streams, aquatic organisms are exposed to a complex array of factors which lead to degradation of stream structure, function and ecological values (Walsh, 2000). In addition to diffuse and point sources of contaminants, habitat alterations, including changes in stream bed characteristics, hydrological regime, and loss of riparian vegetation can all alter biotic community structure and function (Walsh *et al.*, 2005). In extreme situations this can result in changes to fundamental life-support functions (e.g., availability of dissolved oxygen). In such cases, contamination effects, such as those that may be associated with stormwater, are likely to be secondary. However, the more common situation in urban streams is where stream function is only partially impacted and biotic communities retain some measure of intactness. Under such conditions, contaminants may represent a significant stressor to aquatic organisms. Such effects will be seen as changes in community structure and ecosystem functioning (Parker *et al.*, 1999). Depending on the level and duration of contamination events, such changes may result from: (i) loss of sensitive species due to direct toxic effects, (ii) replacement of these species by less sensitive ones as a consequence of reduced competition, (iii) modifications to food-web structures, as a consequence of decreased predation and/or grazing of susceptible species (iv) acclimation (physiological adaptation), and (v) selection of genetically inherited tolerance (genetic adaptation). In spite of the increased awareness and number of studies related to the effects that contamination exerts on different types of ecosystems, biomonitoring has largely focused on the first three types of response (e.g., Maxted *et al.*, 2003; Maxted 2005; Inglis *et al.*, 2008).

There are many examples of rapid genetic changes or small-scale evolutionary processes (micro-evolution) resulting from exposure to contaminants (see Belfiore and Anderson, 2001 for a review; Martins *et al.*, 2007). These changes result in an increase in contaminant-tolerant genetic variants, with loss of more sensitive forms. As these changes are genetically inherited, they form the basis for evolutionary changes which occur within years or after a few generations, rather than over of centuries or millenia (Klerks and Levinton, 1989). While the selection of tolerant genotypes may appear beneficial at first, it may result in increased susceptibility to other selection pressures due to reduced genetic variability (Gillespie and Guttman, 1989, 1993), extinction of species or decline in overall diversity and associated ecosystem functioning. Selection may be measured as a reduction in fitness of individuals (for example survival, reproduction, mobility) within a population as well as extinction of less-tolerant genetic variants. Contamination may also alter the frequency of genetic variants over time, a response which can be used as an early warning of longer-term evolutionary-scale events. The strong selection pressure caused by metal exposure, its persistence and the fact that it is relatively easy to measure has resulted in metal pollution being one of the best demonstrated classic examples of natural selection in action.

In a recent review, Medina *et al.*, (2007) highlighted the importance of integrating evolutionary considerations into the ecological risk assessment process (ERA), given the fact that "genetic variation is one of the pillars of biodiversity and evolution". The goal of ERA is to characterise and estimate the likelihood of adverse ecological effects resulting from past, present or future exposure to toxic substances released into the environment (Suter, 1993). Forbes (1998) and Barata *et al.*, (2002) demonstrated how certain aspects of micro-evolutionary genetic effects resulting from pollution could be incorporated into the ERA process by quantifying the genetic variability of tolerant traits and incorporating those changes into existing ERA safety margins. This project aimed to develop a method suitable for detecting micro-evolutionary-scale effects of contamination on aquatic biota, which could be incorporated into a monitoring framework. Such a method could then be used to derive data suitable for inclusion in an ERA framework.

2.2 Project aims

This project aims to provide a tool for detecting the effects of chronic, low-level contamination in streams on aquatic biota, using changes in genetic structure as a marker. In order to develop such a tool it is necessary to answer two key questions, namely:

1. Does stormwater contamination contribute to reduced fitness in populations of aquatic organisms?
2. Does exposure to stormwater result in selection for tolerant genetic variants?

In this report we present the results of a number of discrete experiments which collectively address these key questions. We have combined field and laboratory experiments ranging over acute (4 days) to chronic (38 days) exposure periods. We have examined effects on both adult and juvenile populations produced during the experiments and have assessed both physiological and reproductive measures of fitness. We have also examined changes in genetic structure between adults used and juveniles produced during the experiments to determine whether or not stormwater contamination can induce selection of specific genetic variants over one generation.

The approach we have taken is to use a "model species", one which provides us with the opportunity to examine long-term questions relating to inter-generational effects, within a realistic timeframe and budget. Such an approach is common in ecological and ecotoxicological studies and provides the basis for extrapolation of results to, for example, functionally similar species (e.g., the zebrafish *Danio rerio* is a prominent model vertebrate in studies across a wide range of disciplines, Hill *et al.*, 2005) .

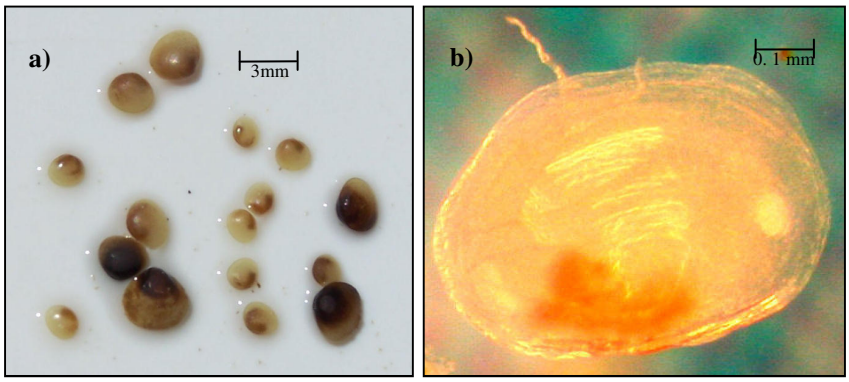
3 Methods

3.1 Study animal

We have chosen as our "test species" the native freshwater bivalve *Sphaerium novaezelandiae*. It is an ideal "model" species for developing this tool, as its filtering activities mean it is exposed to both sediment and water column contamination. It also has a relatively rapid life-cycle and produces live young, starting at around 3 months of age. It is therefore amendable to investigation of inter-generational effects within a two year period of research. In addition, it has been the subject of a number of studies investigating the toxicity of metals and other contaminants (Hickey and Martin, 1999). The freshwater clam *S.novaezelandiae* Deshayes 1853 (Figure 1a) is one of three representatives of the family Pisidiidae found in New Zealand freshwaters (Kuiper 1963). It is endemic to New Zealand and is common and widespread, being found in most freshwater habitats (Kuiper, 1963). Freshwater clams are known to play a key role in energy and nutrient cycling within aquatic environments (Hornbach and Wissing, 1984; Lopez and Holopainen, 1987). They can be numerically dominant in streams and ponds (e.g., Eckblad *et al.*, 1977). The Pisidiidae have a highly specialised reproductive system and functional or simultaneous hermaphrodism is the norm (Heard, 1977), with reproduction occurring as long as feeding and growing occurs (Mackie 1979). Juvenile clams (fully formed miniature adults, Figure 1b) are released into the environment following larval development within specialised brood pouches, which are formed within the inner demibranch of the ctenidia (gills) (Heard 1965). Adult *Sphaerium* rarely exceed 6mm in shell length and produce juveniles of an average of 2mm in shell length (Roa, 1997). They start reproducing at shell length of greater than 3mm (Roa, 1997). Pisidiids have the smallest adult sizes of freshwater bivalves but they release by far the largest young (Mackie, 1979).

Members of the Pisidae are seldom collected using standard stream sampling protocols employed in stream assessments undertaken throughout New Zealand and Auckland is no exception (M. Neale, pers. comm.). This is because the methods generally sample habitats where Pisidae are less likely to be found or using methods which do not specifically target sub-surface dwelling organisms (Maxted *et al.*, 2003).

Figure 1:
a) Adults individuals of the freshwater clam *Sphaerium novaezelandiae* and b) an *S. novaezelandiae* juvenile (Photos: N Phillips).



For this project, all clams were sourced from Hamurana, Lake Rotorua, New Zealand (188437E, 5785145N, NZTM 2000). We chose a single source site as localised conditions can influence the genetic composition of populations. In this way we standardised our experimental population. Animals were collected by scooping approximately the top 2cm of sediment from the lake bed (lake depth 0.5-1.0m) with a plastic scoop. Samples were returned to shore and subsequently passed through a 1mm sieve. The remaining sediment was then visually searched for *Sphaerium*, with individuals (approximately 2mm shell length) collected into a small (2L) bucket filled with lake water. Individuals were transported in aerated buckets to the laboratory. All animals were acclimatised to the experimental set-up for up to 1 week prior to running the experiments. Acclimatisation and experimentation were all undertaken in a constant temperature room (20±1.5 °C) with a light:dark cycle of 16:8 hours.

3.2 Experimental design

In order to examine our two key questions we devised a set of experiments involving both field and laboratory exposure to "natural" stormwaters and zinc-contaminated test solutions. These experiments provided us with the opportunity to examine existing stormwater contamination exposure scenarios, as well as controlled exposures to known concentrations of a key stormwater contaminant, namely zinc. The original design (Phillips and Croker, 2008) involved:

Phase 1: exposing animals to stream stormwater by placing them in cages in 10 field sites for a period of 3–4 weeks. Sites were selected in conjunction with the Auckland Regional Council (ARC) and reflect a range of stormwater contaminated sites, as well as low impact (reference) sites. All sites are currently part of ARC's water quality and/or macroinvertebrate sampling programmes (M. Neale pers. comm.). Sites were designated as either "contaminated" or "uncontaminated" using water quality data provided by the ARC, along with discussions with a council scientist. This phase represented pre-exposure of animals to natural

stormwater conditions, with our hypothesis being that animals pre-exposed to stormwater contamination would be more susceptible to additional stressors.

Phase 2: retrieving the exposed cages, recording mortalities and number of juveniles produced in the field and further exposing surviving a selection of adults and juveniles to low level zinc contamination in a controlled laboratory environment for up to 6 months to enable production of at least one generation. We used a crossed exposure design for this phase of the experiment, with some animals exposed to reference field conditions being exposed in the laboratory to clean water, while others were exposed to zinc contamination. A similar design was used for animals exposed to stormwater contamination in the field.

Results of the field experiment and initial laboratory exposure indicated poor survival and reproductive capacity, compromising interpretation of any genetic analyses due to potentially very low numbers of animals that would be produced by continuing the original design. There was high rainfall immediately in the Auckland region immediately following deployment of our experimental cages and is possible that elevated inorganic suspended solids and chemical contaminants resulting from this event may have impacted on the fitness of the deployed organisms to such an extent that survival was compromised. Changes to the original experimental design were adopted, following discussion with ARC staff (M. Neale, pers. comm.).

The design change was to re-start the experiment using freshly collected animals and undertake all original components within the laboratory. The revised experimental design reproduced the orginal field-based programme within a controlled laboratory environment. The specific components of the revised design involved:

a) pre-exposure to field-collected stream water (three stormwater contaminated sites and two reference sites) – this replaces the field-exposure component of the original design;

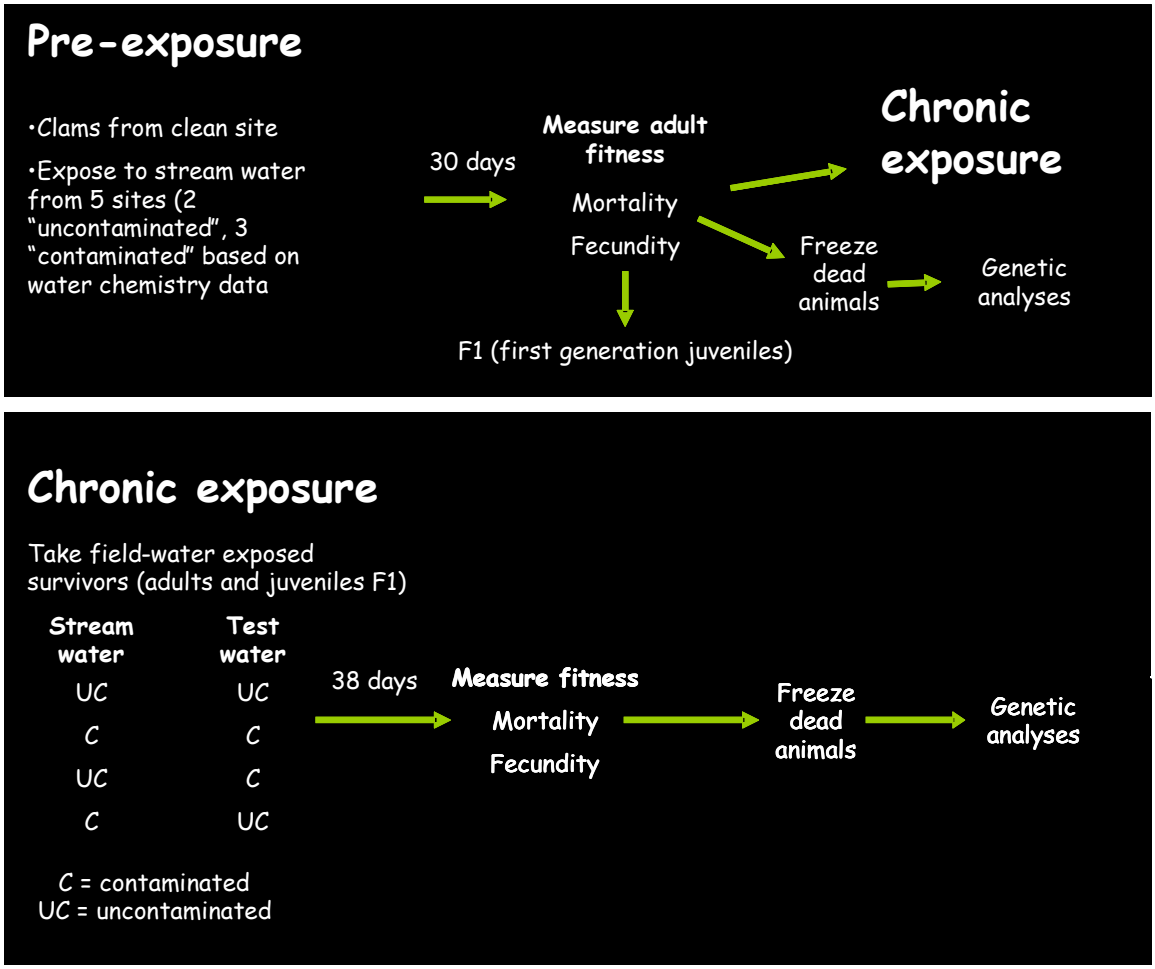
b) a chronic toxicity test with zinc-contaminated water – this is unchanged from the original design.

Figure 2 summarises the key components of the overall experimental design.

An additional acute (4 day) experiment was also undertaken to investigate the genetic basis to tolerance associated with high levels of zinc (120mg/L zinc). This experiment was designed to test the hypothesis that exposure to very high levels of zinc would result in not all individuals dying and that the genetic variants that survived would be most tolerant, with this tolerance being reflected in physiological tolerance (activity levels), as well as fecundity.

The modified experimental design allowed us to address all aspects of our original project aim i.e to assess whether or not exposure to stormwater contamination results in chronic, inter-generational effects on populations of exposed organisms. The following section details each of the experimental phases separately.

Figure 2:
Experimental design for investigation of inter-generational responses to stormwater contaminants.



3.2.1 Site selection

Survey sites were chosen based on a reconnaissance survey of 11 sites (29 February and 3 March 2008) and discussion with ARC staff (K Park, pers. comm.), 5 sites were selected for use in the study (Table 1). Sites included a range of stormwater contaminated sites, as well as "low impact" (reference) sites. All sites are currently part of ARC's water quality and/or macroinvertebrate sampling programmes (M. Neale pers. comm.). Sites were designated as either contaminated or uncontaminated using water quality data provided by the ARC, along with discussions with council scientists. Analysis of sediment and water samples from each site in April and May 2008 (Phillips and Croker, 2008) provides support for the designation of sites as contaminated or uncontaminated, although the differences are more evident from the sediment quality than the water quality data (Tables 2 and 3). Zinc levels in solution in particular are

comparable across all sites on one sampling occasions. It should be noted, however, that the levels recorded for the contaminated sites, while exceeding ANZECC guidelines, were well below the annual mean values recorded by the ARC (Table 1). Water samples were returned to the laboratory on the day of collection and the experiments were initiated within 2 days of collection.

Table 1:

Key characteristics of stormwater collection sites.

Stream name[1]	Site Code	Disturbance[1]	Category[1]	Treatment category[3]	NZTM X	NZTM Y	Location	Zn (mg/L) (2006 annual mean)[4]	Zn (mg/L) (Phillips and Croker 2007)
Mahurangi Reference @ Trappitt	MH	NA	reference	UC	1748968	5965367	Warkworth Reserve	n/a	0.008
Tawharanui	TW	NA	reference	UC	1765924	5972931	Tawharanui Regional Park	n/a	0.018
Otara LTB	OT	high	urban	C	1768326	5908371	Otara Creek Reserve	0.047	0.021
Oakley LTB	OK	high	urban	C	1751914	5917503	Oakley Creek Esplanade	0.033	0.012
Oteha LTB	OE	low	urban	C	1754873	5925353	Clemmows Orchard	0.042	0.010

[1] ARC (2008) Sites represent up to 4 major land use classes (bush, forestry, rural, urban) and two disturbance gradients (high and low). Reference sites with native forest catchments were selected to represent pre-human occupation.

[2] n/a – data not available.

[3] C = contaminated, UC = uncontaminated.

[4] unpublished data, M. Neale, ARC, 2007.

Table 2:

Sediment quality (total recoverable as mg/kg dry weight) at field sites on two sampling occasions. The red cells indicate exceedances of the Environment Canada (EC) TEL (Threshold effects level). Red cells with borders exceed both EC TEL and Auckland background levels. No ISQG-low guidelines were exceeded. See Table 1 for site codes.

Site Code	Date collected	Iron (Fe)	Total Organic Carbon (TOC)	Arsenic (As)	Cadmium (Cd)	Chromium (Cr)	Copper (Cu)	Lead (Pb)	Mercury (Hg)	Nickel (Ni)	Zinc (Zn)
Contaminated sites											
OE	04/04/08	18000	1.4	2.8	0.05	13	7.2	8.3	0.063	9	50
OE	23/04/08	41000	0.43	6.8	0.096	35	21	18	0.069	34	120
OK	04/04/08	35000	2.1	18	0.47	41	54	150	0.18	76	400
OK	23/04/08	33000	2.8	15	1	31	69	200	1.8	32	350
OT	04/04/08	23000	0.9	5.1	0.12	21	16	110	0.059	28	150
OT	23/04/08	30000	0.8	5.3	0.11	23	21	48	0.043	68	140
Uncontaminated sites											
MH	18/04/08	74000	1.5	3.8	0.071	34	12	5.3	0.029	26	69
MH	05/05/08	60000	1.9	3.8	0.084	38	12	5.8	0.03	28	77
TW	18/04/08	45000	1.3	7.9	0.027	12	24	18	0.055	6.4	41
TW	05/05/08	20000	0.9	2.5	0.034	7	11	15	0.058	7	62
EC TEL[1]				7.24	0.68	52.3	18.7	30.2	0.13	15.9	124
ANZECC ISQG-Low[2]				20	1.5	80	65	50	0.15	21	200
Auckland region background levels[3]			14	12	0.65	55	45	65	0.45	104	180

[1] Smith *et al.* (1996); McDonald *et al.* (1996). The TEL was derived from the geometric mean of the 15th percentile concentrations of toxic effects data, and the median of the no-effect data set.

[2] ANZECC (2000) Interim Sediment Quality Guideline –Low, the value at which adverse effects are expected rarely.

[3] ARC (2001) values for non-volcanic Auckland soils.

Table 3:

Water quality (total recoverable as mg/L) at field sites on 2 sampling occasions. The red cells indicate exceedances of the ANZECC water quality criteria (95% level of protection) for low hardness waters (30mg $CaCO_3$/L). See Table 2 for site codes.

Site Code	Date sample collected	Iron (Fe)	Arsenic (As)	Cadmium (Cd)	Chromium (Cr)	Copper (Cu)	Lead (Pb)	Mercury (Hg)	Nickel (Ni)	Zinc (Zn)	Zn^2 (S.E.)
Contaminated sites											
OE	04/04/08	1.6	n.d.	n.d.	n.d.	n.d.	0.0013	0.0003	0.0011	0.0094	0.0512 (0.0142)
OE	23/04/08	1.5	n.d.	n.d.	n.d.	0.00054	0.0012	0.00028	0.0012	0.011	0.0512 (0.0142)
OK	04/04/08	0.38	n.d.	n.d.	n.d.	n.d.	0.0017	0.00074	0.00067	0.0081	0.0428 (0.0119)
OK	23/04/08	0.4	n.d.	n.d.	n.d.	n.d.	0.013	0.00094	0.0011	0.015	0.0428 (0.0119)
OT	04/04/08	0.65	n.d.	n.d.	n.d.	n.d.	0.0021	0.0004	0.00092	0.014	0.0423 (0.0244)
OT	23/04/08	0.78	n.d.	n.d.	n.d.	n.d.	0.002	0.00048	0.00076	0.027	0.0423 (0.0244)
Uncontaminated sites											
MH	18/04/08	0.66	n.d.[1]	n.d.	n.d.	0.0011	0.0011	0.0002	0.0013	0.0074	n/a[3]
MH	05/05/08	0.85	n.d.	n.d.	n.d.	0.0021	0.0021	0.00034	0.002	0.01	n/a
TW	18/04/08	1.9	n.d.	n.d.	n.d.	n.d.	0.0014	0.00036	0.00059	0.0032	n/a
TW	05/05/08	1.4	n.d.	n.d.	n.d.	n.d.	0.0048	0.00097	0.0019	0.033	n/a
ANZECC 95% criterion (ANZECC, 2000)		1.0[4]	0.0006	0.024	0.0002	0.001	0.0014	0.0034	0.011	0.008	0.008

[1] n.d. = below detection limits. Detection limits: Hg = 0.000080, As = 0.0011, Cd = 0.000053, Cr = 0.00053, Cu = 0.00053, Pb = 0.00011.

[2] ARC annual means (ARC, 2007).

[3] n/a = comparable data not available.

[4] US EPA (2006).

3.3 Laboratory protocol

3.3.1 Pre-exposure to field-collected stream water

Clams were exposed to field-collected stream water in the laboratory for a period of 30 days. Approximately 20L of stream water was collected on 1 October 2008 from five sites throughout the Auckland region in acid washed plastic containers lined with plastic bags. The aim of this experiment was to test the hypothesis that pre-exposure to contamination enhanced the tolerance of clams to an additional stressor (zinc in solution), which is tested in the chronic experiment (section 4.1.2). Fifty individual clams per field site were placed in 40ml polystyrene cups and housed on trays placed in stacking racks (Figure 3). Clams were randomly allocated to each of five replicates of 10 animals within each of the treatments (see Table 2). No aeration was supplied. Dissolved oxygen levels were checked periodically in random polystyrene cups and found to be above 8.5 mg/L on all occasions. Animals were fed twice weekly at a rate of 0.006ml YCT (yeast, cerophyll and trout chow, US EPA 1993) and 0.18ml green algae culture (*Pseudokirchneriella subcapiata*)[1] per animal per feeding. Water was replaced weekly from stored, aerated stream water collected in acid-washed containers. Water samples were collected at the beginning and end of the experimental period and analysed for a range of metals. The number of dead clams and the number of juveniles produced were recorded weekly. All dead animals were frozen at -80°C for subsequent genetic analysis.

[1] Species previously known as *Selenastrum capricornutum*

Figure 3:
Experimental setup for laboratory-based experiments (Photo: G Croker).

3.3.2 Chronic toxicity to low level zinc exposure

Clams that had previously been exposed to either uncontaminated or contaminated stream water were divided into two sub-groups of up to 25 individuals. Table 4 describes the allocation of clams from each pre-exposure group. Twenty five clams were placed in Waikato river tap water, while the remaining 25 were placed in a zinc-contaminated solution. Zinc was be added in solution (as $ZnSO_4$) to produce an exposure concentration of 0.050 mg/L. This value is consistent with the higher 2007 values recorded for the study sites and is therefore of direct ecological relevance. Individual clams were placed in 40ml polystyrene cups and housed on trays placed in stacking racks. No aeration was supplied. Dissolved oxygen levels were checked periodically in random polystyrene cups and found to be above 8.5 mg/L on all occasions. Animals were fed twice weekly at a rate of 0.006ml YCT (yeast, cerophyll and trout chow, US EPA 1993) and 0.18ml green algae aulture (*P. subcapiata)* per animal per feeding. Water was replaced weekly from freshly made zinc sulphate solution (0.050 mg/L). A water sample was collected at each water change and analysed for total zinc levels. The number of dead clams and the number of juveniles produced were recorded weekly. All dead animals were frozen at -80°C for subsequent genetic analysis.

Table 4:

Allocation of clams from the pre-exposure experiment to the chronic experiment.

Pre-exposure site	Site Code	Pre-exposure designation	Laboratory solution	
			Uncontaminated	Contaminated
Mahurangi Reference @ Trappitt	MH	Uncontaminated	23[1]	25
Tawharanui	TW	Uncontaminated	24	24
Otara LTB	OT	Contaminated	22	21
Oakley LTB	OK	Contaminated	25	24
Oteha LTB	OE	Contaminated	24	24

[1] Numbers differ as some animals died in the pre-exposure experiment.

3.3.3 Acute toxicity to high level zinc exposure e

This experiment was designed to test the hypothesis that exposure to very high levels of zinc would result in not all individuals dying and that the genetic variants that survived would be most tolerant, with this tolerance being reflected in physiological responses (activity levels), as well as fecundity. The acute exposure used 50 clams per site that had also been pre-exposed to either contaminated or uncontaminated stream water collected from field sites. Zinc was added in solution (as $ZnSO_4$) to produce an exposure concentration of 120 mg/L. Clams that had previously been exposed to either uncontaminated or contaminated stream water were divided into 2 sub-groups. Animals were exposed for a period of 4 days, after which time reburial rates were measured and clams frozen at -80°C for subsequent genetic analysis. The number of dead clams and the number of juveniles produced were recorded daily. A water sample was collected and analysed for total zinc concentration.

3.3.3.1 Reburial test

In order to determine fitness we measured individual clam reburial into standard sediment. This behavioural-response endpoint has been found to be a suitable measure of response for shellfish species (Hickey and Martin, 1995). Approximately 2 cm of clean lake sand (sourced from Lake Rotorua and passed through a 1mm mesh sieve) was placed into clean 40ml polystyrene cups and filled with clean water. Individual clams were introduced into each cup and the number buried at 15, 30 and 60 minutes following introduction was recorded. Individuals not reburied after 60 minutes were deemed to be "not reburied". All tests were undertaken in a constant temperature room (20±1.5 °C). Aeration was not used during the reburial measurements.

3.4 Genetic analyses

The technique of allozyme electrophoresis was employed to determine genotype-specific responses to contamination, as well as to determine whether or not selection for tolerant genotypes had occurred during the course of the experiments (by comparing adult and juvenile populations produced). Allozyme electrophoresis is a relatively straighforward technique and is less consuming than other biochemical techniques, as difficult, expensive and time-consuming DNA extraction and purification processes are not required. This, combined with the small sample size required (5-10µl of tissue extract), allows rapid processing of large numbers of samples. The technique assumes that genetic variation will result in protein (or enzyme) variation and is therefore a relatively conservative approach to determining genetic variation, as not all genes produce proteins (Richardson *et al.*, 1986). Alternative forms of enzymes are known as allozymes. Allozymes display slight variations in their abilities to catalyze biochemical reactions. This variability in function provides links to the physiological mechanisms underlying differential response of allozyme genotypes to contaminants. Equally, some differences in fitness measures (e.g., reproductive characteristics, physical activity level) are influenced by genotype. Thus it is possible to link physiological to whole organism to population level responses using this method. A sample of tissue is loaded onto a porous medium (gel) and placed within an electric field. Enzymes migrate within the electric field depending on their size and structure. Histochemical stains specific to each enzyme are then applied to the gel, with the resulting patterns of genetic variation being reflected in slight variations in movement along the gel. Allelic and genotypic frequencies can then be determined and genetic variability calculated.

For this study, whole clams were homogenised in a grinding solution comprised of 50µl of Tris-HCl pH 8.0 (Hebert and Beaton, 1999). Once homogenised, the samples were centrifuged at 10,000 rpm for 1 minute and 10µl of each sample was immediately transferred to the sample gels for electrophoretic analysis. Allozymes were separated by cellulose acetate electrophoresis (Helen Super Z-12 applicator kit, 76mm x 76mm Titan III Cellulose Acetate plates) using techniques described in Hebert and Beaton (1999). We focused on two functionally important enzymes–GPI (glucose-6-phosphate isomerase (GPI, EC 5.3.1.9)) and PGM (phosphoglucomutase (PGM, EC 5.4.2.2)). For each resolvable locus, the fastes migrating alleles (i.e., those migrating the greatest distance toward the anode) were designated as 1, with sequentially slower migrating alleles designated as 2, 3, 4 etc. Line-up gels (*sensu* Richardson *et al.*, 1986) were employed to compare the relative mobility of alleles for each enzyme recorded on different gels.

3.5 Data analyses

3.5.1 Toxicity estimates

Measures of toxicity were calculated for the acute and chronic toxicity exposures using ToxCalc V5.0.22A (Tidepool Scientific Software, 1994):

a) Acute toxicity - Time to 50% morbidity (LT_{50}) was calculated and provides a measure of the resilience of the exposure population. This measure was calculated using the Maximum Likelihood-Probit Method (USEPA, 1991). In addition, LT_{50} were also calculated for individual genotype to examine genotype-dependent responses.

b) Chronic exposure - Time to 20% morbidity (LT_{20}) and 50% morbidity (LT_{50}) was also calculated for animals exposed to zinc for each site separately. This measure was calculated using the Maximum Likelihood-Probit Method (USEPA, 1991).

3.5.2 Tests for significance

Significant of differences between treatments, times, life stages (juveniles and adults) and genotypes was tested using Analysis of Variance (ANOVA) (factorial or one-way where appropriate) within Statistica v7.1 (Statsoft Inc, 2008). Tukeys post-hoc tests were used to determine the significance of pair-wise comparisons.

3.5.3 Population genetic structure

Genetic data were analysed using the online version of GenePop version 3.4 (http://wbiomed.curting.edu.au/genepop/) to provide an overall description of the genetic structure of the study population. The mean number of alleles (across loci) was calculated, along with allele and genotype frequencies for each locus. A locus was considered polymorphic if the frequency of its most common allele was <95%. Heterozygosity provides a measure of the genetic diversity within a population. Observed heterozygosity (direct count) for each locus were also calculated, along with heterozygosity expected under Hardy Weinberg (H-W) equilibrium. Tests for deviations from H-W equilibrium predictions (i.e., random mating) were undertaken for each locus (using the exact test of Haldane, 1954). Alternative hypotheses of heterozygote deficiency or excess were tested using U tests (Rousset and Raymond, 1995). This provides the likely explanation for any observed deviation from H-W equilibrium.

4 Results

4.1 Does stormwater contamination contribute to reduced fitness in populations of aquatic organisms?

This question was addressed by examining the effects that contamination from natural stormwater or from zinc-dosed solutions had on the fitness of freshwater clams. Fitness was measured as survivorship, activity level (reburial rate) or fecundity (number of offspring). Below, the results of each experimental phase are examined separately and an overall summary of the key effects on fitness is presented at the conclusion of this section.

4.1.1 Pre-exposure phase

4.1.1.1 Water quality

Metals levels were measured in field collected water from five sites at the beginning (1/10/08) and end (21/11/08) of the experiment. The results are presented in Table 5. The three sites designated as contaminated had zinc levels well above the ANZECC 95% guidelines and are comparable with the ARC annual mean values for these sites. The designated uncontaminated sites also recorded marginal exceedances for zinc levels, with the elevated level at Tawharanui most likely representing contamination of the test sample. All contaminated sites also recorded exceedances for copper, with Oakely Creek (OK) also exceeding guideline values for chromium, lead and arsenic. These water quality results generally support the site designations of contaminated and uncontaminated.

Table 5:

Water quality (total recoverable as mg/L) results for field collected water samples used in the pre-exposure experiment. Cells shaded in red indicate exceedances of the ANZECC 95% guidelines for low hardness waters (30 mg$CaCO_3$/L).

Site Code	Date sampled	Arsenic (As)	Cadmium (Cd)	Chromium (Cr)	Copper (Cu)	Lead (Pb)	Nickel (Ni)	Zinc (Zn)	Zn^2 (S.E.)
Contaminated sites									
OE	1/10/08	n.d.[1]	n.d.	n.d.	0.0026	0.00042	0.0015	0.023	0.0512 (0.0142)
	2/11/08	n.d.	n.d.	0.00060	0.0022	0.00071	0.0017	0.020	0.0512 (0.0142)
OK	1/10/08	n.d.	n.d.	0.00058	0.0028	0.0016	0.0011	0.023	0.0428 (0.0119)
	2/11/08	0.0011	0.00011	0.0017	0.0065	0.0096	0.0027	0.065	0.0428 (0.0119)
OT	1/10/08	n.d.	n.d.	n.d.	0.0019	0.00032	0.0010	0.027	0.0423 (0.0244)
	2/11/08	n.d.	n.d.	0.00054	0.0023	0.0013	0.0011	0.034	0.0423 (0.0244)
Uncontaminated sites									
MH	1/10/08	n.d.	n.d.	0.0012	0.00099	0.00034	0.0010	0.0057	n/a[3]
	2/11/08	n.d.	n.d.	0.0011	0.0023	0.00022	0.0015	0.0082	n/a
TW	1/10/08	n.d.	n.d.	n.d.	0.0010	0.00022	0.00065	0.0092	n/a
	2/11/08	n.d.	0.000072	0.00081	0.0074	0.0013	0.0020	0.026[4]	n/a
ANZECC 95% guideline (ANZECC, 2000)		0.0006	0.024	0.0002	0.001	0.0014	0.011	0.008	0.008

[1] n.d. = below detection limits. Detection limits: As = 0.0011, Cd = 0.000053, Cr = 0.00053, Cu = 0.00053, Pb = 0.00011

[2] ARC annual means (ARC, 2008).

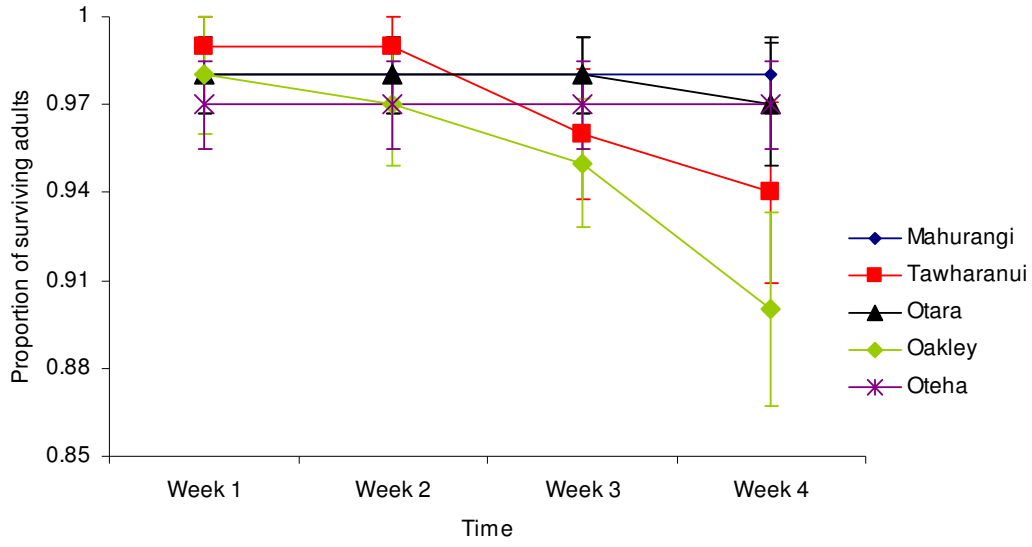
[3] n/a = comparable data not available.

[4] Possible contamination of sample.

4.1.1.2 Survival and reproduction

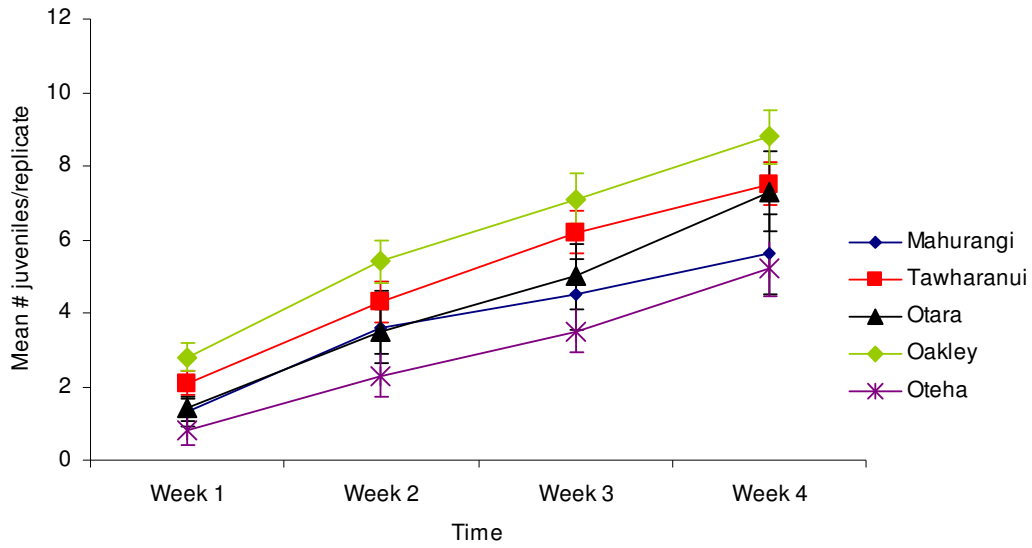
The mean number of adult survivors per replicate at the end of each week for each site is presented in Figure 4. There was no significant difference in the total number of adults alive per replicate at the end of week 4 between sites (One-way ANOVA, p=0.14) or between pre-defined groupings of uncontaminated (Tawharanui, Mahurangi) and contaminated (Otara, Oteha, Oakley) sites (One-way ANOVA, p=0.56). Interestingly, animals exposed to Oakley Creek stream water showed poorer, but not statistically significant, survival than other sites.

Figure 4:
Mean number of live adults/replicate for each pre-exposure group. Error bars are ± 1 S.E.



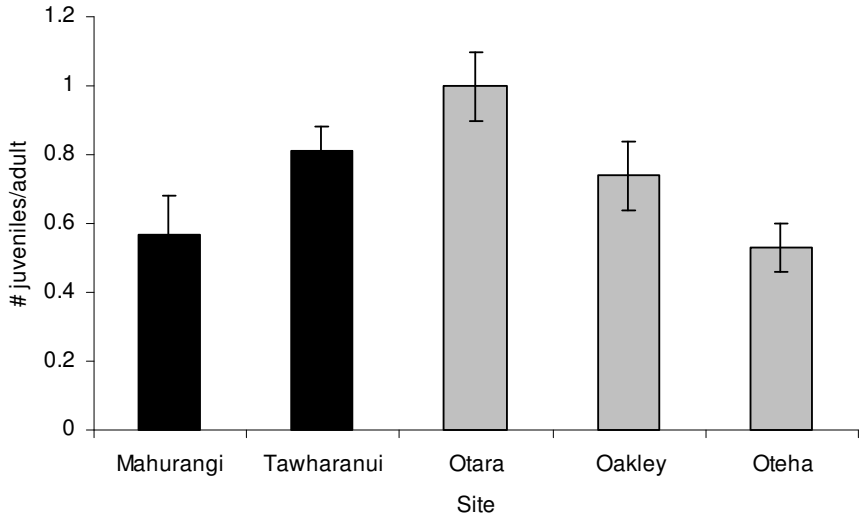
The mean number of juveniles per adult for each site is presented in Figure 5. Mutliple regression was used to investigate difference in trends between sites over time. There was no significant difference between sites (p=0.67), but a significant difference between weeks (p=0.01).

Figure 5:
Mean number of juveniles/replicate for each pre-exposure group. Error bars are ± 1 S.E.



The total number of juveniles as a proportion of the number of adults is presented in Figure 6. There was a significant difference in the number of juveniles/adult produced at the end of week 4 between sites (One-way ANOVA, p=0.03), with significantly higher numbers at Oakley compared with Oteha, but not with other sites. There was no signficant difference between pre-defined groupings of uncontaminated (Tawharanui, Mahurangi) and contaminated (Otara, Oteha, Oakley) sites (One-way ANOVA, p=0.52).

Figure 6:
Total number of juveniles/adult at the end of the pre-exposure experiment. Sites in black are designated as uncontaminated, while those in grey are designated as contaminated. Error bars are ± 1 S.E.



4.1.2 Chronic toxicity

4.1.2.1 Water quality

A water sample was collected at the beginning of the experiment and on several additional occasions. On average, total zinc levels were measured at 0.087 mg/L (±0.00006, n=3), which was slightly above our nominal exposure concentration of 0.050 mg/L.

4.1.2.2 Survival and reproduction

Figure 7 shows the proportion of adults alive at the termination of the chronic exposure. A large proportion of animals exposed to zinc contamination died by this stage. In contrast, survivorship in uncontaminated treatments was greater than 60% at all sites. A factorial ANOVA indicated a significant difference between treatments ($p < 0.001$) but not between sites ($p = 0.21$) or between pre-exposure groupings ($p = 0.21$). There was no significant interaction term ($p = 0.56$), indicating the response was similar for all sites.

While survivorship at termination of the chronic exposure is a useful measure, the comparative rate of mortality provides insight into the likely resilience of the different clam populations (i.e., as defined by their pre-exposure regime). Figure 8 shows the number of adults alive at each recording time. Contaminated (C) animals showed a marked reduction in survivorship compared with uncontaminated animals (UC) from

week 2 on, and even in week 1 for some sites. Animals pre-exposed to Oakley Creek water and then zinc-contaminated water showed a marked reduction in survivorship after week 2 in comparison to other sites.

Figure 7:
Proportion of adults surviving at termination of the chronic exposure. Treatment codes: UCUC = pre-treatment uncontaminated, chronic uncontaminated; UCC = pre-treatment uncontaminated, chronic contaminated; CUC = pre-treatment contaminated, chronic contaminated; CC = pre-treatment contaminated, chronic contaminated.

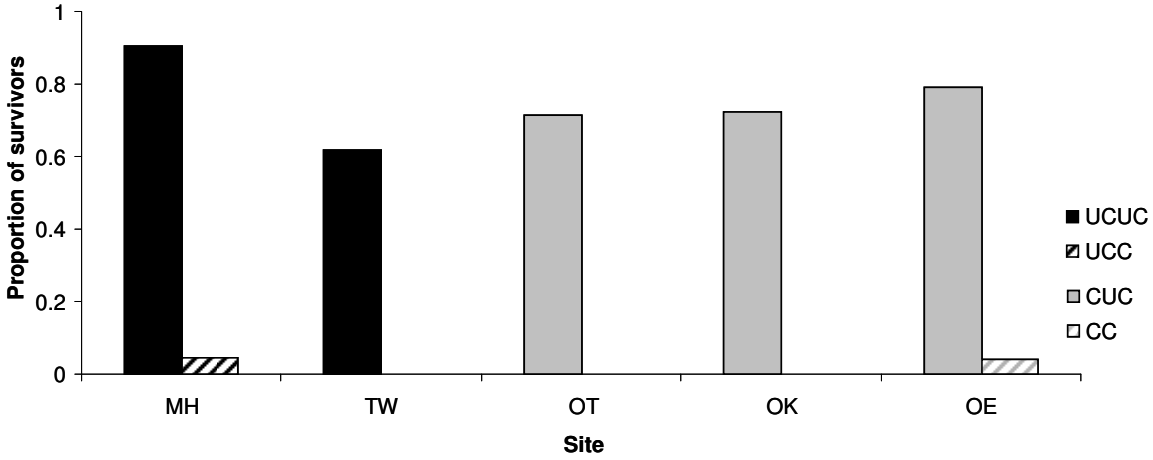
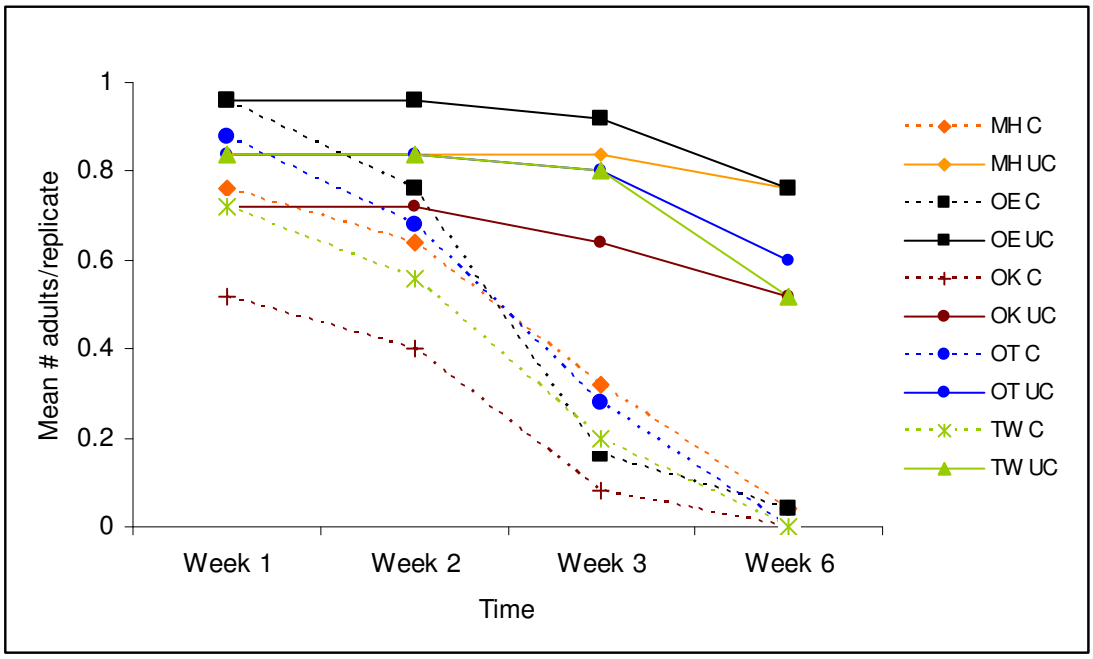


Figure 8:
Mean number of adults per replicate alive at each recording time. The legend indicates the pre-exposure site (MH, OE, OK, OT, TW) and the chronic exposure regime (C = contaminated, UC = uncontaminated).



The LT_{20} and LT_{50} (time taken to observed 20% and 50% mortality respectively, in the test population) are presented in Figures 9 and 10 for animals exposed to zinc contamination in the laboratory. The LT_{20} gives an indication of the likely early response and comparison with the LT_{50} provides insight into both the susceptibility and resilience of populations pre-exposed to differing stormwater contamination levels. It is evident that animals pre-exposed to Oakley Creek water had reduced survivorship at early and later stages of the chronic exposure, when compared to other sites. In contrast, animals pre-exposed to Mahurangi stream water (uncontaminated) were initially more susceptible but showed greater resilience (as indicated by the higher LT_{50}).

Figure 9:
Time to 20% mortality (LT_{50}) at each site (contaminated animals only). Site codes refer to pre-exposure site designations.

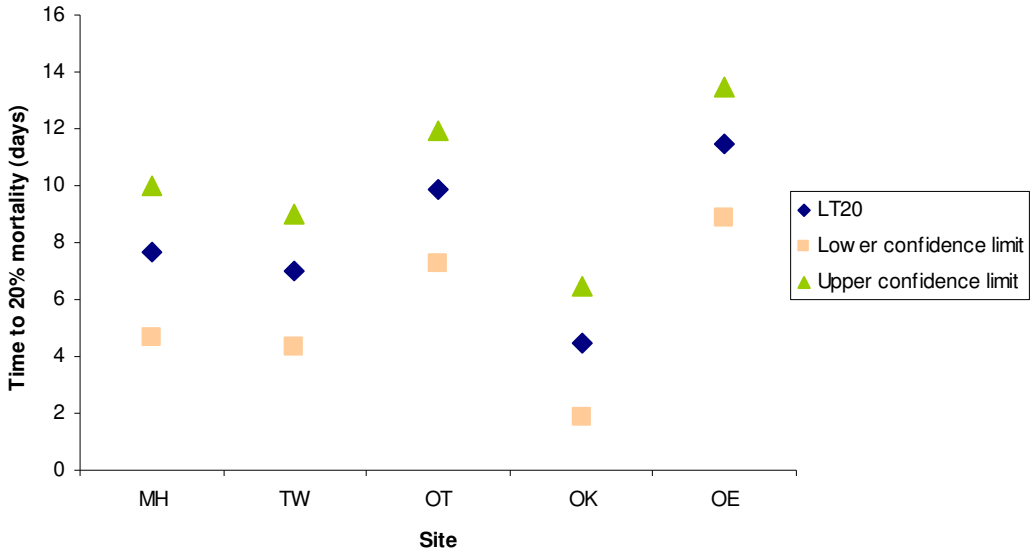
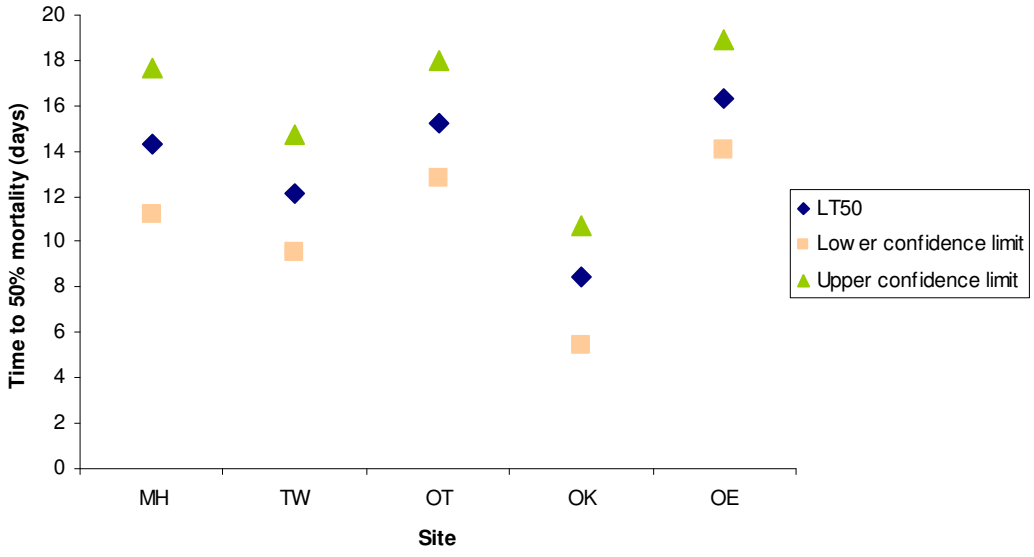
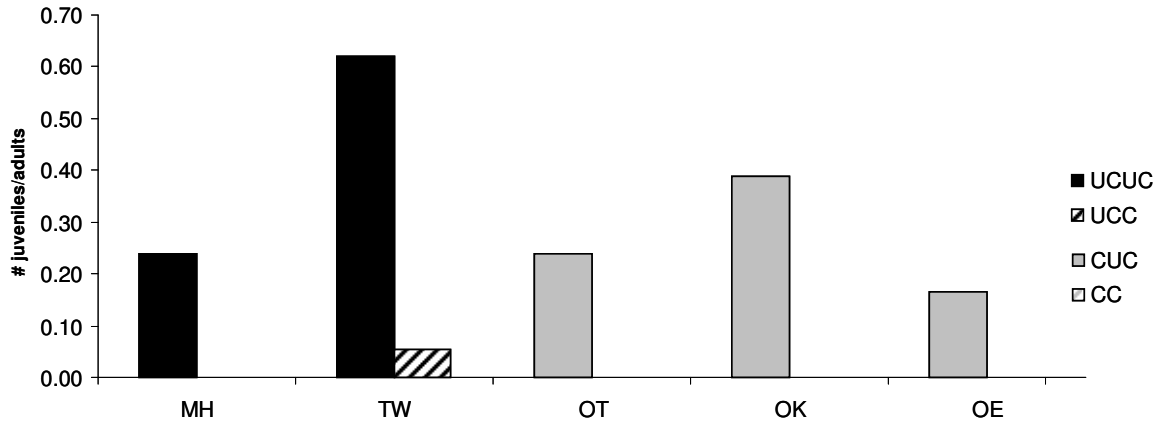


Figure 10:
Time to 50% mortality (LT_{50}) at each site (contaminated animals only). Site codes refer to pre-exposure site designations.



The total number of juveniles/adult produced by the end of the chronic exposure is presented in Figure 11. Very few juveniles were produced by animals exposed to zinc-contaminated water. In contrast, juveniles were produced in all uncontaminated treatments, with significantly higher numbers per adult than contaminated populations (Factorial ANOVA, $p<0.001$). There was no significant interaction between site and treatment ($p=0.42$), although animals pre-exposed to Tawharanui stream water produced significantly more juveniles per adult in the uncontaminated treatment when compared to all contaminated sites (Tukey's post-hoc test $p<0.001$).

Figure 11:
Total number of juveniles/adult at termination of the chronic experiment. Treatment codes: UCUC = pre-treatment uncontaminated, chronic uncontaminated; UCC = pre-treatment uncontaminated, chronic contaminated; CUC = pre-treatment contaminated, chronic contaminated; CC = pre-treatment contaminated, chronic contaminated.



4.1.3 Combined exposure regime

The preceding sections have discussed the results of each component of the overall exposure regime in isolation. By considering the combined exposure regime we are able to assess the overall effect of field and laboratory exposure to contaminated water on our test organism. Figure 12 illustrates an example of the combined exposure regime for a given group of test animals.

Figure 12:
Combined exposure regime for individual pre-exposure site.

Figure 13 shows the proportion of survivors at the end of the experiment for each combined exposure regime. When compared on the basis of combined pre-exposure group (uncontaminated = Tawharanui, Mahurangi; contaminated = Otara, Oakley, Oteha) and subsequent chronic exposure group (zinc contaminated or uncontaminated), there is a significant treatment effect (One way ANOVA, $p<0.001$). Tukey's post-hoc pairwise comparions revealed significant differences in survivorship associated with exposure to zinc contamination (PECCHC, PEUCCHC) ($p<0.001$). Pre-exposure to contaminated stream water had little effect on subsequent survivorship.

Figure 13:

Proportion of adult survivors at the end the combined experimental phases for each combined exposure regime. Codes refer to combined exposure regimes (PECCHC = pre-exposure contaminated, chronic contaminated; PECCHUC = pre-exposure contaminated, chronic uncontaminated; PEUCCHC = pre-exposure uncontaminated, chronic contaminated; PEUCCHUC = pre-exposure uncontaminated, chronic contaminated). Vertical bars denote 95% confidence interval.

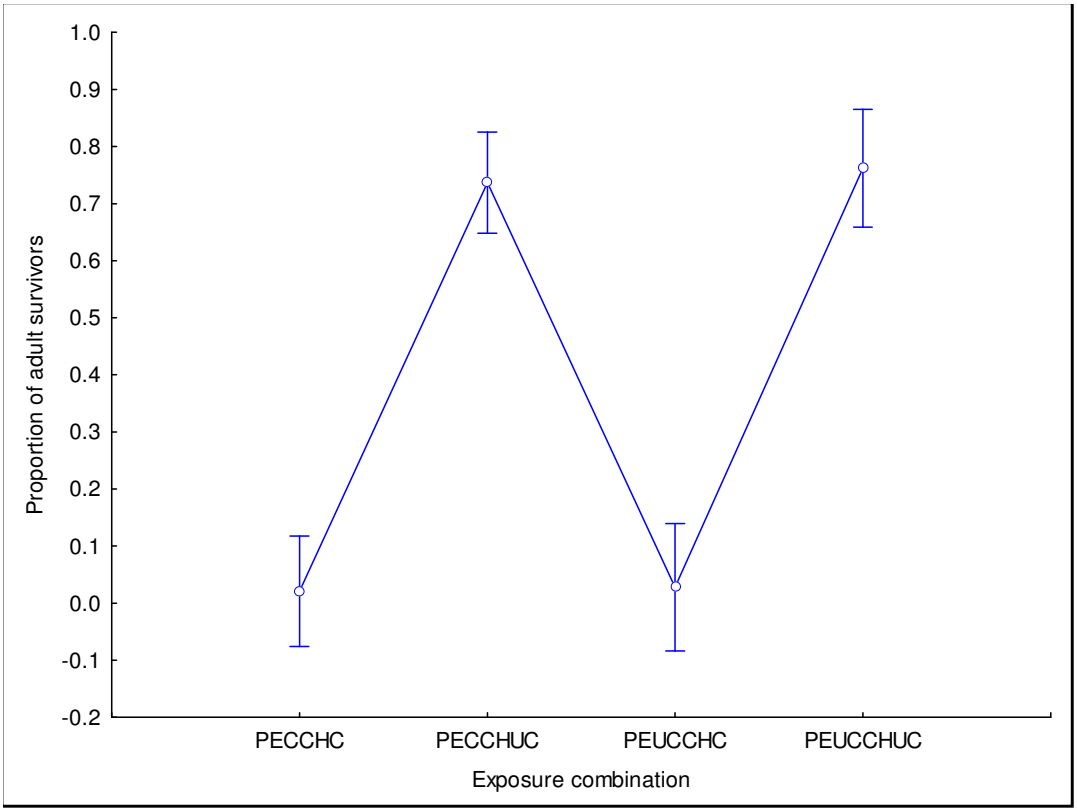
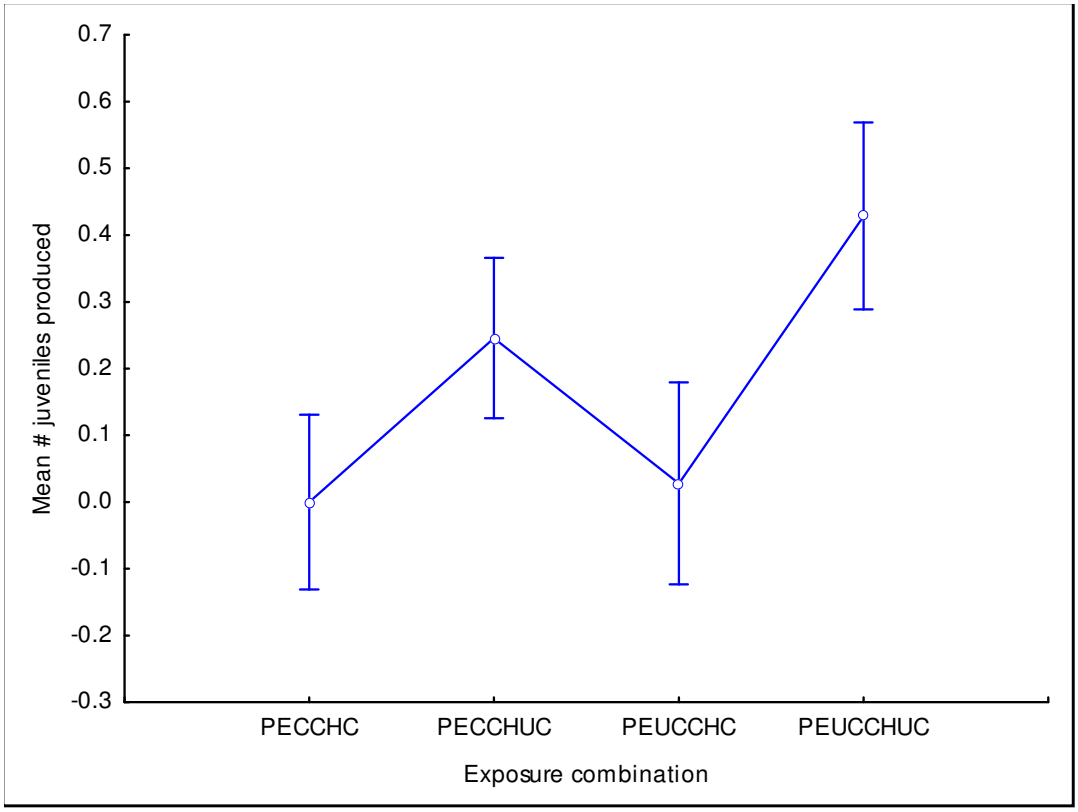


Figure 14 shows the mean number of juveniles produced by the end of the experiment for each combined exposure regime. When compared on the basis of combined pe-exposure group (uncontaminated = Tawharanui, Mahurangi; contaminated = Otara, Oakley, Oteha) and subsequent chronic exposure group (zinc contaminated or uncontaminated), there is a significant treatment effect ($p<0.001$). Tukey's post-hoc pairwise comparions subsequently revealed signficant effects on fecundity associated with exposure to zinc contamination (PECCHC, PEUCCHC) ($p<0.001$). In addition, pre-exposure to contaminated stream water reduced fecundity of clams further exposed to uncontaminated water ($p<0.001$).

Figure 14:

Mean number of juveniles produced at the end the combined experimental phases for each combined exposure regime. Codes refer to combined exposure regimes (PECCHC = pre-exposure contaminated, chronic contaminated; PECCHUC = pre-exposure contaminated, chronic uncontaminated; PEUCCHC = pre-exposure uncontaminated, chronic contaminated; PEUCCHUC = pre-exposure uncontaminated, chronic contaminated). Vertical bars denote 95% confidence interval.



4.1.4 Acute toxicity test

The purpose of this experiment was to test the hypothesis that exposure to very high levels of zinc would result in not all individuals dying and that the genetic variants that survived would be most tolerant, with this tolerance being reflected in physiological tolerance (activity levels), as well as fecundity.

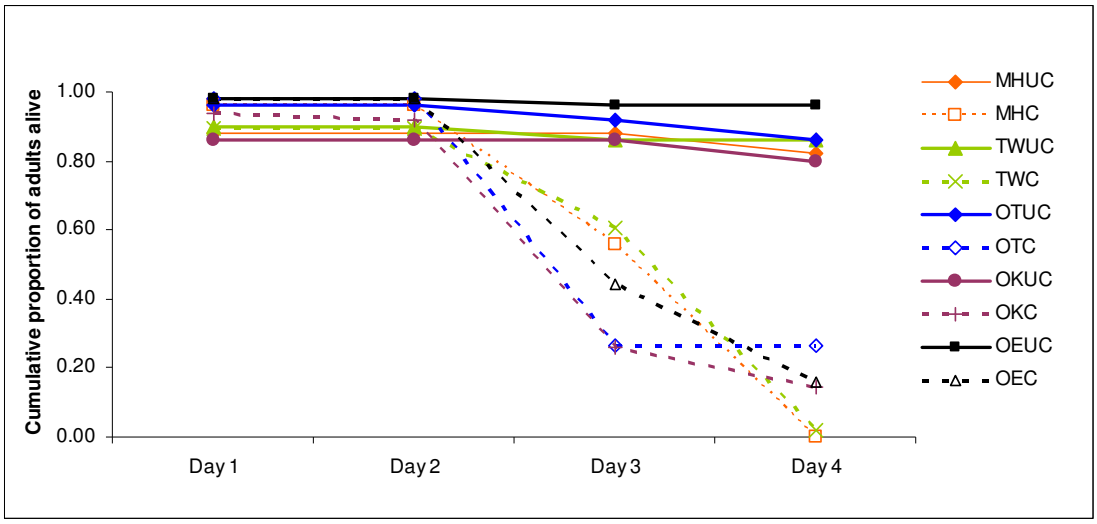
4.1.4.1 Water quality

A water sample was collected at the beginning of the experiment, with the sample measuring 120 mg/L total dissolved zinc.

4.1.4.2 Survival and reproduction

Figure 15 shows the proportion of adults alive in contaminated (C) and uncontaminated (UC) treatments over a four day period. A Factorial ANOVA revealed significant differences between treatments ($p<0.001$) but not between sites ($p = 0.18$) at Day 4. However, there was also a significant site x treatment interaction ($p = 0.005$), with significantly higher numbers of live adults pre-exposed to Mahurangi stream water, when compared to Tawharanui (but not other sites). There was a near-significant difference between days in treatment effect ($p = 0.08$).

Figure 15:
Proportion of adults alive during the 4 day acute toxicity exposure.



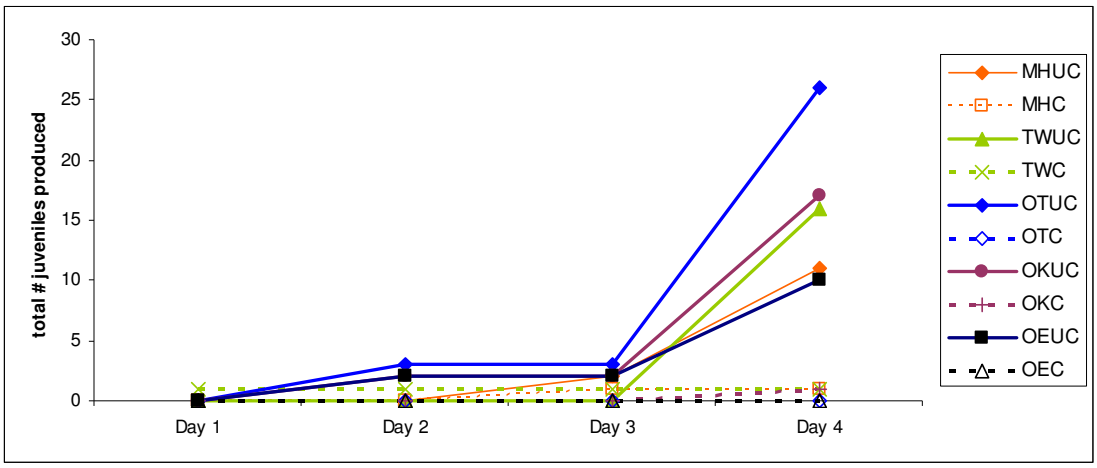
Average time to death (hours) was calculated as the mean of replicates within each site and treatment. Survivors were assigned an arbitrary time to death of 192 hours (twice the length of the experiment). Table 6 presents the results of this analysis. Main effects ANOVA indicated a significant difference between treatments ($p < 0.001$), but not between sites ($p = 0.37$). However, animals pre-exposed to Otara and Oakley stream water displayed slightly lower average time to death than those exposed to other stream waters, with those from Tawharanui displaying the longest average time to death.

Table 6:
Average time to death (hours) for animals exposed to acute levels of zinc.

Pre-exposure site	Site Code	Pre-exposure designation	Average time to death (hours)
Mahurangi Reference @ Trappitt	MH	Uncontaminated (UC)	80.7
Tawharanui	TW	Uncontaminated (UC)	88.6
Otara LTB	OT	Contaminated (C)	72.0
Oakley LTB	OK	Contaminated (C)	76.4
Oteha LTB	OE	Contaminated (C)	80.2

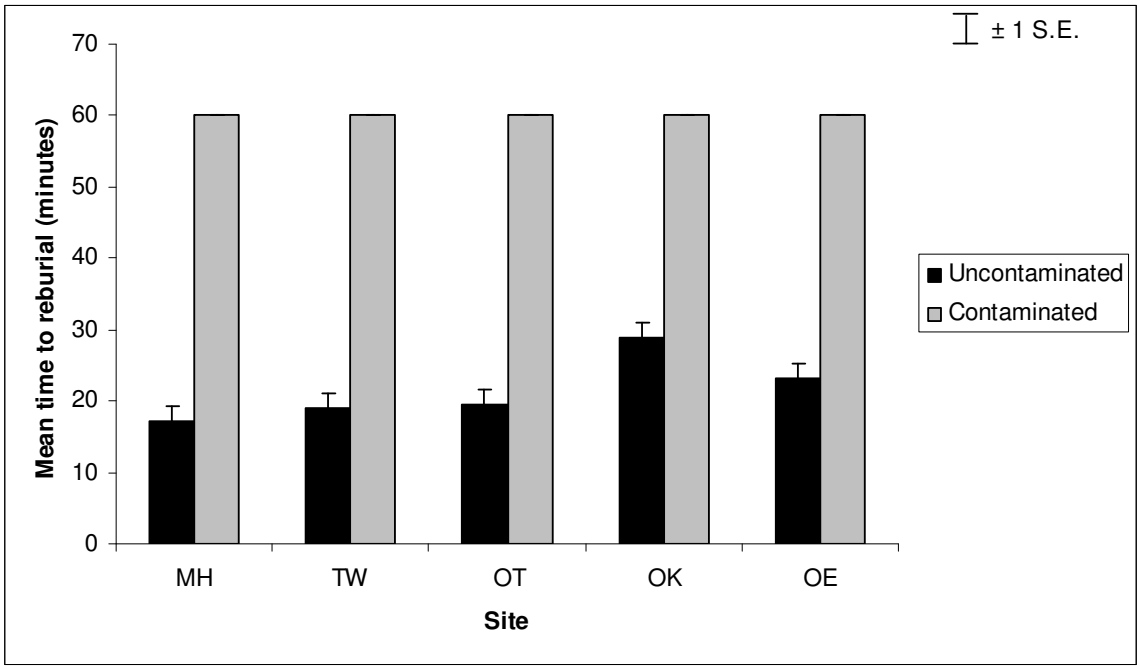
Figure 16 shows the total number of juveniles produced during the experiment in contaminated (C) and uncontaminated (UC) treatments. A Main effects ANOVA revealed significant differences between treatments ($p<0.001$) but not between sites ($p = 0.08$) at Day 4. There were very few juveniles produced in any of the contaminated treatments.

Figure 16:
Number of juveniles produced during the 4 day acute toxicity test.



Reburial rates of survivors were measured at the termination of this experiment (Figure 17). In all cases where animals survived the contaminated treatment, reburial rates were greater than 60 minutes. In comparison, animals in the uncontaminated treatment generally reburied within 30 minutes. There was a significant difference in time to reburial among sites ($p < 0.001$), with animals from Oakley (OK) taking longer to rebury than all other sites.

Figure 17:
Comparative reburial rates of adults after 4 days.



4.1.5 Summary – effects on fitness

Does exposure to stormwater contamination affect fitness of aquatic organisms? The combined results of the above experiments indicate that exposure to relatively low levels of zinc, an important contaminant associated with stormwater, can indeed affect survivorship and fecundity when that exposure is beyond a few days. We have also shown that acute exposure to very high zinc levels can induce significant mortality and effects on fecundity, and even on behavioural activities such as reburial. However, some individuals are able to tolerant even these doses. There is also some evidence to indicate that pre-exposure to stormwater contamination, at concentrations found in naturally contaminated stream waters, can influence the subsequent fitness of aquatic organisms, at least through sub-lethal or possibly indirect effects such as impacts on fecundity. This is indicated by the apparent reduction in survivorship of clams in the chronic exposure which had been pre-exposed to Oakley Creek stream water. What remains to be determined is whether or not such effects on fitness are manifest as selection for tolerant forms at the expense of more sensitive individuals.

4.2 Does exposure to stormwater result in selection for tolerant genetic variants?

In order to address this question we compared the genetic composition of the adult population used in the toxicity tests and juvenile populations produced from each exposure. The overall hypothesis is that there will be a change in the genetic composition of juveniles exposed to contaminants as a consequence of reduced fecundity or increased mortality of sensitive adult genotypes.

4.2.1 Population genetic structure

The genetic composition of the test population was determined to ensure that there was an even spread of genetic variants throughout our test groups, which could otherwise bias the interpretation of the results. Allele frequencies are presented in Table 7 for each locus for the entire sample population (n=110). The GPI has a very low level of variation (polymorphism) and thus could not be used for subsequent comparative analyses (using the 95% criterion for characterisation of a polymoprhic loci, Hartl, 1981). In contrast, the PGM locus was highly polymorphic, with five alleles recorded, including three alleles that were equally common in frequency. In addition, 11 genotypes were recorded for the PGM locus (Figure 18). These results are consistent with a previous investigation of the population genetic structure of the Lake Rotorua *S. novaezelandiaea* population (Phillips and Hickey, in press). All subsequent genotype-specific analyses are therefore based only on the PGM allozyme.

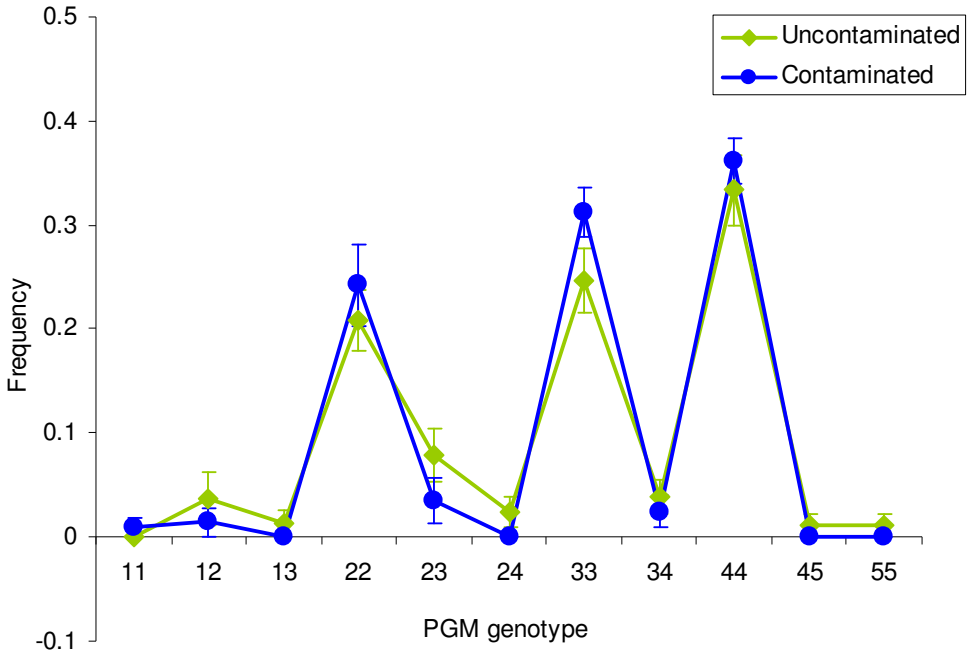
Table 7:

Allele frequencies recorded for adult clams for two allozymes.

Allele/Allozyme	GPI	PGM
1	0	0.022
2	0.013	0.255
3	0.960	0.379
4	0.018	0.332
5	0.018	0.012

Figure 18 shows the genetic composition of adults used for the chronic exposure based on the PGM locus. The mean number of adults used per replicate in these analyses was 16.4 (±1.17) and 18.5 (±1.83), respectively for uncontaminated and contaminated treatments, from a starting population of 25 individuals per replicate. The reduction in numbers reflects difficulties in assigning a genotype to some individuals due to deterioration of tissue. A Factorial ANOVA indicated no significant interaction between genotype frequency and treatment (p=0.275), indicating no significant difference in genotype frequencies between sub-groupings (contaminated and uncontaminated). The greatest difference between adult populations was at PGM 33, which showed a 6.5% difference between sub-groupings.

Figure 18:
Genetic composition of *S. novaezelandiae* adult populations exposed to uncontaminated and contaminated test solutions for 38 days. Error bars are ± 1 S.E.

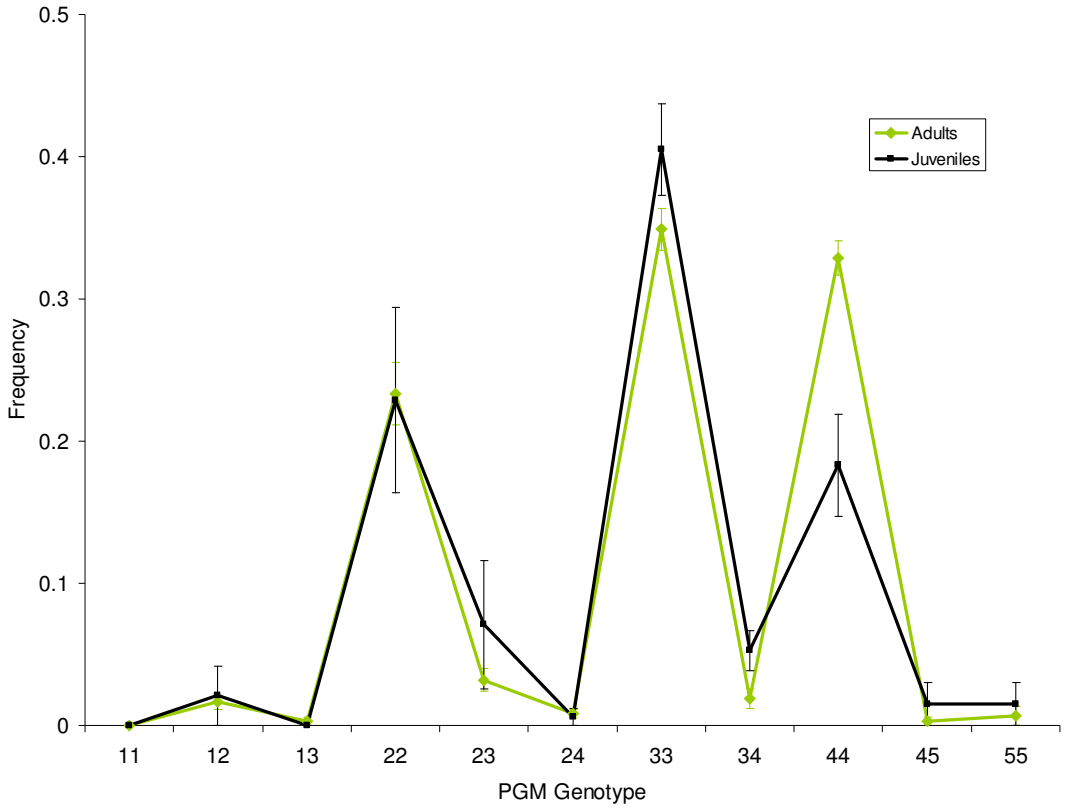


4.2.2 Does exposure to field-collected test waters result in selection?

While the results of the initial pre-exposure test did not indicate any effect on adult survival, there was evidence for a reduction in fecundity related to pre-exposure to stormwater. To further investigate the chronic genetic effects we compared the genotype composition of juveniles produced during pre-exposure with the entire adult population initially used (Figure 19). This shows a statistically significant reduction in the frequency of PGM 44 between adults and juveniles. Results of a Factorial ANOVA of life stage and genotype indicates a significant interaction between these two factors on genotype frequency ($p= 0.007$). Post-hoc analyses indicated a significant difference in the frequencies of PGM 44 between adult and juvenile populations. This represents a 14.6% decrease in the frequency of juveniles with this genotype.

Figure 19:

Mean genotype frequencies in adult and juvenile populations produced during pre-exposure to field-collected stormwater. Error bars are ± 1 S.E.

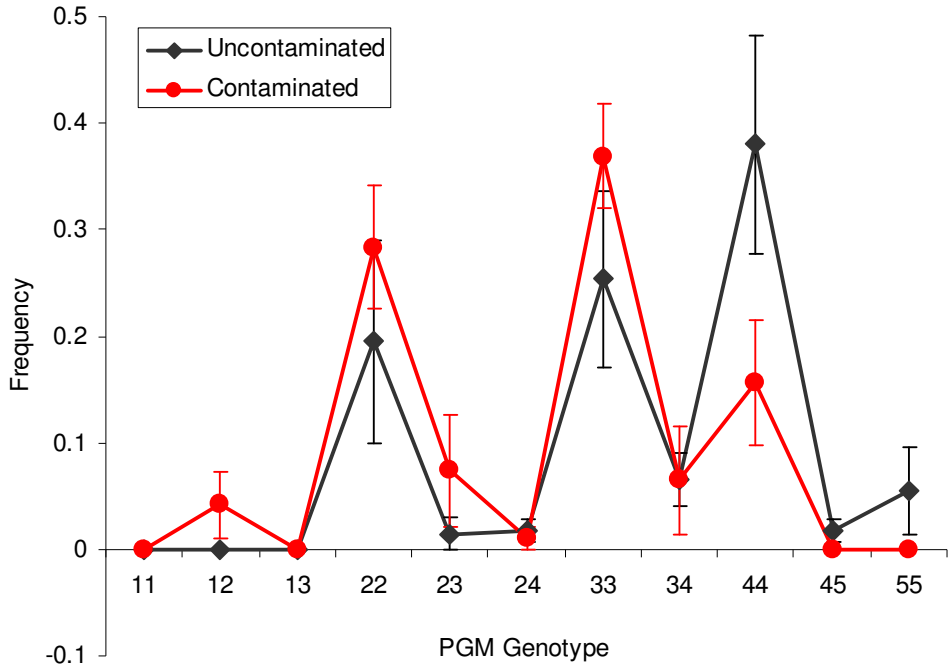


4.2.3 Do genotype frequencies differ between populations of juveniles produced in zinc-contaminated and clean test solutions exposed for 6 weeks?

The chronic exposure indicated a significant difference in adult survival and juvenile production in uncontaminated and contaminated treatments. By comparing the genetic composition of the adult and juvenile populations from this experiment, it is possible to determine whether or not there has been a shift in population structure between generations and therefore provide evidence for selection. Figure 20 presents the mean genotype frequencies for the juvenile populations produced during the chronic exposure. The mean number of juveniles used per replicate in this anlaysis was 21.6 (±2.14) and 16.0 (±2.59) for uncontaminated and contaminated respectively. There is a marked difference between the two populations, with individuals with genotype 44 significantly decreasing relative to other genotypes in the zinc exposed populations. A Factorial ANOVA revealed a significant interaction between genotype and treatment ($p=0.056$). Tukey's post hoc pairwise comparison indicated significant differences

between contaminated and uncontaminated treatments for genotype 44 (p=0.001). This difference represents a 22.4% decrease in frequency of PGM 44 in zinc contaminanted juvenile populations compared with uncontaminated populations. Genotypes 22 and 33 also showed some differences between treatments (8.8% and 11.5% increase respectively in contaminated populations), although this was not significant due to variability within these genotype responses.

Figure 20:
Frequency of individual genotypes for juveniles produced from adults exposed to contaminated or uncontaminated test solutions (chronic exposure). Error bars are ± 1 S.E.

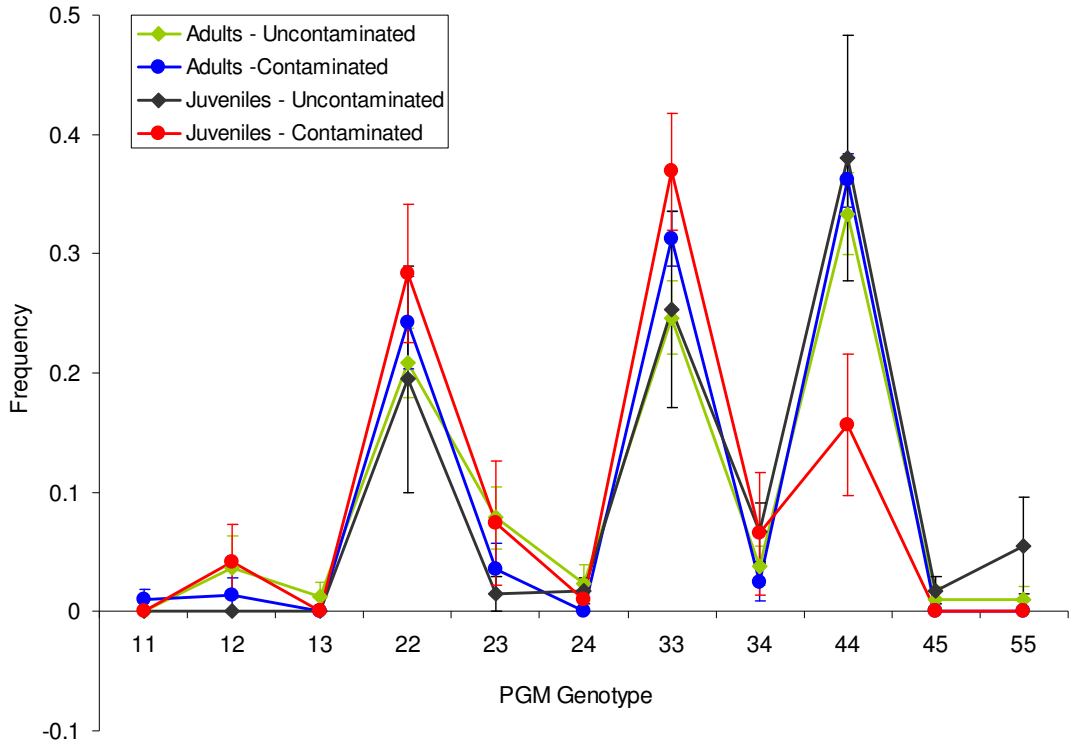


4.2.4 Do genotype frequencies differ between populations of adults and juveniles within treatments?

It is important to determine whether or not the differences observed between juvenile genotype composition is an artefact of differences in the initial adult population genetic composition, the effect of some other selection agent (such as food availability) or the effects of the zinc treatment. Figure 18 indicated that the two initial adult sub-populations did not differ significantly. Figure 21 presents the combined data for the adult and juvenile populations. A Factorial ANOVA across the entire data set indicates a marginally significant interaction between life stage (adult versus juvenile), treatment and genotype (p=0.067). Additional Factorial ANOVAs were undertaken to test for differences between life stage and genotype for each treatment separately. For the uncontaminated treatment, there was no significant difference in genotype frequency

of adults and juveniles (p=0.94). In contrast, there was a significant difference between adults and juveniles in the zinc contaminated treatment (p=0.008). Post hoc pairwise comparisons indicated a significant difference in the frequency of PGM 44 (p=0.008). This difference represents an average decrease in frequency of PGM 44 of 20.2% (±1.35%) compared to the adult and juvenile populations.

Figure 21:
Frequency of individual genotypes in adults and juveniles in chronic uncontaminated and contaminated exposures (chronic zinc exposure). Vertical bars indicate 95% significance criterion. Error bars are ± 1 S.E.

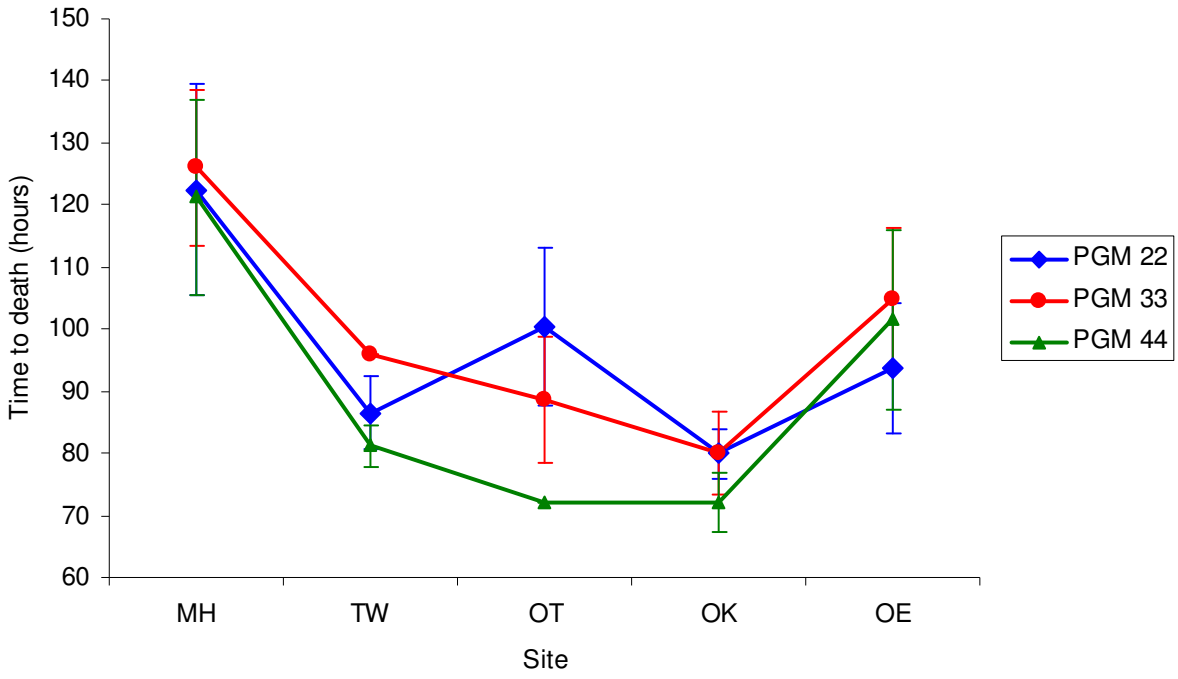


4.2.5 Is there a difference in the way genotypes respond to different exposure regimes?

Consistency of response by specific genotypes across differing exposure regimes provides insight into the resilience of specific genotypes. Although there were clear genotype-specific responses to contamination in adults exposed to zinc at low levels in the chronic exposure, this is not the case when the time to death of common genotypes (22, 33 and 44) in the acute exposure are assessed. A Factorial ANOVA found no significant difference between genotypes (p=0.32). There is, however, an overall significant difference between sites in time to death (regardless of genotype). Figure 22 shows the time to death of the 3 most commonly occuring genotypes. There is a generally consistent trend indicating that genotype 44 has a lower stress tolerance (i.e., shorter time to death) than other genotypes, especially in sites OK, OT and TW,

where time to death was on average 38% faster for individuals with this genotype at these 3 sites. This is shown when we examine genotype differences across all sites. However it is not statistically significant (Factorial ANOVA, p = 0.348).

Figure 22:
Frequency of the most commonly occurring genotypes in adults in acute contaminated treatments. Error bars are ± 1 S.E.



4.2.6 Summary – selection effects

Does exposure to stormwater contamination result in selection for or against specific genetic variants (genotypes)? The combined analyses above indicate that exposure to relatively low levels of zinc results in selection for tolerant genotypes at the expense of less tolerant individuals. These results are consistent with the observations of reduced fecundity and increased mortality observed in the toxicity exposures. These selection effects are evident even in association with field-collected stormwater.

5 Discussion and conclusions

Does stormwater contamination result in inter-generational effects on aquatic organisms? We have examined this question by undertaking short and long-term exposures to stormwater-contaminated stream waters and zinc-dosed laboratory test solutions. We have examined both lethal (mortality) and sub-lethal (fecundity) measures of response to this contamination. In addition, we have examined these effects beyond a single generation by comparing changes in the genetic structure of adult and juvenile populations.

We have found that pre-exposure to natural streamwaters contaminated with stormwater does not affect adult mortality but may have some effect on fecundity, at least for those animals exposed to Oakley Creek. Importantly, we found a significant reduction in the frequency of one genetic variant (PGM 44) when we compared the genetic composition of adult and juvenile populations used for and produced during the pre-exposure experiment.

When the test organisms were subsequently exposed to low levels of zinc in solution for 38 days we observed significant differences in both adult mortality and fecundity. There was also some suggestion of an influence of pre-exposure to contamination on the susceptibility of organisms to further contamination, with pre-exposure to Oakley Creek stream water reducing both overall survival and rate of mortality. We observed differences in the genetic composition of juvenile populations produced during the chronic exposure, with significantly lower frequencies of PGM 44 in the contaminated population. In addition, PGM 22 and PGM 33 were increased in frequency in contaminated populations, although these results were variable and therefore not statistically significant. These results are also supported by the finding of significant effects on fecundity when considering the overall exposure regime (pre-exposure and chronic exposure). This supports our original hypothesis that increased susceptibility to additional stressors is likely with organisms pre-exposed to stormwater contamination.

Genotype-dependent responses to contamination have been widely reported in a range of organisms (see Belfiore and Anderson, 2001 for a review). However, few investigations have been undertaken on bivalves within the Pisidiae. Sloss *et al*. (1998) reported elevated frequencies of a pollution-tolerant genotype at the GPI locus in populations of the fingernail clam *Musculium transversum*. Both GPI and PGM have commonly been reported to have alleles under selection by metals in a range of taxa (Lavie and Nevo, 1982; Mulvey *et al*., 1995; Roark and Brown, 1996; Sloss *et al*., 1998; Gale *et al*., 2003). GPI is a key enzyme for the energy production pathways and mobilisation of energetic reserves (Riddoch, 1993). PGM is important for gluconeogenesis and glucogenesis. The different enzyme kinetics, metabolic efficiency, and sensitivity to metals of PGM and GPI allozymes (Nevo, 2001), may therefore

provide selection advantage for increased fitness under stressful, energy-demanding conditions (Calow, 1991).

The results of this study are consistent with previous laboratory experiments undertaken on *S. novaezelandiae* exposed to zinc at sub-lethal concentrations for 96 hours (Phillips and Hickey in press). In that experiment, PGM 33 was found to be able to recover faster from sub-lethal concentrations of zinc when compared with other genotypes, with this difference being measured by increased reburial rate following 24 hours post-experiment exposure to clean water. In contrast, neither PGM 22 or PGM 44 individuals recovered to any great extent.

The acute toxicity exposure provided an opportunity to investigate the extent to which genetically-based tolerance can act. While a considerable proportion of the test population died, there were some survivors. There were no statistically significant differences in time to death of genotypes when examined across all pre-exposure sites, suggesting that genetic tolerance may be "over-ridden" in extreme stressor events. However, there is a generally consistent trend indicating that genotype 44 has a lower stress tolerance (i.e., shorter time to death) than other genotypes, especially in individuals pre-exposed to Oakley, Otara and Tawaharanui stream water, where time to death was on average 38% faster for individuals with this genotype at these three sites.There were no statistically significant differences in time to death of genotypes when examined across all pre-exposure sites. However, individuals with genetic variant PGM 44 which had been pre-exposed to Otara, Oakley and Tawaharanui stream water died on average faster than other genotypes, although this measure was quite variable and not statistically significant. This suggests that genetic tolerance may be "over-ridden" in extreme stressor events.

Adaptation to stress implies not only that selection for tolerant genotypes has resulted, but also that effects on traits associated with life history processes such as survival, growth and reproduction have occurred (Shirley and Sibly, 1999; Bubliy and Loeschcke, 2005). As energy available for such processes is limited, resource allocation to one trait generally means that other traits are affected as a consequence (van Noordwijk and de Jong, 1986; Reznick *et al.*, 2000). Physiological defence mechanisms such as increased metal excretion or the production of detoxifying enzymes of tolerant genotypes (Maroni *et al.*, 1987; Van Straalen *et al.*, 1987) can therefore be expected to reduce resource availability for growth and reproduction (Calow, 1991). For example, Shirley and Sibly, (1999) found that a fruit-fly (*Drosophila melanogaster*) exposed to a cadmium-contaminated medium over multiple generations displayed a significant decrease in female weight and fecundity compared with those reared in unpolluted environment. In addition, Hendrikx *et al.* (2008) observed differential responses in life history traits of the wolf spider *Pirata piraticus* (egg size, growth rates) depending on metal pre-exposure. They found that heritability for growth rate in cadmium pre-exposed populations was very low, even when subsequently exposed to reference conditions. In

contrast, heritability for growth rate was large for the reference population under reference conditions, but much lower under metal-stressed conditions. In this study we have provided evidence for selection associated with reduced fecundity and increased mortality and, to a less extent, activity levels (as indicated by reburial rates) on adult clams. Ideally, we would measure such traits, as well as others, in juvenile populations, to support the hypothesis of heritability of traits.

While our work has focused at the individual organism level, it is evident that responses observed at this level will have direct and indirect effects at higher levels of organisation (population, community, ecosystem), the extent of which will be influenced by the functional role that the species plays within its environment, as well as the nature and extent of the contamination event (Medina *et al.*, 2007). Medina *et al.* (2007) have suggested that, while there is some evidence to indicate that current approaches to the protection of aquatic ecosystems may account for genetic variation in tolerance, further investigation is required on the longer-term effects on higher levels of organization. Approaches utilizing multi-generational studies on model organisms such as has been conducted in this study, as well as those which examine variability of functional response traits (e.g., Medina *et al.*, 2007) offer much promise in this regard.

Collectively these results provide strong evidence for selection effects of stormwater resulting in heritable shifts in genetic composition. The characterisation of the extent of genetic variability associated with contaminant exposure provides the first step towards incorporating micro-evolutionary changes into an ecological risk assessment framework. This study has demonstrated a sensitive and relatively simple method for assessing the chronic effects and genetic resposes to stormwater contamination on aquatic communities. We have identified significant chronic effects of stormwater contamination on aquatic organisms using an experimental design which could be applied over only a few months. Development of a monitoring programme based around this method would be a desirable next step and would complement existing ecologically-based stormwater monitoring programmes. Such a method could then be used to derive data suitable for inclusion in an ERA framework.

6 Acknowledgements

We thank Ian Hogg and Arvina Ram of the Pacific Biosystematics Laboratory (University of Waikato) for use of their genetic facilities, as well as fruitful discussions on genetic analyses. We thank Peter Smith (NIWA) for valuable advice on allozyme electrophoresis. We are particularly grateful to Martin Neale and Kylie Park (ARC) for their assistance in providing data, obtaining site access and assisting with fieldwork in this project. We thank Gateway Construction for providing assistance in accessing sites on Numukea, Otanerua and West Hoe Streams and to ARC staff at Tauwharanui Regional Park. Much of the specialized field and laboratory equipment was constructed by Mark Smith and his contribution is gratefully acknowledged. Mike Martin provided helpful hints on the use of ToxCalc and Chris Hickey provided valuable advice on equipment design, as well as providing useful comments on the draft report. We are grateful for of the funding support provided by the ARC for this project.

7 References

ANZECC (2000). *Australian and New Zealand guidelines for fresh and marine water quality. Volume 1. The guidelines*. Australian and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand.

Auckland Regional Council (ARC) (2008). *State of the Environment Monitoring: River and Stream Water Quality Data Report 2006.* ARC Technical Publication 342. Auckland. 83 pages.

Barata, C., Baird, D.J., Medina, M., Albalat, A., and Soares, A.M.V.M. (2002a). Determining the ecotoxicological mode of action of toxic chemicals in meiobenthic marine organisms: stage-specific short tests with *Tisbe battagliai*. *Marine Ecology Progress Series* **230**; 183–194.

Belfiore, N. and Anderson, S. (2001). Effects of contaminants on genetic patterns in aquatic organisms: a review. *Mutation Research* **489;** 97–122.

Bubliy, O.A. and Loeschcke, V. (2005). Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. *Journal of Evolutionary Biology* **18**; 789–803.

Calow, P. (1991). Physiological costs of combating chemical toxicants: ecological implications. *Comparative Biochemistry and Physiology* **1/2**; 3–6.

Eckblad, J.W., Petersen, N.L., Ostlie, K. and Tempte, A. (1977). The morphometry, benthos and sedimentation rates of a floodplain lake in Pool 9 of the upper Mississippi River. *American Midland Naturalist* **97**; 433–443.

Forbes, V.E. (1998). *Genetics and Ecotoxicology*. Taylor and Francis, Philadelphia, PA, USA.

Gale, S.A., Smith, S.V., Lim, R.P., Jeffree, R.A. and Petocz, P. (2003). Insights into the mechanisms of copper tolerance of a population of black-banded rainbowfish (*Melanotaenia nigrans*) (Richardson) exposed to mine leachate, using $^{64/67}$Cu. *Aquatic Toxicolology* **62**; 135–153.

Gillespie, R. and Guttman, S. (1989). Effects of contaminants on the frequencies of allozymes in populations of the Central Stoneroller. *Environmental Toxicology and Chemistry* **8**; 309–317.

Gillespie, R. and Guttman, S. (1993). Correlations between water quality and frequencies of allozyme genotypes in Spotfin Shiner (*Notropis spilopteris*) populations. *Environmental Pollution* **81**; 147–150.

Gow, J.L., Noble, L.R., Rollinson, D. and Jones, C.S. (In press). Polymorphic microsatellites in the African freshwater snail, *Bulinus forskalii* (Gastropoda, Pulmonata). *Molecular Ecology* **1;** 237–240.

Haldane, J.B.S. (1954). An exact test for randomness of mating. *Journal of Genetics* **52**; 631–635.

Hartl, D. (1981). *A primer of population genetics*. Sinauer Assoc. Ltd, Sunderland, Massachusetts.

Heard, W.H. (1977). Reproduction of fingernail clams (Sphaeriidae: *Sphaerium* and *Musculium*). *Malacologia* **16**; 421–455.

Hebert, P.D.N. and Beaton, M.J. (1999). *Methodologies for Allozyme Analysis Using Cellulose Acetate Electrophoresis*. A Practical Handbook, Helena Laboratories, Beaumont, Texas.

Hendrickx, F., Maelfait, J.P. and Lens, L. (2008). Effect of metal stress on life history divergence and qualitative genetic architecture in a wolf spider. *Journal of Evolutionary Biology* **21**; 183–193.

Hickey, C. and Martin, M. (1995). Relative sensitivity of five benthic invertebrate species to reference toxicants and resin-acid contaminated sediments. *Environmental Toxicology and Chemistry* **14**; 1401–1409.

Hill, A.J., Teraoka, H., Heideman, W. and Peterson, R.E. (2005). Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. *Toxicological Sciences* **86;** 6–19.

Hornbach, D.J. and Wissing, T.E. (1984). Energy budget for a stream population of the freshwater clam, *Sphaerium striatinum* Lamarck (Bivalvia: Pisidiidae). *Canadian Journal of Zoology* **62**; 2410–2417.

Inglis, L., Boothroyd, I.K.G. and Brierley, G. (2008). Effectiveness of the river environment classification in the Auckland Region. *New Zealand Geographer* **64**; 181–193.

Klerks, P.L. and Levinton, J.S. (1989). Rapid evolution of metal resistance in a benthic oligochaete inhabiting a metal-polluted site. *Biological Bulletin* **176**; 135–141.

Kuiper, J.G.J. (1966). Critical revision of the New Zealand Sphaeriid clams in the Dominion Museum, Wellington. *Records of the Dominion Museum* **5**; 147–162.

Lavie, B. and Nevo, E. (1982). Heavy metal selection of phosphoglucose isomerase allozymes in marine gastropods. *Marine Biology* **71**; 17–22.

Lopez, G.R. and Holopainen, I.J. (1987). Intersitial suspension feeding by *Pisidium* sp (Pisidiidae: Bivalvia): a new guild in the lenthic benthos? *American Malacological Bulletin* **5**; 21–30.

Mackie, G.L. (1979). Growth dynamics in natural populations of Sphaeriidae clams (*Sphaerium*, *Musculium*, *Pisidium*). *Canadian Journal of Zoology* **57**; 441–456.

Maroni, G., Wise, J., Young, E. and Otto, E. (1987). Metallothionein gene duplications and metal tolerance in natural populations of *Drosophila melanogaster*. *Genetics* **117**; 739–744.

Martins, N., Lopes, I., Harper, R., Ross, P. and Ribeiro, R. (2007). Differential resistance to copper and mine drainage in *Daphnia longispina*: relationship with allozyme genotypes. *Environmental Toxicology and Chemistry* **26**; 1904–1909.

Maxted, J.R. (2005). *Summary of the Ecological Health of Auckland Streams based on State of the Environment Monitoring 2000-2004*. Technical Publication 304. 15 p.

Maxted, J.R., Evans, B.F. and Scarsbrook, M.R. (2003). Development of standard protocols for macroinvertebrate assessment of soft-bottomed streams in New Zealand. *New Zealand Journal of Marine and Freshwater Research* **37**; 793–807.

MacDonald, D.D., Carr, R.S., Calder, F.D., Long, E.R. and Ingersoll, C.G. (1996). Development and evaluation of sediment quality guidelines for Florida coastal waters. *Ecotoxicology* **5**; 253–278.

Medina, M.H., Correa, J.A. and Barata, C. (2007). Micro-evolution due to pollution: Possible consequences for ecosystem responses to toxic stress. *Chemosphere* **67**; 2105–2114

Mulvey, M., Newman, M.C., Chazal, A., Keklak, M.M., Heagler, M.G. and Hales, L.S. (1995). Genetic and demographic responses of mosquitofish (*Gambusia holbrooki* Girard 1859) populations stressed by mercury. *Environmental Toxicolology and Chemistry* **14**; 1411–1418.

Nevo, E. (2001). Evolution of genome-phenome diversity under environmental stress. *Proceedings of the National Academy of Sciences (USA)* **98**; 6233–6240.

Parker, E.D., Forbes, V.E., Nielsen, S.L., Ritter, C., Barata, C., Baird, D.J., Admiraal, W., Levin, L., Loeschchke, V., Lyytikäinen-Saarenmaa, P., Hogh-Jensen, H. and Calow, P. (1999). Stress in ecological systems. *Oikos* **86**; 179–184.

Phillips, N.R. and Hickey, C.W. (in press). Genotype-dependent recovery from acute exposure to heavy metal contamination in the freshwater clam *Sphaerium novaezelandiae*. *Aquatic Toxicology* **99**, 507-513.

Phillips, N. and Croker, G. (2008). *Intergenerational responses to low level contamination in urban streams – Year 1 report*. Prepared by NIWA for Auckland Regional Council. Auckland Regional Council Working Report No. 136.

Reznick, D., Nunney, L. and Tessier, A. (2000). Big houses, big cars, superfleas and the cost of reproduction. *Trends in Ecology and Evolution* **15**; 421–425.

Richardson, B.J., Baverstock, P.R. and Adams, M. (1986). *Allozyme electrophoresis: a handbook for animal systematics and population studies*. Sydney, Academic Press.

Riddoch, B.J. (1993). The adaptive significance of electrophoretic mobility in phosphoglucose isomerase (Pgi). *Biological Journal of the Linnean Society* **50**; *1*–17.

Roa, S.A. (1997). *Studies on the Pea Mussel Sphaerium novaezelandiae (Deshayes, 1853) in Lake Rotorua*. MSc. Thesis, University of Waikato, New Zealand.

Roark, S. and Brown, K. (1996). Effects of metal contamination from mine tailings on allozyme distributions of populations of Great Plains Fishes. *Environmental Toxicology and Chemistry* **15**; 921–927.

Rousset, F. and Raymond, M. (1995). Testing Heterozygote Excess and Deficiency. *Genetics* **140**; 1413–1419.

Shirley, M.D. and Sibly, R.M. (1999). Genetic basis of a between-environment trade-off involving resistance to cadmium in *Drosophila melanogaster*. *Evolution* **53**; 826–836.

Sloss, B.L., Romano, M.A. and Anderson, R.V. (1998). Pollution-tolerant allele in fingernail clams (*Musculium transversum*). *Archives of Environmental Contamination and Toxicology* **35**; 302–308.

Smith, S.L., MacDonald, D.D., Keenleyside, K.A., Ingersoll, C.G. and Field, L.J. (1996). A preliminary evaluation of sediment quality assessment values for freshwater ecosystems. *Journal of Great Lakes Research* **22**; 624-638.

Statistica Software (2008). *Statistica v7.1*. Statsoft Inc, Tulsa, OK.

Suter, G.W. (1993). *Ecological Risk Assessment*. Lewis Publishers, London, UK.

Tidepool Scientific Software (1994). *ToxCalc® Users Guide. Vol. 5*. McKinleyville, CA.

US EPA (1991). *Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms*. U.S. Environmental Protection Agency, Cincinatti, Ohio. EPA/600/4-90/027.

US EPA (1993). *Methods for the measuring of acute toxicity of effluents and receiving waters to freshwater and marine organisms*. U.S. Environmental Protection Agency, Cincinatti, Ohio. EPA/600/4-90/027/F.

US EPA (2006). *National Recommended Water Quality Criteria: 2006*. In Washington D.C.: United States Environmental Protection Agency, Office of Water.

van Noordwijk, A.J. and de Jong, G. (1986). Acquisition and allocation of resources: their influence on variation in life history tactics. *American Naturalist* **128**; 137–142.

Van Straalen, N.M., Burghouts, T.B.A., Doornhof, M.J., Groot, G.M., Jansens, M.P.M., Joose, E.N.G., Van Meerendonk, J.H., Theeuwen, J.P.J.J., Verhoef, H.A. and Zoomer, H.R. (1987). Efficiency on lead and cadmium excretion in populations of *Orchesella cincta* (Collembola) from various contaminatrd forest soils. *Journal of Applied Ecology* **24**; 953–968.

Walsh, C.J. (2000). Urban impacts on the ecology of receiving waters: a framework for assessment, conservation and restoration. *Hydrobiologia* **431**; 107–114.

Walsh, C.J., Roy, A.H., Feminella, J.W., Cottingham, P.D., Groffman, P.M. and Morgan, II R.P. (2005). The urban stream syndrome: current knowledge and the search for a cure. *Journal of the North American Benthological Society* **24**; 706–723.