

**Assessing the effect of a laundry detergent ingredient (LAS) on
organisms of a rural South African river**

This thesis is submitted in fulfilment of the
requirements for the degree of

Doctor of Philosophy
of
Rhodes University

By

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Submitted
December 2011

Abstract

Powdered laundry detergents are consumed in high volumes worldwide. Post use, they are directed toward water resources via wastewater treatment works or, as is the situation in many rural areas of South Africa, they enter the environment directly as a result of laundry washing activity undertaken alongside surface waters. Within wastewater treatment works, the main ingredient in powdered laundry detergents, the narcotic toxin linear alkylbenzene sulfonate (LAS), is mostly removed, rendering the waste stream a negligible risk to the aquatic biota of receiving waters. In contrast, the biological and ecological impacts of direct LAS input to the aquatic environment, as a consequence of near-stream laundry washing, are yet to be fully realised. Consequently, this thesis posed two research questions: 1) 'What are the LAS concentrations in a small rural South African river?' and 2) 'Is the in-stream biological community negatively affected at these concentrations?' The chosen study area, the community of Balfour in the Eastern Cape Province, is like many rural areas of South Africa where inadequate provision of piped water to homesteads necessitates laundry washing alongside the nearby Balfour River.

The first research question was addressed in two ways: by predicting LAS concentrations in Balfour River water by assessing detergent consumption and laundry washing behaviour of residents living alongside the river; and measuring actual in-stream LAS concentrations on different days of the week and during different seasons. Results indicated that LAS concentrations were highly variable temporally and spatially. High peak concentrations of LAS occurred infrequently and were limited to the immediate vicinity of near-stream laundry washing activity with the highest measured concentration being $342 \mu\text{g.L}^{-1}$ and the average $21 \mu\text{g.L}^{-1}$ over the sampling period.

The second research question was addressed by integrating the chemical evidence, determined from the first research question, with the biological evidence of stress responses measured in macroinvertebrates collected downstream of near-stream laundry washing activity on the Balfour River. Predicted and measured LAS exposure concentrations from the Balfour River were compared to a water quality guideline for LAS ($304 \mu\text{g.L}^{-1}$), specifically derived in this thesis. Biological stress responses were measured at different levels of organisation: two sub-cellular responses (lipid

peroxidation and cholinesterase activity); three measures of macroinvertebrate tolerance to water quality impairment; five measures of community composition; three measures of community richness; and a surrogate measure of ecosystem function (functional feeding groups). Weight-of-evidence methodology was utilised to assess, integrate and interpret the chemical and biological evidence, and at its conclusion, determined no effect on the in-stream biological community of the Balfour River downstream of laundry washing activity.

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List of abbreviations

AChE	acetylcholinesterase
AEV	acute effect value
AF	assessment factor
ANOSIM	analysis-of-similarity
ANOSIM2	two-way crossed analysis-of-similarity
ANOVA	analysis of variance
cAMP	adenosine 3',5'-cyclic monophosphate
CAT	catalase
CCC	chronic criterion concentration
CEV	chronic effect value
ChE	cholinesterase
CTAB	cteryl trimethyl ammonium bromide
EC	electrical conductivity
DBS	dodecyl benzyl sulphonate
DCBS	sodium dodecylbenzenesulfonate
EPT	Ephemeroptera, Plecoptera and Trichoptera
FAV	final acute value
FCV	final chronic value
FFGs	functional feeding groups
FPV	final plant value
GC-MS	gas chromatography – mass spectrometry
GSM	gravel/sand/mud
GST	glutathione-S-transferase
HPLC	high-performance liquid chromatography
IHAS	integrated habitat assessment system
LAS	linear alkylbenzene sulfonate
LC-MS	liquid chromatography – mass spectrometry
LOEC	lowest observed effect concentration
LPx	lipid peroxidation
MBAS	methyl blue active substances
MDA	malondialdehyde
NMDS	non-metric multi-dimensional scaling

NOEC	no observed effect concentration
PC	protective concentration
PNEC	predicted no-effect concentration
POD	peroxidase
ROS	reactive oxygen species
SASS5	South African Scoring System Version 5
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SDBS	sodium dodecylbenzene sulfonate
SMCVs	species mean chronic values
SOD	superoxide dismutase
SSD	species sensitivity distribution
WWTW	wastewater treatment works

Acknowledgements

The following organisations are gratefully acknowledged for their financial support in the course of this study: the Safety and Environmental Assurance Centre (SEAC), Unilever Colworth, United Kingdom; the Water Research Commission of South Africa; Unilever South African; the Claude Leon Foundation; and the Rhodes University Joint Research Committee.

I would especially like to thank Dr Nikite Muller and Prof Tally Palmer for their supervision during the course of this study.

I would like to acknowledge the considerable assistance that was provided to me by staff and students (both past and present) of the Institute for Water Research. Without their support on fieldtrips, the collection of chemical and biological data would not have been possible to the extent reported in this thesis. Within the laboratory I received particular assistance from Tarqyn Human. I benefitted from discussions with Dr Neil Griffin on statistics, and with Naomi Richardson and Alexandra Holland on ecotoxicology.

The analyses of LAS concentrations in water and sediment samples from the Balfour River were undertaken by Chris Sparham and Sean O'Connor of SEAC, Unilever Colworth. Thanks to them and also to Dr Stuart Marshall (SEAC) and Dr Mick Whelan (Cranfield University) for assistance in interpreting these data.

Thanks to Profs Brett Pletschke and Greg Blatch and their students, from the Department of Biochemistry, Microbiology and Biotechnology at Rhodes University, for providing laboratory space and advice on the measurement of biochemical stress responses.

I'm also grateful to Dr Sebastian Jooste of Resource Quality Services, Department of Water Affairs, for his willingness to discuss aspects of water quality management in South Africa.

Lastly, I would like to thank my family, especially Kim for her unerring support.

Declaration

In accordance with the regulations for the award of the degree of Doctor of Philosophy, I declare that the work presented in this thesis is my own original research. This thesis has not been submitted to any other university.

Several manuscripts arising from the research work have been published:

Gordon AK, Blatch GL, Daniel S and Muller WJ (2008) Stress protein responses in South African freshwater invertebrates exposed to detergent surfactant linear alkylbenzene sulfonate (LAS). *Water, Air & Soil Pollution* 193: 123-130.

Gordon AK, Muller WJ, Gysman N, Marshall SJ, Sparham CJ, O'Connor SM and Whelan MJ (2009). Effect of laundry activities on in-stream concentrations of linear alkylbenzene sulfonate in a small rural South African river. *Science of the Total Environment* 407: 4465-4471.

Gordon AK, Slaughter AR and Muller WJ (2010) *The application of chronic (sublethal) toxicity endpoints to the development of resource quality objectives*. Report No. 1484/1/09. Water Research Commission, Pretoria, South Africa.

Chapter 1 Introduction

Preface: Information and excerpts of text from this chapter have been published in *Science of the Total Environment* (Gordon et al. 2009) and in a scientific report to the Water Research Commission of South Africa (Gordon et al. 2010).

1.1 Introduction

Large quantities of laundry detergent ingredients enter the environment continuously, either through waste water streams (Mungray and Kumar 2009) or as a direct result of detergent product use in, or near to, surface water bodies (Whelan et al. 2007). Synthetic surfactants are the active ingredient in powdered laundry detergents with linear alkylbenzene sulfonate (LAS) being the primary anionic surfactant used in laundry detergents worldwide (HERA 2007). At the end of the 20th century, annual worldwide use of surfactants was estimated between 4 and 5 million tons per year (Van de Plassche et al. 1999). Linear alkylbenzene sulfonate (LAS) has the highest production of all surfactants (Karsa 1998) with approximate estimates of annual production during the late 1990s ranging from 1.77 million tons (Britton 1998) to 2.4 million tons (Karsa 1998). More recent estimates of global annual LAS production range from 2.9 million tons in 2003 (Hauthal 2004) to 2.7 million tons in 2005 (Adami 2009) to 3.5 million tons in 2006 (Vora et al. 2009).

Risk assessments conducted for LAS in developed countries — where wastewater is usually treated before entering receiving water bodies — indicate acceptable risks, i.e. the predicted no-effect concentration (PNEC) is not likely to be exceeded by the environmental concentrations of LAS (Van de Plassche et al. 1999; Sanderson et al. 2006; HERA 2007). Low LAS concentrations are attributed to high removal rates of LAS through precipitation, adsorption and biodegradation (Fox et al. 2000) within the sewer system (10-68% in the case of a Dutch investigation; Matthijs et al. 1999) and within wastewater treatment works, where levels of between 70 and 99% have been recorded (McAvoy et al. 1993; Feijtel et al. 1995; Waters and Feijtel, 1995; Holt et al. 1998; McAvoy et al. 1998; Matthijs et al. 1999; Holt et al. 2003). LAS biodegradation in

wastewater treatment works by means of anaerobic processes are not as effective as those using aerobic processes (Mungray and Kumar 2009).

In urban areas of the developing world, however, poor provision of wastewater treatment means that municipal and household wastewater is often discharged directly to receiving waters. For example, Ding et al. (1999) estimate that less than 5% of all municipal waste in Taiwan is treated by wastewater treatment works, and in Brazil the sewage effluents from only 10% of the urban population are subjected to proper treatment (Eichhorn et al. 2002). This means that surfactant concentrations are often high in urban water courses close to emission points, although there is evidence that concentrations decrease rapidly beyond the urban fringe (e.g. McAvoy et al. 2003; Whelan et al. 2007).

Very little is known about the fate of laundry detergents in rural areas of developing countries. In rural areas of South Africa, some homesteads and villages do not yet have piped water, which necessitates the utilisation of nearby water resources. Consequently, laundry washing is undertaken alongside rural rivers, resulting in the direct introduction of detergent ingredients, such as LAS, into rivers. In-stream LAS concentrations have not however been determined in South African water courses, although the impact of the phosphorus component of laundry detergent on eutrophication has been assessed (Pillay and Buckley 2001; Quayle et al. 2010). Pillay and Buckley (2001), in an investigation (based on data collected in 1988) into the specific impact of detergent phosphorus on eutrophication in the Mgeni catchment in KwaZulu-Natal, determined that the rural per capita consumption rate of powdered laundry detergent consumption was $1.63 \text{ kg.annum}^{-1}$. Using 1991 census data from the rural areas in the Mgeni catchment and data from a 1993 Lever Brothers survey — which indicated that 16% of the rural population washed their laundry directly in a watercourse — Pillay and Buckley (2001) estimated that the direct input rate of laundry detergent into natural waters of this catchment was $159.2 \text{ tons.annum}^{-1}$. The specific contribution of LAS was not determined. More recently, Soviti (2002) investigated laundry-washing practices of residents of the small rural village of Balfour, situated alongside the Balfour River in the Eastern Cape, South Africa. Utilising questionnaires, Soviti (2002) determined that the 150-household village consumed approximately 479 kg powdered laundry detergent per month, with 48% of households washing laundry alongside the river and that for 96% of the respondents, this took place on a Saturday. Consequently, it appeared that on any

given Saturday approximately 55.2 kg of laundry detergent was entering the watercourse of this small river. Again, the specific contribution of LAS was not determined. The potential effects of these periodic, large-volume detergent inputs into the Balfour River on in-stream biota were not determined. Despite the potential for in-stream biodegradation of the LAS component of the detergent to less harmful shorter-chained homologues by microorganisms (Eichhorn et al. 2002) and the potential reduction in LAS concentration through dilution and adsorption to sediments and organic matter, the intensity of the detergent load input suggests a potential for high LAS concentrations downstream of laundry washing sites.

Consequently, two research questions are posed in this thesis:

- 1) what are the LAS concentrations in a small rural South African river;
- and
- 2) is the in-stream biological community negatively affected at these concentrations?

There are a number of reasons why the Balfour River is a suitable location for investigating the above-mentioned research questions:

- Soviti (2002) confirmed that near-stream laundry washing took place along the river;
- no other land use activity has a significant contribution towards LAS levels in the river;
- no other point-source water-quality impacts (that would potentially confound biological monitoring) are discernable within the catchment;
- there are clearly-identifiable washing sites as well as suitable upstream and downstream biological and water chemistry monitoring sites;
- the presence of a hydrological gauging weir for measuring river discharge is an important advantage.

Various ecological or environmental assessment methods are available for answering research question such as the ones posed in this thesis. Cormier and Suter grouped these into four types (2008), which they integrated into a framework that shows how they are conceptually linked (Figure 1.1). Within the framework, the four different types of environmental assessment are organised as a 2x2 matrix. There are two columns, with

the left consisting of assessments that detect problems and the right column consisting of assessments that attempt to solve problems. The two rows represent the activities undertaken as part of the assessment. The top row consists of assessments that try to explain what has happened (environmental epidemiology) while the bottom row represents assessments that try to estimate what could happen, given various management actions (environmental management) (Cormier and Suter 2008).

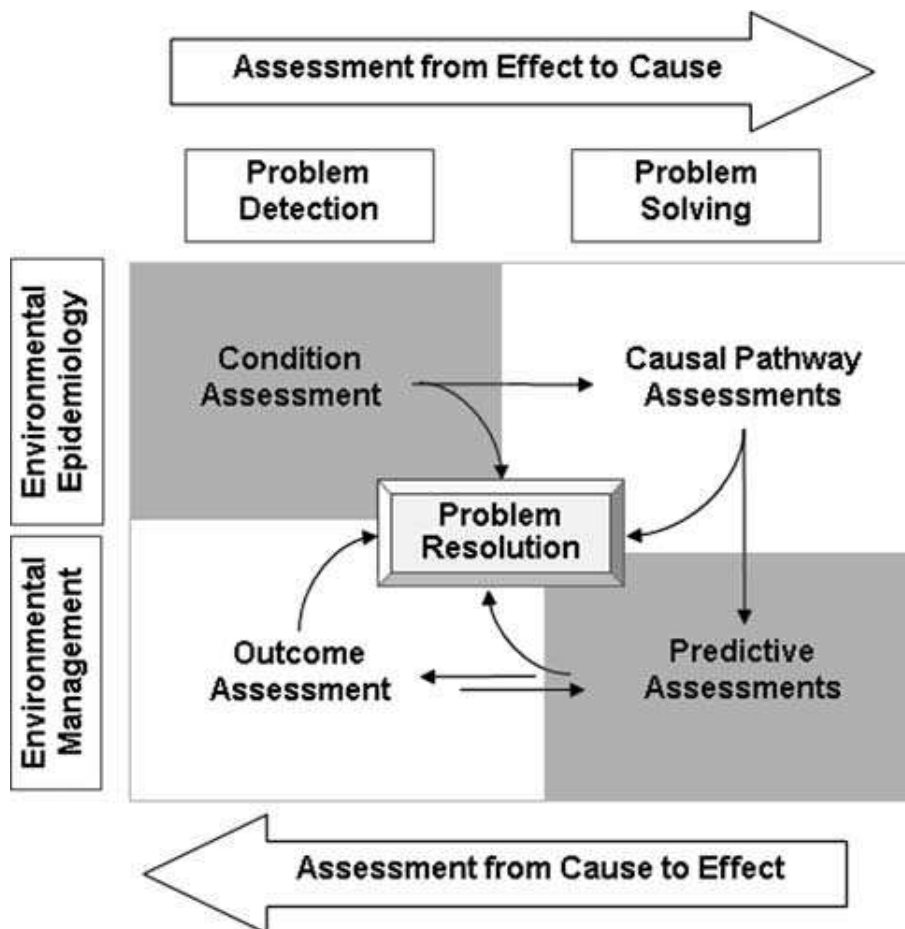


Figure 1.1 Framework integrating the four major types of environmental assessments (figure taken from Cormier and Suter 2008).

The four types of assessment are outlined below.

- Condition assessment

This type of assessment attempts to answer the question, 'Is there a problem?' (Cormier and Suter 2008). The assessment utilises biological and chemical monitoring data to determine if there has been a change from some desired state. Biological monitoring at the site of interest can include measures from resident individual organisms, population measures of specific taxa, and/or a measure of the biological community as a whole. Chemical monitoring data can be compared to specific water quality guidelines (WQGs) to identify potential problems. If no impairment is determined the process is ended, i.e. the problem is resolved (centre block in Figure 1.1). However, if an impaired condition is identified, then a causal assessment is initiated.

- Causal assessment

This type of assessment attempts to answer the question, 'What caused the problem?' (Cormier and Suter 2008). The aim is to identify the probable causes of environmental impairment and the source of the cause. Sometimes the cause is obvious (e.g. the WQG of a particular chemical is exceeded or there is an oil spill) and, if the source is also obvious, the causal assessment may be unnecessary (Cormier and Suter 2008). In some cases, identification of the cause of impairment can result in the resolution of the environmental problem, thus ending the process (Cormier and Suter 2008). If action is required to address the environmental problem, then a predictive assessment is undertaken.

- Predictive assessments

These include risk assessment and management assessment. They attempt to answer the question, 'What will be the consequences of addressing (or not addressing) the problem?' (Cormier and Suter 2008). Risk assessments attempt to determine the positive and negative consequences associated with alternative management actions relating to an environmental problem or potential impact, including no action (Cormier and Suter 2008). A management assessment attempts to predict the acceptability of the various management actions with regard to social, economic and legal values (Cormier and Suter 2008).

- Outcome assessment

An outcome assessment asks, 'Did the solution work?' (Cormier and Suter 2008). The aim is to evaluate the success of a management action in achieving environmental goals. If the desired outcome has not been achieved the predictive assessments can be re-engaged to determine a potentially more beneficial management action.

An assessment is initiated by an environmental problem or as a result of a prior assessment. It ends with a resolution of the problem, initiation of a further assessment, or implementation of a management action (Cormier and Suter 2008). In reality, however, assessments are rarely undertaken in such an integrated manner, with the lack of integration of national monitoring programmes cited as a major reason (Cormier and Suter 2008). In terms of addressing the research questions posed in this thesis, it appears logical to begin with a condition assessment. The initiator of the assessment is the perceived high potential LAS input to rural South African rivers, such as the Balfour River, as a consequence of near-stream laundry washing.

All the assessment methods have a common three-step process: plan, analyse, and synthesize (Cormier and Suter 2008). Although the terminology used in different assessments from different countries may change slightly, the essential actions required by each stage or procedure remain the same. The common three-step approach was used in the condition assessment undertaken in this thesis:

- The first 'plan assessment' step defines the scope of the assessment in terms of the research questions and describes how the assessment will be undertaken.
- The second 'analyse' step characterises the biological and chemical data relevant to the research questions.
- The third step synthesizes these data to facilitate a decision regarding the research questions. Suter and Cormier (2011) view the weight of evidence (WoE) approach as an ideal method for analysing and synthesizing information in all types of assessment.

Details of the common three-step process, applied here, and associated actions are presented in Table 1.1, as discussed below.

The thesis begins with the formulation of the research questions, identification of the stressor and its associated chemical and biological traits, identification of the ecosystem component that is to be protected, and identification of the biological stress responses to be monitored in order to determine if the particular ecosystem component is at risk (these actions are presented in Chapter 1). Materials and methods are described in Chapter 2.

Within the analysis step of the assessment process, the activities associated with response characterisation and exposure characterisation can be examined in any order, or concurrently. The sequence and methods of presentation of the results are outlined below:

- Toxicity test responses of biota to LAS (response characterisation) are presented first (Chapter 3) and integrated to produce a WQG for LAS for South African freshwaters (Chapter 4).
- The exposure characterisation stage is presented next. The concentrations of LAS in the Balfour River, a small river in the rural Eastern Cape Province of South Africa, are investigated (Chapter 5). This activity addresses the first research question: 'What are the LAS concentrations in a small rural South African river'?
- To ensure that the LAS chemical testing program of Balfour River water, undertaken for the exposure characterisation, adequately reflects the actual exposure of aquatic biota (continually resident in the river), comprehensive biological monitoring of this river is undertaken (Chapter 6).
- Lastly, in Chapter 7, the response and exposure characterisation data presented in previous chapters are combined to address the second research question: 'Is the in-stream biological community negatively affected at these concentrations'?

Table 1.1 Outline of the approach utilised to answer research questions. The procedure of a typical environmental assessment has been adopted. These procedural steps are associated with research actions and aligned to a particular thesis chapter.

Environmental assessment procedure or research stage	Actions	Chapter
Plan assessment	Formulate the research questions Identify the stressor and it's associated chemical and biological traits Identify what is to be protected Identify which biological responses to the stressor are to be monitored	Chapter 1
Characterise biological response data	Determine responses of biota to stressor exposure using toxicity test methodology.	Chapter 3
	This information leads to the development of a water quality guideline for LAS for South African fresh waters	Chapter 4
Characterise exposure data	Determine the environmental concentration of the stressor and how it comes into contact with in-stream biota. Addresses the first research question	Chapter 5
Characterise biological response data	Determine responses of biota to stressor exposure from field biological monitoring	Chapter 6
Synthesize information	Combine biological response data and exposure data utilising a weight of evidence approach in order to address second research question	Chapter 7

Chapters 1 and 3-6 will thus lead the reader through the various stages of a condition assessment, culminating in the synthesis of the evidence in Chapter 7, utilising a weigh of evidence (WoE) approach. The result of the WoE process will indicate whether an impairment or effect is observed. If there is no impairment then the identified problem (potential hazard to aquatic biota from the direct input of LAS to a rural river) would be resolved by the condition assessment. If an impairment is observed, then a causal assessment must be initiated to confirm that LAS input is indeed the causal factor.

1.2 Problem formulation

Although the reasons for undertaking this study, as well as the consequent research questions, have been described earlier in this chapter, they are briefly repeated here in order to follow the condition assessment process. In essence, the practice of near-stream laundry washing in rural areas of South Africa appears to be directing large quantities of detergent ingredients into natural waters in these areas (Soviti 2002). The

biological and ecological impacts of this direct input of detergent into rural rivers are yet to be fully realised. Consequently, two research questions are posed: 1) 'What are the LAS concentrations in a small rural South African river'? and 2) 'Is the in-stream biological community negatively affected at these concentrations?'

1.3 Identification of stressor and associate chemical and biological traits

Determination of ecological impacts of laundry detergents has usually focused on the active ingredient, with LAS being the most widely-used active ingredient worldwide (HERA 2007). The input of phosphates into the environment via laundry detergents is likely to become less of a concern with the increased use of phosphate-free builders in powder detergents. Linear alkylbenzene sulfonate is a surfactant (surface active ingredient), a group of chemicals designed to have cleaning and solubilising properties. It has both hydrophobic and hydrophilic properties, being characterised by a hydrophilic polar head group attached to a nonpolar hydrophobic hydrocarbon tail (Mungray and Kumar 2009). Commercial LAS is not a single compound but comprises a mixture of homologues of various alkyl chain lengths (generally 10 to 14 carbons) with a phenyl group that can be attached at various positions along the carbon chain (Figure 1.2) (Belanger et al. 2002). Commercial LAS compounds used in Europe and the United States usually have alkyl chains with average lengths of 11.6 or 12.3 carbons, respectively (Belanger et al. 2002). Toxicity levels increase with increasing alkyl chain length (Blok and Balk 1993) and as the phenyl position moves closer to the external carbons (Fendinger et al. 1994). Longer alkyl chains and external isomers are, however, more sorptive, which decreases their bioavailability and rates of biodegradation (Belanger et al. 2002).

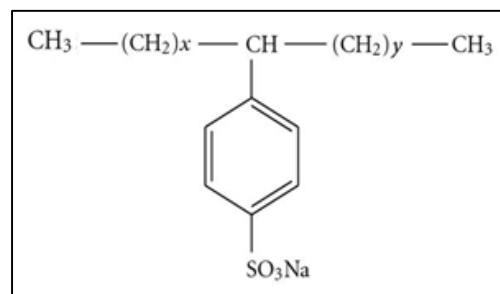


Figure 1.2 General structure of linear alkylbenzene sulfonate. Symbols x and y refer to the number of alkyl groups (CH_2) or 'carbons' either side of the phenyl group (SO_3^-Na^+) (after Eichhorn et al. 2002).

Linear alkylbenzene sulfonate has a polar narcotic mechanism of toxicity (Hodges et al. 2006, Roberts 1991). Polar narcosis acts by nonspecific disruption of the functioning of cell membranes; characteristic symptoms of toxicity are progressive lethargy, followed by unconsciousness and death (Veith and Broderius 1990). Anionic surfactants like LAS bind to various bioactive macromolecules, such as starch (Merta and Stenius 1999) and proteins (Nielsen et al. 2000), by inserting into various phospholipid membranes, thus causing malfunction (Cserhati et al. 2002), or by accumulating in lysosomes and inhibiting lysosomal enzymes (Bragadin et al. 1996). These interferences can alter the folding of polypeptide chains, change the surface charge of molecules, and modify the activity of various enzymes or other cell constituents (Cserhati et al. 2002). Sirisattha et al. (2004) investigated the toxicity of LAS using *Saccharomyces cerevisiae* microarray analysis and determined that LAS caused damage to membranes, alterations to carbon metabolism, induced the oxidative stress response, and also induced the pleiotropic drug-resistance network.

Routledge and Sumpter (1996) tested a range of surfactant classes (anionic, nonionic, cationic and amphoteric) for estrogenic activity, using a recombinant yeast screen. Besides nonionic surfactant alkylphenol polyethoxyate, whose degraded metabolites were found to be weakly estrogenic, none of the other surfactants were found to be specifically estrogenic. In a study testing LAS and two of its metabolites (sulfophenyl carboxylate C5 and C11) for estrogenicity, using the yeast estrogen receptor assay and the vitellogenin assay with cultured trout hepatocytes, no estrogenic effects were observed (Navas et al. 1999). Nevertheless, Harris et al. (2009) reported a transient induction of vitellogenin after exposure to $362 \mu\text{g}\cdot\text{L}^{-1}$ LAS, in fathead minnow (*Pimephales promelas*). By the end of the 14-day trial, however, the levels of vitellogenin had returned to pre-exposure levels.

Bioconcentration factors for LAS are low (Comber et al. 2003; Versteeg and Rawlings 2003). However, LAS has been shown to indirectly enhance bioaccumulation of some dietary xenobiotics (Tan et al. 2010). Catfish (*Ictalurus punctatus*) that were exposed to 0, 100 and $300 \mu\text{g}\cdot\text{L}^{-1}$ LAS for 12 days and concurrently fed a diet containing carcinogen benzo(a)pyrene (BaP) and pharmaceutical ivermectin (IVM) showed enhanced bioaccumulation of these chemicals at higher LAS exposure levels (Tan et al. 2010).

1.4 What is to be protected?

Environmental assessments can be specific: for example, they could involve determining the effect of an effluent from a new industry on a rare fish species occurring downstream. In such a case, the population viability of the fish species is what needs protection. In terms of the second research question posed in this thesis, the likely fate of all aquatic biota comprising the in-stream biological community is of interest. This is a more complicated problem as it is not possible, in this thesis or otherwise, to determine the effects of stressor exposure on each individual organism within an ecosystem (Connell et al. 1999). Therefore, in the response characterisation stage of this thesis, specific organisms are identified, and their responses used as surrogate measures to represent the responses of the biological community within the ecosystem in question. Two particular approaches were used to characterise responses: toxicity test data, and biological monitoring of in-stream organisms. Toxicity data existed for organisms from a range of taxonomic groupings (outlined in Chapter 3) and these data are used to derive a WQG for LAS, representing a concentration that would have no undesirable effect on the aquatic ecosystem as a whole. The biological monitoring component of the response characterisation stage, however, relied on measuring only macroinvertebrate responses in the Balfour River, up and downstream of near-stream laundry washing. Fish were not considered as no fish have been found in the upper reaches of the Balfour River (Lerotholi 2005). Since macroinvertebrates tend to be more sensitive to LAS exposure than taxa from the periphyton community (Belanger et al. 2002), the latter organisms were not monitored.

The use of aquatic invertebrates to detect anthropogenic impacts in lotic systems has been extensively discussed in Rosenberg and Resh (1993) and more recently in Bonada et al. (2006). Ollis et al. (2006) also provide a review, focusing on application in a South African context. According to these authors, the advantages of using aquatic macroinvertebrates in biomonitoring relate to aspects of their ecology, the way in which they lend themselves to efficient sampling methods, and the fact that data analysis methods associated with these taxa are well established. As a whole, invertebrates form an important component of most lotic food webs. Most aquatic macroinvertebrates are relatively sessile and can thus reflect impacts occurring at local spatial scales. The

relatively long lifecycle of some species allows temporal stress impacts to be integrated and detected. Macroinvertebrates are also widespread and occupy most habitats. Their high abundance and species diversity enhances the ecological relevance of bioassessment approaches based on this group as well as the statistical analyses thereof. The sensitivity of macroinvertebrate taxa to stressors is highly variable and for many common macroinvertebrate taxa these sensitivities have been established, leading to the development of numerous biotic indices. Sampling of macroinvertebrate taxa can be easy and relatively inexpensive, and identification to family and genera levels is generally well researched. Consequently, these taxa are considered reliable response-indicators for the biological monitoring component of this study.



1.5 Biological stress responses

1.5.1 Stress responses at different levels of biological organisation

In both forms of bioassessment (in-stream biomonitoring and toxicity testing) biological stress responses can be measured at various levels of biological organisation, from sub-organism to organism, population, community and eventually at ecosystem level (Munkittrick and McCarthy 1995; Adams et al. 2000). Some examples of stress responses at each level are summarised in Table 1.2. The stress response times to contaminant exposure at the cellular and sub-cellular are generally short. Thus, they can be used as early warning indicators of toxic stress (Clements 2000). Such responses also reflect contamination effects at a small spatial scale (Landis and Yu 2004). In addition, they can provide information on the underlying causal mechanisms between stressor and biological effect i.e. they have great specificity (Adams and Tremblay 2003). A serious limitation encountered when studying stress responses from these lower levels of biological organisation is that their impact on the survival of the individual organism, and thus population viability of that taxon, can rarely be determined. The effects of such stress on community composition and overall ecological significance are thus generally unknown (Clements 2000). Sibley et al. (2000) have, however, shown that the responses of acetylcholinesterase to organophosphorus insecticides is a reliable predictor of effect at an organism level in selected invertebrates and fish, and could be a useful predictor of population-level effects in the selected invertebrates. In contrast, stress responses measured at the higher levels of organisation are considered more

ecologically relevant (Clements 2000). It is however difficult to determine a mechanistic basis for these responses, particularly since they can take a long time to manifest.

Table 1.2 Types of biological and ecological stress responses used in bioassessment (adapted from Clements 2000; Downes et al. 2002; Suter et al. 2005).

 Mechanistic understanding and specificity	Level of organisation	Stress responses measured	 Ecological relevance and spatiotemporal scale
	Organ, cellular and sub-cellular	Sub-lethal responses measuring molecular and biochemical changes within cells and histopathological damage to cells and organs	
	Organism	Sub-lethal responses measuring deformities and fluctuating asymmetry, reproductive success, growth and behaviour. Lethal responses such as mortality rates	
	Population	Abundance, sex ratios, age structure and production (recruitment)	
	Community	Taxon richness, diversity indices, ratios of one or more taxa to other taxa, biotic indices using scores related to stressor intolerance, functional group ratios e.g. feeding groups, matrices of (dis)similarity indices	
	Ecosystem	Detrital processing, oxygen production, nutrient fluxes, respiration or photosynthetic rates	

One of the most useful aspects of employing aquatic invertebrates in bioassessment is that their responses can easily be measured at all levels of biological organisation (Hodkinson and Jackson 2005). At the lower end of biological organisation, histopathological, cellular and sub-cellular responses to contaminant exposure can be measured within individual organisms. The use of these stress responses in bioassessments is controversial with extensive arguments having been made for (Handy and Depledge 1999; Adams et al. 2001; Handy et al. 2003) and against (Clark et al. 1999; Tannenbaum 2005; Forbes et al. 2006) their use. As some biological stress responses are part of the defence, repair or detoxification machinery of cells targeted by the contaminant (Bierkens 2000), there are concerns about the difficulty of determining whether the response is an indicator of impairment or just part of a homeostatic response, indicating that the organism is successfully dealing with the exposure (Forbes et al. 2006). The research worker must therefore ask two questions:

- Whether the measured biological stress response is an indication of toxic effect or just a normal response to exposure to the stressor;
- and

- if it is a measure of effect, is this effect ecologically significant, i.e. will it have an effect at population and community levels of biological organisation?

Some authors (e.g. Forbes et al. 2006) term sub-organism responses (histopathological, cellular and sub-cellular) as 'biomarkers', while others include organism-level responses (physiological and behavioural) in the term (Peakall 1994; Handy et al. 2003). To avoid confusion, the term 'biomarker' will not be used in this thesis and biological stress response will be identified according to the level of organisation (Table 1.2).

Organism-level stress responses are easily applied to macroinvertebrate bioassessments. In a critical analysis of available bioassessment tools according to 12 criteria that should ideally be fulfilled (based on criteria addressing the rationale, implementation and performance of the tool), Bonada et al. (2006) concluded that invertebrate organism-level stress responses meet 10 of the 12 criteria (Appendix 1: Table A). No current bioassessment tool meets all 12 criteria and sub-organism level stress responses meet only 6 of the 12 criteria.

Population-level stress responses are measured in multiple individuals of a single species (Hodkinson and Jackson 2005). However, as Suter et al. (2005) point out, there is confusion amongst scientists and water resource managers regarding this concept of organism-level responses being measured in multiple individuals for population level assessments. For example, organism-level stress responses — such as survival, growth and/or fecundity — measured in multiple individuals in a geographic location (i.e. a population) can be used to derive population-level stress responses such as abundance, production and/or population growth or decline/extinction (Suter et al. 2005). A further example is presented by Bonada et al. (2006) who assessed the use of fluctuating asymmetry in multiple individuals as a population level stress response and determined that it fulfilled 7 of the 12 criteria of the ideal bioassessment tool (Appendix 1: Table A).

Considerable effort has been applied to the development and application of community-level stress responses for aquatic macroinvertebrates exposed to assorted stressors. Community structure, measured as taxonomic richness and diversity, has been the approach most readily investigated and applied (Peru and Doledec 2010). Taxonomic

richness can include all taxa at a site, or focus on pollution-sensitive groups such as the Ephemeroptera, Plecoptera and Trichoptera (EPT), or pollution-tolerant groups such as Chironimidae (Peru and Doledec 2010). Biotic indices can also be based on the relative tolerances of macroinvertebrates to water quality degradation (an extensive review is provided by Ollis et al. 2006). Such an index is the South African Scoring System (SASS), now in Version 5 (Dickens and Graham 2002), the most popular macroinvertebrate rapid bioassessment tool in use in South Africa. Chapman (2002) suggests that protecting ecosystem structure should, in general, protect ecosystem function. It has however been suggested that utilising biotic measures of functional diversity could provide an indirect measurement of ecosystem functioning (Peru and Doledec 2010). A functional guild is a group of taxonomically unrelated species that perform similar functions or use similar resources within the community (Simberloff and Dayan 1991). One of the most widely used measures of functional diversity is the assessment of macroinvertebrate functional feeding groups (FFG), defined by the size and type of food particle consumed and the mode of feeding, which reflects the trophic functioning of stream ecosystems (Downes et al. 2002). The use of FFGs to assess water quality has been undertaken using South African macroinvertebrate biota (Palmer et al. 1996).

In addition to being assessed individually, structural and functional community stress responses (i.e. measures of abundance, trophic composition, species richness and species composition) are commonly integrated to form an index by which to measure community and ecosystem impairment (Hughes et al. 2010). Suter (1993; 2001) has criticised these types of indices for a number of reasons, particularly because individual metrics are arbitrarily combined to form a single unit/score, which obscures individual community stress responses and limits diagnostic power. An alternative to multimetric indices is the use of predictive multivariate approaches, which rely on multivariate statistical analyses, to compare patterns of invertebrate communities at reference sites with those at test sites. Such a comparison enables the determination of whether impairment has occurred. Examples of such statistical packages include RIVPACS, AUSRIVAS and BEAST (Wright et al. 2000). Bonada et al. (2006) rate these measures of community impairment fairly highly — particularly FFGs, multimetric and multivariate approaches — since they, respectively, meet 8, 9 and 10 of the 12 criteria that distinguish an ideal bioassessment tool (Appendix 1: Table A).

1.5.2 Desirable attributes in biological stress responses used for bioassessment

A desirable attribute of biological stress responses chosen to detect contaminant effects would be that they can be causally linked, or strongly associated with, the toxic mode of action of the contaminant, i.e. that the biological response to contaminant exposure is well understood and predictable (Downes et al. 2002). Ideally, measurement of the biological stress response should also be cost-effective and the methodology reliable. Furthermore, the stress response should be capable of signalling 'important' changes, defined by Downes et al. (2002) as being ecologically or/and socially significant. The assessment should therefore be ecologically relevant, i.e. it should have the potential to provide reasonably accurate predictions, or to signal changes to the aquatic ecosystem. Indeed, legislative goals in South Africa (National Water Act No. 36 of 1998) and internationally (Suter et al. 2005) generally focus on the protection of the aquatic ecosystem as a whole rather than focusing on a particular species or population. There is, however, no current bioassessment approach that possesses all the attributes desired (Bonada et al. 2006). Furthermore, contaminant effects are often manifested at all levels of biological organisation (Clements 2000). To obtain the necessary information for a reasonably accurate assessment of contaminant impacts, stress responses across all levels of biological organisation are often investigated (Clements 2000; Hodgkinson and Jackson 2005).

1.5.3 Biological stress responses utilised in this study

In characterising the biological stress response data for LAS determined from toxicity tests (Chapter 3), responses measured at all levels of organisation were identified. Data that are determined to be of suitable quality (assessed according to the methodology described in Chapter 3) were considered for use in the derivation of a WQG for LAS (Chapter 4). The use of sub-organism stress responses in water resources management has been questioned due, in part, to concerns regarding the variability of their responses in toxicity tests (Clark et al. 1999; Tannenbaum 2005; Forbes et al. 2006). The quality of sub-organism data available in the literature, and the challenge of generating these data, is therefore subjected to critical assessment in Chapter 3.

In the literature, the statistical measure describing biological responses to LAS is in most cases the no observed effect concentrations (NOECs). However, this measure of expressing toxicity has been severely criticised by Landis and Chapman (2011) and Jager (2012), with these authors recommending the use of the exposure concentration with x% effect approach instead. A more in depth discussion of this issue as pertaining to the derivation of water quality guidelines is presented in section 4.2.2, page 110.

Biological stress responses measured in macroinvertebrates, collected from sites upstream and downstream of laundry-washing sites on the Balfour River, represent various levels of organisation (Chapter 6). The stress responses utilised from low levels of biological organisation include the commonly-applied sub-cellular response assay of cholinesterase (ChE) inhibition and cellular response assay lipid peroxidation (LPx). At higher levels of biological organisation, measures of macroinvertebrate tolerance to water quality impairment, community composition and richness, together with macroinvertebrate functional feeding groups (FFG) (a measure of ecosystem function) were utilised (Table 6.1). The implications of response data generated from the different levels of organisation are discussed (Chapter 6).

1.6 Synthesis of biological and chemical data

A large quantity of disparate response data were collected as part of this study. Characterisation of toxicity test response data (Chapter 3) allowed for the derivation of a WQG for LAS (Chapter 4), which was compared to the environmental concentrations of LAS determined in the Balfour River (Chapter 5). Data at various levels of organisation were also collected by monitoring various macroinvertebrate stress responses at sites upstream and downstream of the laundry-washing site in the Balfour River (Chapter 6). The merits of these different biological stress response data are discussed below, and a case made for their integration, in order to provide an assessment of the likely effect of LAS to the in-stream biological community.

Chemical measures are important components of aquatic impact assessments (Adams 2005). For example, measuring the environmental concentrations of a chemical can give an indication of the spatial extent of contamination and also indicate the temporal nature of contaminant exposure to in-stream organisms (Galloway et al. 2004). The measured

environmental concentration, however, only reflects the level of the particular chemical at the time of sampling. Since it is not possible to measure continuous exposure concentration, an accurate assessment of the effects of a particular chemical on in-stream organisms cannot be determined.

In-stream biological monitoring of organisms has the advantage of providing a time-integrated indication of responses to temporally-variable concentrations of the potential contaminant (Karr and Chu 2000). In addition, biological response data provide an integrated indication of the effects of the many potential physical, biological and chemical stressors that coexist in aquatic ecosystems and that might interact with the potential contaminant (Adams 2005). Assessments of biological responses can therefore be used to identify environmental impacts that may be missed or underestimated if tests relied exclusively on chemical data (Adams 2005; Chapman 2007).

Since different biological stress response measures provide independent assessments of the effect of the stressor, the case for integrating disparate response data as evidence in an environmental assessment is compelling (Suter and Cormier 2011) and is now well established. The sediment quality triad (SQT) approach (Long and Chapman 1985) has traditionally been used to integrate biological responses at community (benthic community surveys) and organism levels (usually mortality and reproductive measures) with chemistry data (compared to sediment quality guidelines) in order to identify sediment-associated environmental impacts. The SQT approach formed the basis of the WoE approach (Chapman et al. 2002), which is a framework for analysis of multiple types of biological response and chemical data (termed 'Lines of Evidence'). To address the second research question of this thesis (are LAS concentrations in rural South African rivers negatively affecting the in-stream biological community?), the various exposure-response data (biological evidence) collected in the course of this research are integrated with chemical measures (chemical evidence) and assessed within a WoE framework in Chapter 7.

1.7 Application of this research to South African water resource management

The National Water Act of South Africa (No. 36 of 1998) requires the sustainable management of water resources, taking account of both water resource protection and

use. This approach is effected though the National Water Resource Strategy (NWRS) (DWAF 2004a). The NWRS introduces the concept of water resource classification, implemented through a process termed the 'Ecological Reserve', allowing management that can favour different levels of use and protection (although never at a level that would destroy the ecological functioning of the associated ecosystem). The NWRS acknowledges that successful implementation requires a great deal of information on the country's water resources. Consequently, in addition to the existing monitoring programmes at the time of its establishment, a number of new monitoring programmes were proposed by the NWRS (DWAF 2004b) for the purpose of collecting this information. The interpretation of the water chemistry data — collected during various research and monitoring programmes mentioned below — require guidelines against which the data are compared in order to assess the potential hazard to the biological components of the resource. Such data have been collected via the National (surface) Chemical Monitoring Programme and National Toxicity Monitoring Programme, or as part of assessment geared towards meeting in-stream flow objectives (incorporating resource quality objectives and source directed controls) in terms of the Ecological Reserve. Although a number of guidelines for this purpose have been developed in South Africa, no such guideline has been developed for assessing the effects LAS. The development of a WQG for LAS, as outlined in this thesis, contributes towards improved water resources management of South Africa.

The National Aquatic Ecosystem Health Monitoring Programme details accepted biological response methodology for assessing ecosystem stress (DWAF 2008a). The responses of fish, macroinvertebrates, riparian vegetation and diatoms are all determined at a community level. The investigation of biological responses to LAS at cellular and sub-cellular levels in this thesis provides further input into the ongoing debate regarding the application of these data to routine monitoring programmes.

While there is likely to be some regional and cultural variation, it is likely that near-stream laundry washing and the associated effects of LAS in rural areas of the world are not country specific. Consequently, this study is likely to have considerable relevance outside South Africa too.

Chapter 2 Materials and methods

2.1 Introduction

This chapter outlines the general methods employed and materials used in generating data required to address the two research questions posed in this study. In particular, the methods and materials, described here, pertain to the toxicity testing undertaken and measurement of biological responses outlined in Chapter 3. The materials and methods employed in the determination of both in-stream LAS concentrations (Chapter 5) and biological monitoring (Chapter 6) of the Balfour River are presented within those particular chapters. This approach was adopted because details of the materials and methods used in those chapters are referred to frequently in the results and discussion sections. Thus, including the relevant methods in Chapters 5 and 6 facilitates easy reading.

2.2 Study site

The Balfour River is a small tributary of the Kat River, situated within the Eastern Cape Province of South Africa (Figure 2.1). The river is approximately 9 km long with a catchment area of 120 km² (Hosking & Du Preez, 2002) and a mean annual discharge of 329 L.s⁻¹. The river morphology is typical of low order streams: the depth is generally shallow (ranging between about 0.2 m and 1 m in riffles and pools, respectively) and the bed material is coarse (boulders, gravel and sand). Land use within the catchment has not been comprehensively assessed but broadly consists of limited livestock and subsistence agriculture. Land use in the headwaters of the catchment is dominated by a mixture of indigenous forest and commercial forestry. The Balfour village is the only settlement situated alongside the Balfour River and is composed predominantly of residential buildings. There are also a few homesteads scattered throughout the catchment.

A number of features of the Balfour River mean that this site is highly appropriate for the present study:

- No other land uses or activities to which LAS input can be attributed;

- no other point source water quality impacts that could confound biological monitoring results;
- clearly-identified laundry washing sites;
- a suitable reference site upstream of washing sites and potential downstream monitoring sites; and,
- the presence of a Department of Water Affairs hydrological gauging weir, which records river discharge.

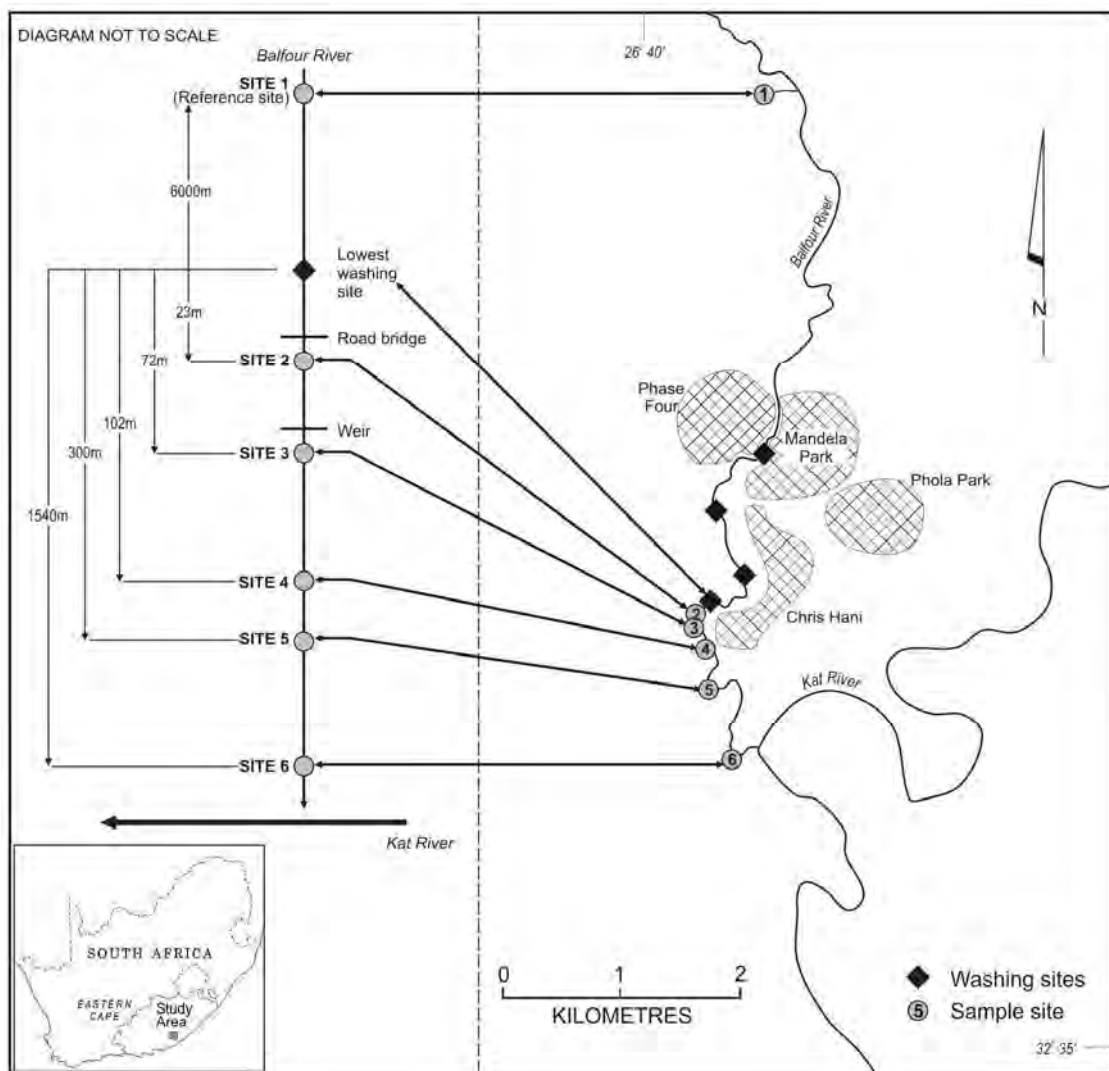


Figure 2.1 Schematic map showing monitoring sites in the Balfour River and the four sub-villages that comprise the Balfour Village.

2.3 Measurement of water quality parameters

Selected water quality parameters for toxicity tests were measured in the laboratory using desktop meters: dissolved oxygen (DO) (mg.L^{-1}) was measured using a Cyberscan® 1500, pH was measured using a Cyberscan® 5000, electrical conductivity (EC) (mS.m^{-1}) was measured using the Cyberscan® 200. Certain parameters were measured in the field, such as water temperature ($^{\circ}\text{C}$), measured using a glass mercury thermometer. Selected in-stream water quality parameters were also measured in the field using hand-held meters: DO (Cyberscan® 300), pH (Cyberscan® 300) and EC (Cyberscan® 200).

The concentration of LAS was determined either by using liquid chromatography/mass spectrometry (LC/MS) or by the Spectroquant® Surfactants (anionic) Cell Test. The LC/MS method was utilised during the comprehensive LAS analysis of Balfour River water, outlined in Chapter 5, and was undertaken at the Safety and Environmental Assurance Centre (SEAC), Unilever Colworth, United Kingdom. The LC/MS method is described in detail in Chapter 5. The costs of shipping water samples to the United Kingdom or contracting a local laboratory to undertake LC/MS analyses precluded the use of this analytical method for LAS determination during laboratory toxicity tests (Chapter 3) and biological monitoring of the Balfour River (Chapter 6). Instead, the Spectroquant® Surfactants (anionic) Cell Test was utilised. In this test, anionic surfactants of the sulfonate and sulfate type react with the cationic dye methylene blue to form an ion pair, which is then extracted with chloroform. The blue colour of the chloroform phase is then determined photometrically using a Merck Spectroquant® NOVA 60. The test does not specifically measure LAS but instead measures any sulfonate or sulfate-type surfactant present in the sample. Consequently, the output of the test is the concentration of methylene blue active substances (MBAS). Since LAS was the only surfactant utilised in the laboratory toxicity tests described in Chapter 3, it is assumed that the MBAS measurement provides a reliable indication of LAS concentration. MBAS concentrations measured in the Balfour River during the collection of organisms for cholinesterase activity and lipid peroxidation assays may, however, be a less reliable estimation of LAS in-stream concentrations, since various substances may have interfered with the test (Pedraza et al. 2007). These substances include sulfonate surfactants other than LAS, naturally occurring cations, anions, and organic

compounds such as phenols and humic acids, which react with the methylene blue dye causing MBAS readings that exceed levels that would be expected from the presence of LAS alone (Pedraza et al. 2007). The Spectroquant® Surfactants (anionic) Cell Test has a detection limit of 50–2000 µg.L⁻¹ MBAS.

The LAS utilised in the toxicity exposure tests with indigenous organisms was obtained from Unilever South Africa (Pty) Ltd. It was provided in powder form and determined by the Safety and Environmental Assurance Centre (SEAC), Unilever Colworth, to be 80% active with a homologue composition of C9 (0.4%), C10 (12.2%), C11 (32.9%), C12 (40.6%) C13 (13.5%) and C14 (0.4%) and an average chain length of C11.6.

2.4 Short-term (96 hr) toxicity tests

Short-term (96 hr) lethal toxicity trials were undertaken exposing four indigenous aquatic invertebrates to LAS. These were laboratory-reared *Dugesia* sp. flatworms (Turbellaria), laboratory-reared shrimp *Caridina nilotica* (Atyidae), wild-caught mayfly *Adenophlebia auriculata* (Leptophlebiidae) and wild-caught freshwater limpet *Burnupia stenochorias* (Ancylidae). A trial with the standard test organism, *Daphnia pulex* (Cladocera) was also undertaken.

2.4.1 General methods

Linear alkylbenzene sulfonate is readily biodegraded and absorbs to a range of surfaces. Consequently, a flow-through system (for all species except *D. pulex*) was utilised to ensure that in-stream concentrations of LAS remained constant (OECD 2000). A LAS stock solution (prepared by mixing a predetermined amount of LAS with deionised water) was added at a specific volume to 1 m lengths of white PVC roof guttering. A specific volume of diluent (tap water dechlorinated by activated carbon and passed through a metal filter) was added to the input end of the vessel which was positioned higher than the output end, allowing LAS and diluent to mix as they flowed down the length of the guttering to form a 50 cm long pool at the output.

The accuracy of the dosing system was tested by collecting the mixed LAS and diluent medium from the test vessel at the beginning and end of the trial. (This was only

undertaken for vessels from exposure treatments that lay within the detection range of the Surfactant Cell test i.e. 50–2000 $\mu\text{g.L}^{-1}$). Analysis for LAS was determined using the Spectroquant® Surfactants (anionic) Cell Test. If the geometric mean values of the measured concentrations were within 80–120% of nominal concentrations, then the nominal concentration value was utilised for data analysis and presentation (OECD 2000). As the dosing system for all channels was the same, and flow rates were monitored to ensure proper functioning, the accuracy in vessels associated with treatments with LAS levels greater than 2000 $\mu\text{g.L}^{-1}$ was considered to be reliably represented by vessels with LAS concentrations within the MBAS detection limits. In addition, water temperature, pH, EC and DO were measured daily from each channel.

Mortality counts were undertaken at 12 hourly intervals. Mortality was indicated by immobility: *C. nilotica*, *A. auriculata*, *Dugesia* sp. and *B. stenochorias* organisms not responding to slight prodding with a paintbrush were considered dead and removed from experimental vessels. Calculation of LC50 values was based on the US EPA Probit Analysis Program Version 1.5. If the data did not meet the assumptions of the Probit model, the Trimmed Spearman-Kärber Method was utilised.

2.4.2 *Caridina nilotica* toxicity test methods

LAS input for all treatments was set at a rate of 0.5 mL.min^{-1} , using an Ismatec IP-24 peristaltic pump. Actual LAS input was measured daily and the average over the 96 hr of the study determined as $0.49 \pm 0.01 \text{ mL.min}^{-1}$ (\pm standard deviation). Diluent input volume for all treatments was calculated as 29.8 mL.min^{-1} prior to commencement of the study. Dosing stock estimations were based on these calculations. During the study, daily diluent input volumes were measured from all treatments and averaged at $28.8 \pm 0.25 \text{ mL.min}^{-1}$ (\pm standard deviation). The volume of each vessel was 1.6 L, resulting in complete test-medium exchange 26.4 times every 24 hr. The experiment was conducted in a controlled environment room and maintained at 17–21°C on a 12 hr light : 12 hr dark photoperiod.

The trial consisted of seven treatments, including a control with three replicates each. Nominal test concentrations were 0, 1000, 2000, 4000, 8000, 16000, 32000 $\mu\text{g.L}^{-1}$ LAS. The specimens used in this trial were laboratory reared at UCEWQ and aged between

20–30 days, with a total length of 5–10 mm. Individuals were introduced into the test vessels 24 hr before the start of the trial. At the beginning of the trial *C. nilotica* were re-counted and dead specimens removed.

2.4.3 *Adenophlebia auriculata* toxicity test methods

The LAS exposure trials for *A. auriculata* and *C. nilotica* were undertaken together in the same experimental system. Individuals from each species were placed in the same vessel for exposure to the selected LAS concentrations. These species do not naturally interact, and it was assumed that the large volume of medium within the vessel and its high turnover rate would reduce the chance of the organisms experiencing stress. Thus, the methods used for the *C. nilotica* trial apply to this section too, with the following exception: *A. auriculata* were collected from the Fish River at the Carisle Bridge site (33°04'59.8"S ; 26°13'31.8"E) and transported in cooler boxes with aeration to the laboratory. The test organisms (of 5–8mm total length) were introduced into vessels 40 hr before start of the trial. Organisms with wingbuds were not included in the trial, eliminating the occurrence of exposed organisms emerging during the exposure period and confounding results. At the beginning of the trial, organisms were re-counted and any dead specimens removed.

2.4.4 *Dugesia* sp. toxicity test methods

LAS input for all treatments was set at a rate of 1.0 mL.min⁻¹ using an Ismatec IP-24 peristaltic pump. Diluent input volume for all treatments was calculated to be 29.6 mL.min⁻¹ before the study commenced and dosing stocks were calculated accordingly. The volume of each vessel was 1.6 L, resulting in complete test-medium exchange 26.6 times every 24 hr. The experiment was conducted in a controlled environment room maintained at 20–22°C, on a 12 hr light : 12 hr dark photoperiod.

The trial consisted of seven treatments, including a control, with three replicates each. Nominal test concentrations were 0, 500, 1000, 1500, 2000, 4000, 8000 µg.L⁻¹ LAS. Five organisms were introduced into each of the test vessels 24 hr before the trial began, resulting in 15 organisms per treatment. Individuals ranged in total length between 5–10 mm.

2.4.5 *Burnupia stenochorias* toxicity test methods

The trials for *Dugesia* sp. and *B. stenochorias* were undertaken together in the same experimental system. These species do not naturally interact; furthermore, it was assumed that the large volume of medium within the vessel and its high turnover rate would reduce the chance of the organisms experiencing stress. Thus, methods used for the *Dugesia* sp. trial apply to this section too, with the following exception: 35 *B. stenochorias* (3–5 mm diameter) were placed in each of the control replicate vessels and 20 were placed in each of the vessels exposed to LAS. *Burnupia stenochorias* were collected from the Kubusi Stream (32°32'42.8"S ; 26°20'32.1"E), Eastern Cape province.

2.4.6 *Daphnia pulex* toxicity test methods

The static exposure experimental procedure was undertaken according to Slabbert et al. (1998). Treatments were conducted in quadruplicate with five neonates (< 24 hr after hatch) per replicate. Organisms were obtained from laboratory cultures at the Institute for Water Research, Rhodes University, and exposed to LAS at nominal concentrations of 1000, 2000, 4000, 6000, 10000, 15000 and 20000 µg.L⁻¹. Stock concentrations were individually prepared in 1 L glass beakers and decanted into the four experimental vessels per concentration level at the beginning of the trial. Immobility (as a surrogate measure of mortality) was monitored as the biological stress response at 1, 2, 4, 8, 24 and 48 hr during the trial.

2.5 Methods for conducting the long-term sub-lethality *Caridina nilotica* experiment

2.5.1 Experimental design

Because of the tendency for LAS to readily biodegrade and adsorb onto a range of surfaces (OECD 2000), a flow-through system was utilised to ensure concentrations of LAS remained as constant as possible within the exposure vessels. A LAS stock solution of predetermined concentration was prepared for each treatment by mixing a specific quantity of LAS with deionised water in 150 L glass aquaria. The relevant stock solutions

were pumped at a specific volume to each individual 35 L glass aquarium, using an Ismatec IP-24 peristaltic pump (a fresh stock solution of LAS for each treatment was prepared weekly). Diluent was prepared from tap water, which was dechlorinated by filtration through a large activated carbon filter. A specific volume of diluent was added to the exposure vessel using a peristaltic pump. An air stone aided the mixing of the LAS and diluent to the desired exposure concentration. The LAS concentration for each treatment is reported as actual (i.e. measured) concentration and averaged 0 (below detection limit of $50 \mu\text{g.L}^{-1}$), 65, 331 and $1225 \mu\text{g.L}^{-1}$. The experiment was run for 70 days. The dosing system was cleaned every 14 days with methanol to remove biofilm (excessive biofilm breaks down LAS, reducing the concentration delivered to the test vessels). The experiment was conducted in a controlled environment room maintained on a 12 hr light : 12 hr dark photoperiod. There were 12 glass aquaria consisting of four exposure treatments of three replicates, each containing 25 shrimp per tank. Shrimp were fed daily to satiation with TetraMin® fish flakes; remaining uneaten flakes were siphoned from the tank.

Water temperature, pH, EC and DO were measured weekly. The accuracy of the dosing system was tested weekly by collecting the mixed LAS and diluent medium from the exposure vessels and applying the Spectroquant® Surfactants (anionic) Cell Test.

2.5.2 Determination of growth response

Growth rates of control shrimp and LAS-exposed shrimp were determined using a length-based, age structured model, based on the MULTIFAN-CL approach (Fournier et al. 1998) developed for fisheries stock assessment. This was adapted for length data generated from this toxicity trial by Prof. Tony Booth of the Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown.

Approximately every two weeks for the duration of the experiment, carapace length — defined by Hart (1980) as the length from the anterior margin of the carapace behind the insertion of the eyestalk to the most posterior margin of the carapace — of experimental shrimp in each tank was measured under a microscope. At the end of the experiment, the data that had been collected from each tank t , \mathbf{N}^t , comprised a $L \times A$ matrix, denoting A columns (each time period sampled) of length frequencies at each length L .

Due to differential mortality, the column sums were not equal. It was assumed that the progression of the length frequencies over time from each tank would contain sufficient information to allow for the statistical estimation of the tank/treatment-specific growth parameters.

Animals were assumed to grow according to the von Bertalanffy growth model such that

$$\bar{l}_a = \hat{L}_\infty (1 - e^{-\hat{K}a})$$

where \bar{l}_a is the mean length of an animal of age a , \hat{L}_∞ is the theoretical asymptotic length, and \hat{K} is the rate at which \hat{L}_∞ is approached. The 'hat' notation denotes a statistically estimated parameter or function of parameters.

The von Bertalanffy growth model was re-parameterised to improve numerical stability to the following form:

$$\bar{l}_a = \hat{l}_{\min} + (\hat{l}_{\max} - \hat{l}_{\min}) \left(\frac{1 - e^{-\hat{K}a}}{1 - e^{-\hat{K} \max}} \right) \text{ such that } \hat{l}_\infty = \frac{\hat{l}_{\max} - \hat{l}_{\min} \hat{K}^{\max}}{1 - \hat{K}^{\max}}.$$

If \hat{K} is extremely small (say $\hat{K} < 0.001$), then growth is effectively linear between \hat{l}_{\min} and \hat{l}_{\max} .

The proportion of animals in a tank t that are of length l and age a were assumed to be normally distributed. The column-normalised proportions were:

$$\Lambda_{l,a}^t = N(\bar{l}_a^t, (\sigma_a^t)^2) \cong \int_{L_l - \Delta L}^{L_l + \Delta L} \frac{1}{\sigma_a^t \sqrt{2\pi}} \exp\left[-\frac{(L - \bar{l}_a^t)^2}{2(\sigma_a^t)^2}\right] dL$$

where σ_a^t is the standard deviation of an animal from tank t and age a , L_l is the mid-point of length class l , and ΔL is half the mid-point of length class l .

The tank-specific standard deviation of an animal of age a , σ_a^t , was calculated from an age-independent $\hat{\lambda}_1$ and age-dependent parameter $\hat{\lambda}_2$, such that

$$\hat{\sigma}_a = \hat{\lambda}_1 \exp\left(\hat{\lambda}_2 \left[-1 + 2 \left(\frac{1 - e^{-\hat{k}a}}{1 - e^{-\hat{k}_{\max}}}\right)\right]\right). \text{ If } \hat{\lambda}_2 = 0 \text{ then } \hat{\sigma}_a = \hat{\lambda}_1.$$

Growth parameters were estimated from the observed \mathbf{N}^t and model-predicted $\hat{\mathbf{N}}^t$ length frequencies.

The predicted length frequency vector was calculated as

$$N_{l,a}^t = \Lambda_{l,a} n_a^t$$

where n_a^t is the number of animals in each age class a in tank t .

The replicated design allocated three tanks to one of four experimental treatments. The growth model parameter vector for each experimental treatment T $\boldsymbol{\theta}^T = \{L_1^T, L_2^T, K^T, \lambda_1^T, \lambda_2^T\}$ was estimated by minimising a negated normal likelihood function of the form:

$$-\ln L = \frac{\sum_t \sum_l \sum_a n_a^t}{2} \ln \left(\frac{\sum_t \sum_l \sum_a [N_{l,a}^t - \hat{N}_{l,a}^t]^2}{\sum_t \sum_l \sum_a n_a^t} \right)$$

This is equivalent to minimising a sum of squares, i.e., $SS = \sum_t \sum_l \sum_a [N_{l,a}^t - \hat{N}_{l,a}^t]^2$ as

$\sum_t \sum_l \sum_a n_a^t$ is a constant. The log-likelihood function was however preferred as it

provided the statistical basis for further tests of inference.

2.5.3 Determination of reproductive stress response measures

The number of gravid females within each tank was recorded when fortnightly length measurements were taken. The total number of juveniles produced in each tank was recorded when the last length measurement of the experiment was undertaken. Results from exposure treatments were compared to those of the control.

2.5.4 Statistical analyses

Environmental parameters were analysed for normality using the Shapiro-Wilk W test. Normally distributed data were statistically analysed using the Student's t test, while non-normally distributed data were analysed using the Kruskal-Wallis ANOVA and Median test. Growth rates of shrimp from each treatment were compared using the likelihood ratio test (LRT) which compares the log-likelihoods of the saturated and reduced models. In this context, the saturated model refers to the model in which the parameters from all different experimental treatments were estimated, while the reduced model has fewer parameters and is based on the null hypothesis that certain treatments have the same growth parameters. Reproductive stress responses measured in LAS-exposed treatments were compared to the control treatment using the Student's t test. A significant difference was reported if $p \leq 0.05$. Point estimates of toxicity were determined using the US EPA Probit Analysis Program Version 1.5.

2.6 Methods for determining the induction of stress protein as a biological stress response

2.6.1 Organisms and stress exposure tests

Organisms surviving the 96 hr toxicity tests outlined in Section 2.5 were utilised for stress protein analysis.

2.6.2 Stress protein sample preparation

Organisms were collected and immediately frozen in 1.5 mL Eppendorf microcentrifuge tubes with liquid nitrogen. Due to the small sizes of the organisms and varying numbers of survivors in the different treatments and their replicates after the 96 hours, individuals from within the same replicate were often grouped into one tube to ensure sufficient biological material for analysis. *Burnupia stenochorias* were stored whole, at -70°C , until protein analysis took place. *Caridina nilotica* samples were further processed by macerating tissue with 50 μL PBS (phosphate buffered saline) 10 mM (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4) using Eppendorf Teflon micro-pestles

(Merck, South Africa), resuspended with 50 μ L PBS, refrozen with liquid nitrogen, and stored at -70°C until further protein analysis (Harlow and Lane 1988). Further processing of *C. nilotica* was undertaken for technical convenience as this species was analysed before *B. stenochorias*. For future studies, it is recommended that the PBS solution be supplemented with protein stabilizer dithiothreitol.

Samples were centrifuged for 15 minutes at 13 000 rpm at 4°C , after which two sub-samples of supernatant were removed to 1.5mL Eppendorf microcentrifuge tubes. Total protein content of each sample was determined by the Biorad DC Protein Assay (Biorad Laboratories, USA) using supernatant from one of the sub-samples. Based on the results obtained, the protein contents of the supernatants from the second sub-sample were equated using PBS dilution. Sample buffer (Laemmli, 1970) was added to the second sub-samples on a 1:1 ratio. These samples were boiled at 100°C for 5 min prior to protein analysis. Eppendorf tubes containing the original supernatant and the first sub-sample were refrozen and stored at -70°C .

2.6.3 Protein analysis

Protein analysis was undertaken by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) using the BioRad Mini-PROTEAN II System (Biorad Laboratories, USA). Samples were resolved on 12% polyacrylamide separating gel at 150 V for approximately 1.5 hr using SDS Running buffer. The size of the proteins resolved on SDS-PAGE was estimated with reference to standard molecular mass markers (Broad Range SDS-PAGE markers, BioRad Laboratories, USA). Gels were resolved in duplicate, with one being stained with Coomassie stain to reveal banding pattern while the second sample was used for Western blot analysis.

Western blotting analysis was carried out according to Towbin et al. (1979). Gels were electroblotted onto nitrocellulose membrane at 100 V for one hour. Three primary antibodies were used to probe the membranes for stress protein synthesis: Mouse monoclonal anti-Hsp70 (Stressgen Bioreagents SPA-810, 1:2500 dilution) for detecting stress-induced Hsp70 (Hsp72) (epitope in the region of amino acid residues 436-503); Rabbit polyclonal anti-Hsp40 (Stressgen Bioreagents SPA-400; 1:3000 dilution); Mouse monoclonal anti-Hsp70/Hsc70 mixture (Stressgen Bioreagents SPA-820, 1:2500

dilution) for detecting both the stress-induced (Hsp72) and constitutive Hsp70 (Hsp73) (epitope in the region of amino acid residues 503-640). The amount of protein from the various shrimp and limpet samples subjected to western analysis ranged between 5–20 µg; equal amounts of protein were always loaded per lane per gel to allow for comparison between lanes for each gel. The positive control used during western detection of Hsp70 was purified human Hsp72 and cell extracts from heat-shocked baby hamster kidney cells were utilized for Hsp40. The quality of each Western blot was checked visually by Ponceau S staining of proteins transferred onto a nitrocellulose membrane. Luminol-based chemiluminescence detection reagents (ECL Advance Western Blotting Detection Kit; Amersham biosciences) were used for immunodetection. Chemiluminescent digital imaging of the nitrocellulose membranes was undertaken using a BioRad Chemidoc 170series.

2.6.4 SDS-PAGE gel data analysis

The protein band intensities of the *C. nilotica* SDS-PAGE gels were analysed using Quantity One V4.4 ID and Analysis Software. Four SDS-PAGE gels, reflecting protein bands for *C. nilotica* samples from replicate treatments, were used (heat shocked bacterial cells — DNA K — were used as a positive control molecular weight indicator for Hsp70). Analysis focused on two bands, with density analysis based on arbitrary units of relative optical density. Although equal amounts of protein were loaded in each of the lanes of the four gels, fold intensity was determined by dividing the optical density units measured for the various LAS exposure treatments by those measured in the unexposed control, to account for possible gel-to-gel variations. Statistical analysis, to determine which treatment differed significantly from the control, was undertaken using the non-parametric Kruskal Wallis Two Sample Test in Statistica 6.1 (Statsoft Inc, USA), with statistical significance set at $p < 0.05$.

2.7 Methods for determining the inhibition of cholinesterase as a biological stress response

2.7.1 Organisms and stress exposure tests

Organisms surviving the 96 hr toxicity tests, outlined in Section 2.5, were subjected to the cholinesterase (ChE) assay.

2.7.2 Cholinesterase assay

At the end of the 96 hr toxicity exposure, organisms were collected and immediately frozen in 1.5 mL Eppendorf microcentrifuge tubes with liquid nitrogen and stored whole at -70°C until ChE analysis took place. Due to the small sizes of the organisms, individuals from the same replicate were sometimes grouped into one tube for analysis. Besides flatworms, in which all individuals were combined into one sample tube, there were usually 3–4 sample tubes for each of the three replicates in each treatment, for use in each biochemical assay. The method for determining ChE activity in the above organisms was based on the method developed by Ellman et al. (1961) and refined by McLoughlin et al. (2000). Organisms were macerated in 30 µL PBS (phosphate buffered saline) 10 mM (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), of pH 8 and containing 1% (v/v) Triton X-100, using Eppendorf Teflon micro-pestles (Merck, South Africa). The sample was then diluted with 270 µL PBS (pH 8) and centrifuged at 4°C at 13 000 g for ten minutes. A 100 µL sub-sample of the resultant supernatant was removed to a 1.5 mL Eppendorf microcentrifuge tube and diluted to 500 µL with 400 µL PBS (pH 8) containing 0.1% (v/v) Triton-x-100, in preparation for the assay.

Enzyme activity was determined by adding 100 µL of 8 mM 5,5'-di-thiobis(2-nitrobenzoic acid) and 50 µL of each sample's supernatant to a well of a 96-well microtiter plate kept on ice. Each sample was analysed in quadruplicate (i.e. four wells per plate). The microtiter plate was incubated at 30°C in a microtiter plate reader (PowerWave X, Bio-Tek Instruments, USA) for three minutes before 50 µL of 16 mM acetylthiocholine iodide was added to each well. The plate was read at 405 nm every 30 sec for 10 min and the change in absorbance recorded. The average value from the four independent

measurements of change in absorbance for each sample was determined and this value used to calculate enzyme activity, using the following formula:

$$\text{Activity } (\mu\text{mole/mL}\cdot\text{min}^{-1}) = \left(\frac{\Delta\text{abs}}{\epsilon \times L} \right) \times (\text{df}) \times 10^3$$

Where Δabs is change in absorbance; ϵ is 1.36×10^4 ; L is the path length; and df is the dilution factor (Ellman et al. 1961).

Standards were prepared daily using commercial acetylcholinesterase (AChE) ($1 \text{ mg}\cdot\text{mL}^{-1}$) at a concentration range of $0\text{--}0.125 \text{ mg}\cdot\text{mL}^{-1}$ to produce a standard curve. Specific standard concentrations were included in subsequent plates and compared against the daily standard curve to ensure consistent accurate readings.

Total protein content of each sample was determined by the Biorad DC Protein Assay (Biorad Laboratories, USA) using the supernatant from the sub-sample. Standards were prepared using commercial bovine albumin serum at concentrations ranging between $0.5\text{--}1.4 \text{ mg}\cdot\text{mL}^{-1}$. The standard curve was used to calculate the protein content of each sample. The ChE activity ($\mu\text{mole}\cdot\text{mL}\cdot\text{min}^{-1}$) was divided by the protein content ($\text{mg}\cdot\text{mL}^{-1}$) and multiplied by 1000, to present ChE activity as $\text{nmol}\cdot\text{min}\cdot\text{mg}^{-1}$ protein.

2.7.3 Statistical analysis

The ChE activities recorded for each of the samples in each replicate (usually three samples for shrimp, mayflies and limpets, with only one for flatworms) were averaged to obtain one ChE activity value for each of the three replicates per LAS exposure treatment. These three data points per treatment were used in the statistical analyses to determine significant differences in ChE activity between exposed and unexposed organisms. The data were checked for homogeneity of variance; if large differences in the variance of ChE activity between treatments were noted, then the data were log transformed. Normality of data was determined using the Kolmogorov-Smirnov test. If data were normal, the Student's t test was used and if the data were non-normal the Mann Whitney U-Test was used.

2.8 Methods for measuring lipid peroxidation as a biological stress response

2.8.1 Organisms and stress exposure tests

Organisms surviving the 96 hr toxicity tests outlined in Section 2.5 were subjected to the lipid peroxidation assay.

2.8.2 Lipid peroxidation assay

One of the products formed during lipid peroxidation is malondialdehyde (MDA), which is used in the thiobarbituric acid (TBA) reactive substances test to infer the level of oxidative stress. At the end of the 96 hr toxicity trials, organisms were collected and immediately frozen in 1.5 mL Eppendorf microcentrifuge tubes with liquid nitrogen. Whole samples were stored at -70°C until LPx analysis took place. Due to the small sizes of the organisms, individuals from the same replicates were sometimes grouped into one tube for analysis. The assay used here is based on the method described in a handbook for estuarine species (Ringwood et al. 2003), with some modifications. For example, in the present study the MDA activity was described in terms of protein concentration rather than in relation to whole body mass, and the assay was undertaken with half the quantities described by Ringwood et al. (2003), to allow the use of 1.5 mL Eppendorf tubes. MDA standards were initially prepared by producing a 10mM stock solution of MDA tetraethylacetal (24% 1,1,3,3-tetraethoxypropane; 1% 1N HCl; 90% deionized H_2O), which was heated at 50°C for 60 min. A $3200\ \mu\text{M.L}^{-1}$ MDA secondary stock solution was then produced (406 μL of 10 mM MDA stock + 192 μL potassium phosphate buffer 50 mM (12.5 mM KH_2PO_4 ; 12.5 mM K_2HPO_4) at pH7). Serial dilutions of the $3200\ \mu\text{M.L}^{-1}$ MDA solution were performed, with the lowest concentration being $6.25\ \mu\text{M.L}^{-1}$. Whole-body homogenates were obtained by macerating test organisms in 200 μL potassium phosphate buffer. Samples were then centrifuged at 4°C at 13 000 g for 10 min and 50 μL of the supernatant was transferred to a new 1.5 tube with 700 μL 0.375% TBA and 7 μL 2% butylated hydroxytoluene (BHT). Samples were then vortexed and both the samples and standards heated for 15 min at 100°C . Thereafter, samples and standards were centrifuged at 13000 g for 5 min at room temperature. 100 μL of sample or standard was placed in a well of a 96-well microtiter plate and read at 532 nm using a microtiter plate reader (PowerWave X, Bio-Tek Instruments, USA). Each sample

and standard was analysed in quadruplicate (i.e. four wells per plate). MDA activity in $\mu\text{mol.L}^{-1}$ was calculated for each sample, using the linear regression obtained from concentration vs absorbance of MDA standards and multiplied by four to represent the activity over an hour. Sample MDA activity was converted from $\mu\text{mol.L}^{-1}$ to nmol.mL^{-1} , which was divided by the protein concentration of the particular sample to produce MDA activity in nmol.hour.mg^{-1} protein. Total protein content of each sample was determined by the Biorad DC Protein Assay (Biorad Laboratories, USA), as described in the ChE section.

2.8.3 Statistical analysis

The MDA activities recorded for each of the samples in each replicate (usually three samples for shrimp, mayflies and limpets, and one for flatworms) were averaged to obtain one MDA activity value for each of the three replicates per LAS exposure treatment. These three data points per treatment were used in the statistical analyses to establish whether significant differences in MDA activity occur between exposed and unexposed organisms. If there were large differences in the variance of MDA activity between treatments the data were log transformed. Normality of data was determined using the Kolmogorov-Smirnov test. If data were normal the Student's *t* Test was used and if the data were non-normal the Mann Whitney U-Test was used.

2.9 Species sensitivity distribution approach

A water quality guideline for LAS was derived in Chapter 4 using a species sensitivity distribution (SSD). A SSD is a statistical distribution describing the variation in toxicity of a range of species to a particular toxicant. From this statistical distribution an estimate of the percentage species affected by a specific concentration of the toxicant can be made. The particular distribution utilised in this thesis belongs to the Burr Type III family and was utilised in the development of the Australian and New Zealand Water Quality Guidelines (ANZECC and ARMCANZ 2000) by applying the specially developed BurliOZ software v1.0.9 CSIRO (2000).

Chapter 3 Characterising responses of freshwater organisms to LAS exposure

Preface: Excerpts of text and selected data from this chapter have been published in *Water, Air & Soil Pollution* (Gordon et al. 2008) and in a scientific report to the Water Research Commission of South Africa (Gordon et al. 2010).

3.1 General introduction

The overall objective of this chapter is to identify suitable toxicological data for use in the derivation of a water quality guideline (WQG) for linear alkylbenzene sulfonate (LAS) for South African freshwaters (Chapter 4). Toxicological data were sourced from scientific literature and produced, as part of the current study, using toxicity testing with indigenous species.

The toxicological data from the literature and those generated in the indigenous toxicity tests were collated in terms of their exposure period and biological response attributes:

- Short-term lethality data (≤ 96 hr exposure);
- long-term lethality data (> 96 hr exposure);
- long-term sub-lethal data (> 96 hr exposure including organism-level, population-level and community-level responses); and,
- cellular and sub-cellular responses over any exposure period.

The quality of these data was then assessed for use in water resources management application. The reliability of WQGs depends to a great extent on the quality of the toxicity data used to produce them (Warne 2001). It was thus necessary to assess the collated toxicity data from the scientific literature to ensure that these were of adequate quality. The assessment scheme employed to do this is outlined in the materials and methods, Section 3.2.2. Toxicological data of acceptable quality are presented in separate sections within this chapter (together with – in the case of data from indigenous organisms – details of the toxicological tests used to generate them), differentiated according to the exposure period and biological response attribute categories identified above.

The use of short- and long-term lethal data, and sub-lethal organism-, population- and community-response data are commonplace in the derivation of WQGs. Indeed, these data have been utilised in the derivation of an LAS WQG for Australia and New Zealand, and two predicted no-effect concentration (PNEC) values for LAS (Van de Plaasche et al. 1999; Dyer et al. 2003). The use of cellular and sub-cellular data is, however, not commonplace, due to concerns regarding response variability in toxicity tests and questionable links to ecosystem impacts (Clark et al. 1999; Tannenbaum 2005; Forbes et al. 2006). Despite the suggestion, from Handy et al. (2003), that an enormous number of published scientific papers and books concerning cellular and sub-cellular biomarkers exist as a potential source of data applicable to water resource management tools such as WQGs, little is known of the quantity and quality of these types of data pertaining to LAS. Thus a detailed assessment of cellular and sub-cellular response data to LAS exposure sourced from the literature is undertaken in Section 3.6.5 of this chapter. The particular suitability of cellular and sub-cellular response data as measures of LAS exposure is assessed. The challenges encountered in generating these data — such as the types of experimental exposure systems used, the accuracy of the biochemical assays employed to measure responses, and the consequent quality of data generated — are discussed in Section 3.6.4.

3.1.1 Description of the LAS chemical and toxic mode of action

As discussed in Chapter 1, the LAS molecule consists of a linear alkyl carbon chain ranging from 10–14 carbon units, with a phenyl group attached to any one of the internal carbon atoms except the terminal carbons (OECD 2005; Ying 2006). Consequently, LAS is not a single compound but consists of a mixture of homologues of various alkyl chain lengths. Toxicity increases with increasing alkyl chain length (Blok and Balk 1993) and as the position of the phenyl group moves closer to the external carbons (Fendinger et al. 1994). The increase in toxicity with increasing LAS chain length has been demonstrated in a number of studies on *Daphnia magna* (Kimerle and Swisher 1977; Verge and Moreno 2000; Hodges et al. 2006), fathead minnow *Pimephales promelas* (Kimerle and Swisher 1977; Holman and Macek 1980) and two species of green algae (Verge and Moreno 1996). The high level of intra-species variation observed in LAS exposure data, noted in the literature, has been ascribed to the use of LAS of different chain lengths in toxicity testing (Blok and Balk 1993; Van de Plaasche et al. 1999).

Consequently, the normalisation of toxicity response data to a common chain length is recommended (OECD 2005; HERA 2007). The approach adopted in this study to achieve this objective is described in Section 3.2.3 of this chapter.

Being an anionic surfactant, the mode of action for LAS is one of polar narcosis (Roberts 1991; Hodges et al. 2006), the effect of which is a nonspecific disruption of the functioning of cell membranes. In animals this condition is characterised by progressive lethargy, unconsciousness and death (Veith and Broderius 1990). At a cellular and sub-cellular level, anionic surfactants like LAS have been found to interfere with proteins (for example, by altering the folding of polypeptide chains (Cserhati et al. 2002)), by modifying the activity of various enzymes (Bragadin et al. 1996; Cserhati et al. 2002), inducing the oxidative stress response (Sirisattha et al. 2004) among other effects. The above examples of cellular and sub-cellular responses to LAS formed the rationale for the choice of biochemical toxicity tests undertaken in this thesis. This topic is described in Section 3.6 of this chapter.

3.2 Materials and Methods

3.2.1 Collation of toxicity response data to LAS exposure

The collation of toxicity response data was achieved by searching the US EPA ECOTOX database (<http://epa.gov/ecotox> accessed 9 February 2011) and the general scientific literature (sourced using the online research databases: ScienceDirect; SAGE Journals online; SpringerLink; Wiley Online Library; Taylor and Francis Informaworld and EBSCOhost, last accessed 11 February 2011). In addition to published data, the short-term lethal responses to LAS of a number of aquatic invertebrates indigenous to the United Kingdom were included in the collation (Blake 2001). A major database of organism responses to LAS exposure was developed by BKH Consulting Engineers (1993), which included both lethality and sub-lethality data. Unfortunately, the archive of the literature/studies BKH used to collate this database is no longer available (OECD 2005).

3.2.2 Quality assessment of toxicological data

Assessment schemes are designed to evaluate the scientific rigor of toxicity tests, and quality of associated toxicity data, reported in scientific publications. Assessment schemes utilise a scoring system in which points are based on a series of questions relating to exposure duration, the biological stress response measured, characteristics of the test organism, test methodology, and type of data reported. The national WQGs developed for South Africa (DWAF 1996) utilised the method developed for the US EPA (Stephan et al. 1985). However, the refined assessment scheme for the Australian Ecotoxicology database, proposed by Hobbs et al. (2005), was chosen as the assessment scheme to be employed in this thesis for data sourced from published literature. The reasons for choosing this scheme are as follows: it is the most recently developed scheme, it is objective, is clearly described and thus easily applied (see Section 4.2.2 for further discussion on data quality and assessment schemes).

Only data in which the original source document could be assessed were included. An exception was made for data from the OECD (2005) report as these data had been validated using the Klimisch et al. (1997) approach, which was considered a suitably reliable measure of data quality. A thesis (Blake 2001) as a whole was also subjected to the assessment process. Blake (2001) details results for some field-collected organisms in which the control mortality exceeded 20% or where 95% confidence limits could not be generated for an LC50 value. In these cases, the data were excluded. In the collation process, where more than one toxicity datum was available for an organism, the geometric mean is reported.

The toxicity tests involving the exposure of indigenous organisms to LAS (presented in this chapter) were undertaken to meet the necessary requirements of the data quality assessment scheme. The materials and methods used in undertaking these toxicity tests are described in Chapter 2.

3.2.3 Normalisation of LAS chain length

The procedure for normalisation is based on the use of quantitative structure-activity relationships (QSARs). A QSAR theoretically relates chemical structure to a biological

response — or in this case, an LAS molecule of a specific chain length — to a level of toxicity. Van de Plaasche et al. (1999) used the following QSAR (originally developed by Saarikoski and Veluksela (1982) to predict the toxicity of phenols to fish):

$$\text{Log (1/LC50)} = 0.63\log K_{ow} + 2.52 \quad (1)$$

More recently, Hodges et al. (2006) determined a QSAR specifically for LAS using *Daphnia magna*:

$$\text{Log (1/LC50)} = 0.77\log K_{ow} + 2.47 \quad (2)$$

The greater relevance of this latter QSAR to the toxicity data in this chapter resulted in its inclusion as part of the normalisation process. The above QSARs are based on short-term lethality data and are thus directly applicable to the normalisation process for those types of toxicity data. Unfortunately, there is no long-term sub-lethal QSAR available. Consequently, this QSAR was also used for long-term sub-lethal data, as in Feijtel and Van de Plassche (1995) and Van de Plassche et al. (1999). This may contribute some uncertainty to the normalised sub-lethal stress response data.

Since the degree of hydrophobicity of narcotic compounds like LAS affects their toxicity to aquatic organisms, the octanol/water partition coefficient (K_{ow}) of the different LAS chain lengths can be utilised to estimate toxicity in the form of an LC50 value. The $\log K_{ow}$ for each LAS homologue was calculated by Hansch and Leo (1979) and is cited in Hodges et al. (2006) (Table 3.1). The normalisation procedure therefore involved calculating the LC50 value for the normalised LAS homologue (chosen to be C11.6 as this is the most abundant homologue in commercial products in South Africa) using QSAR Equation 2, and also for the LAS homologue of the tested structure used in the toxicity test that produced the exposure response datum to be normalised. The ratio between the LC50 value determined for C11.6 and for the tested structure, was multiplied by the exposure response concentration to normalise it to C11.6.

Table 3.1 Log K_{ow} values calculated for LAS homologues by Hansch and Leo (1979).

LAS homologue chain length	Calculated log K_{ow}
C9	1.63
C10	2.15
C11	2.60
C12	3.17
C13	3.62
C14	4.19

3.3 Short-term (\leq 96 hr) lethal toxicity data

A large quantity of short-term lethality data for aquatic organisms exposed to LAS was available. These data have been the basis of a number of comprehensive reviews on the aquatic toxicity of LAS (Feijtel and Van de Plassche 1995; Van de Plassche et al. 1999; HERA 2007; OECD 2005). Short-term lethality data were also available from the US EPA ECOTOX database and from the scientific literature. The quality of these data were assessed using Hobbs et al. (2005) and those found to be suitable are presented in Table 3.2. In addition, a number of short-term lethality tests were undertaken in the course of this research in which various species of indigenous macroinvertebrates were exposed to LAS. These tests are described below and results of acceptable quality included in Table 3.2.

3.3.1 Short-term lethal toxicity tests

Introduction

Short-term lethal toxicity tests were undertaken exposing four indigenous aquatic invertebrate species to LAS. The species exposed were as follows: laboratory-reared flatworms (Class Turbellaria, Genus *Dugesia*); laboratory-reared Atyidae shrimp, *Caridina nilotica*; wild-caught Leptophlebiidae mayfly *Adenophlebia auriculata*; and wild-caught freshwater limpet, *Burnupia stenochorias* (Ancylidae). A trial using the standard laboratory-reared test organism, the cladoceran *Daphnia pulex*, was also undertaken.

Materials and methods

The exposure of *A. auriculata* and *C. nilotica* to LAS was undertaken concurrently within the same experimental vessels. The exposure of *Dugesia* sp. and *B. stenochorias* was also undertaken concurrently. These species do not naturally interact and it was

assumed that the large volume of medium within the vessel, together with its high turnover rate, would reduce the chance of the organisms experiencing stress as a result of being confined together. The general methodology used in these toxicity tests is described in Chapter 2, with specific details relevant to interpreting the results described briefly below.

Laboratory reared *C. nilotica* juveniles, of 20–30 days old (approximately 5–10 mm total length) and wild-caught *Adenophlebia auriculata* nymphs of approximately 5–8 mm total length were exposed to nominal concentrations of 0, 1000, 2000, 4000, 8000, 16000, 32000 $\mu\text{g.L}^{-1}$ LAS for 96 hr. Three replicates were set up at each concentration level, containing 25–30 shrimp and 20 mayflies per replicate. There was a malfunction in the diluent input pipe of one of the replicates designated for the nominal concentration of 8000 $\mu\text{g.L}^{-1}$. Results from this channel were excluded from the data analysis. The accuracy of the dosing system was tested for treatments exposed to 1000 and 2000 $\mu\text{g.L}^{-1}$ LAS (as the Spectroquant® Surfactants (anionic) Cell Test has a detection limit of 50–2000 $\mu\text{g.L}^{-1}$), with measured concentrations ranging from 91–102% of nominal concentrations. As the measured concentrations were within 80–120% of nominal concentrations, results are presented as nominal concentrations (OECD 2000).

Laboratory reared *Dugesia* sp. of approximately 5–10 mm in length and wild-caught *B. stenochorias* (3–5 mm diameter) were exposed for 96 hr to nominal concentrations of LAS of 0, 500, 1000, 1500, 2000, 4000, 8000 $\mu\text{g.L}^{-1}$. Each concentration consisted of three replicates with five *Dugesia* sp. per replicate, while 35 *B. stenochorias* specimens were placed in each control replicate and 20 in each LAS exposure replicate. The accuracy of the dosing system was tested for treatments exposed to 500, 1000, 1500 and 2000 $\mu\text{g.L}^{-1}$ LAS, with measured concentrations ranging from 106–82% of nominal concentrations. Consequently, results are presented as nominal concentrations.

Daphnia pulex neonates (<24 hr old) were exposed to nominal stock concentrations of LAS of 1000, 2000, 4000, 6000, 10000, 15000 and 20000 $\mu\text{g.L}^{-1}$ for 48 hr. Immobility (as a surrogate measure of mortality) was measured as the stress response. The measured LAS concentrations in the 1000 and 2000 $\mu\text{g.L}^{-1}$ treatment vessels ranged from 80–100% of nominal, and consequently nominal concentrations were used to present and

analyse data. Further details of experimental methodology employed in the above toxicity tests are outlined in Chapter 2.

Results

There were no significant variations in temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO) over the period of the toxicity tests for the indigenous organisms, both within and between treatments (Appendix 2, Tables A and B). A slight increase in average EC with increasing LAS concentration was observed.

There were no mortalities within the control treatments of the *A. auriculata*, *Dugesia* sp. and *D. pulex* exposure trials, while *B. stenochorias* and *C. nilotica* experienced 9% and 15% cumulative mortality, respectively (Figure 3.1). As *C. nilotica* is not a standard organism for toxicity testing, control mortalities of up to 20% were allowed, i.e. they did not invalidate the result (DWAF 2000).

At the end of 96 hr, *C. nilotica* experienced 94% mortality and *A. auriculata* 70% mortality at the highest exposure concentration of 32000 $\mu\text{g.L}^{-1}$ (Figure 3.1). As 100% mortality was not recorded, failing one of the assumptions of the Probit model, the Trimmed Spearman-Kärber (TSK) model was used instead to determine the LC50 values. The 96 hr LC50 value for *C. nilotica* was 9196 $\mu\text{g.L}^{-1}$ with lower and upper 95% confidence limits of 7782 and 10867 $\mu\text{g.L}^{-1}$, respectively (data required a 7% trim by the TSK model). For *A. auriculata* a 96 hr LC50 value of 14982 $\mu\text{g.L}^{-1}$ with lower and upper 95% confidence limits of 11059 and 20295 $\mu\text{g.L}^{-1}$, respectively, was determined, although the data required a 31% trim by the TSK model.

The cumulative mortality data for *B. stenochorias* and *Dugesia* sp. did not fit the Probit model, so the TSK model was used to determine respective LC50s of 4559 $\mu\text{g.L}^{-1}$ (with lower and upper 95% confidence limits of 4089 and 5083 $\mu\text{g.L}^{-1}$, respectively; also requiring a 5% trim) and 5401 $\mu\text{g.L}^{-1}$ (with lower and upper 95% confidence limits of 4940 and 5906 $\mu\text{g.L}^{-1}$; no trim required). The Probit model was used to determine a 96 hr LC50 for *D. pulex* of 10383 $\mu\text{g.L}^{-1}$ LAS, with lower and upper 95% confidence limits of 8882 and 11895 $\mu\text{g.L}^{-1}$, respectively.

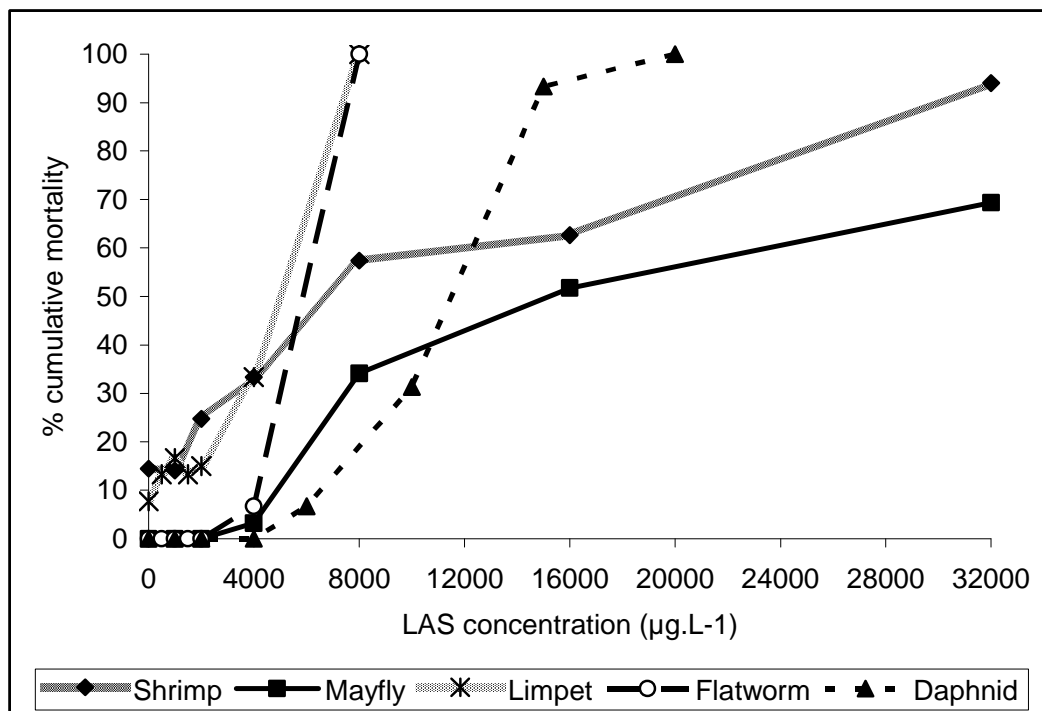


Figure 3.1 Percentage cumulative mortality curves for shrimp *Caridina nilotica*, mayfly *Adenophlebia auriculata*, limpet *Burnupia stenochorias*, flatworm *Dugesia* sp. and daphnid *Daphnia pulex* after 96 hr of LAS exposure.

Discussion

The mayfly *A. auriculata* was considerably more tolerant of LAS exposure than the other South African indigenous organisms tested (Figure 3.1). The crustaceans, *C. nilotica* and *D. pulex*, were more tolerant than the flatworm *Dugesia* sp. and the limpet *B. stenochorias*, perhaps suggesting that possession of an exoskeleton afforded some protection from LAS toxicity. The results also suggest that *B. stenochorias* was not able to use its shell to avoid an LAS exposure during the 96 hr period.

The mayfly *A. auriculata* was also more tolerant than three British mayfly species exposed to LAS: *Ecdyonurus dispar*, *Rhithrogena semicolorata* and *Ephemerella ignita* Blake (2001) Ninety-six hour LC50s for these three mayflies were 3900, 4300, 4900 µg.L⁻¹, respectively.

The LAS exposure concentrations chosen for the *Dugesia* sp. trial in this thesis were based on previous results published by Blake (2001), who reported a 96 hr LC50 value

of 1800 $\mu\text{g.L}^{-1}$ for the flatworm *Polycelis feline*. Lewis and Suprenant (1983) and BKH Consulting Engineers (1993) both reported 48 hr LC50 values of 1800 $\mu\text{g.L}^{-1}$ for flatworms *Dugesia* sp. and *Planaria* sp., respectively. Li (2008) also exposed *D. japonica* to LAS over 96 hr and reported an LC50 value of 1450 $\mu\text{g.L}^{-1}$. The laboratory-reared flatworm utilised in this thesis appeared to be less sensitive, however, with mortalities being first recorded at 4000 $\mu\text{g.L}^{-1}$ (Figure 3.1).

3.3.2 Collation of short-term lethality data

A database of LAS exposure response data was developed by BKH Consulting Engineers (1993) and consisted of 586 effects data for 93 species of freshwater and marine organisms. Feijtel and Van de Plassche (1995) and Van de Plassche et al. (1999) summarised these data, providing geometric means for selected species. For some of these species many data values were available (e.g. *D. magna* $n = 139$, *Lepomis macrochirus* $n = 88$) but with high intraspecies variation, probably a consequence of the different alkyl chain lengths of LAS homologues used in the toxicity tests and the non-standardisation of experimental methods (Van de Plassche et al. 1999). Neither of these authors attempted normalisation of the short-term lethality data. As these were review articles the source of the data was not provided and the archive of the literature/studies BKH used to collate this database is no longer available (OECD 2005). Consequently, it is not possible to confirm the quality of the effects data and ultimately the accuracy of the derived predicted no-effect concentrations (PNEC) derived by Feijtel and Van de Plassche (1995) and Van de Plassche et al. (1999). As Van de Plassche et al. (1999) point out, the interspecies variation decreases considerably when the geometric mean value per species is calculated, i.e. when such a large quantity of exposure data is condensed into a single mean value, the effect of outliers is lessened considerably. Nevertheless, in this thesis it was decided to only include effects data that could be validated, i.e. that met the criteria developed by Hobbs et al. (2005), or met the validation criteria stipulated by OECD (2005). The resultant validation scores for the scientific sources assessed using Hobbs et al. (2005) are presented in Appendix 3. Although this approach excludes some available toxicity data, there are still 29 taxa, representing a wide range of taxonomic classifications, for which reliable-quality data exist. These data are presented in Table 3.2, with all data normalised to C11.6.

In terms of lethality, the most sensitive organism to short-term exposures of LAS was the fish *Lepomis macrochirus* (LC50 of 2000 $\mu\text{g.L}^{-1}$), followed by oligochaete *Dero* sp. (LC50 of 2036 $\mu\text{g.L}^{-1}$), the soft bodied turbellarians *Dugesia* sp. (with LC50 values of 2079 and 2155 $\mu\text{g.L}^{-1}$, respectively) and the remaining two fish species (Table 3.2). Although also soft bodied, the leach and nematode species were considerably less sensitive (with LC50 values of 9340 and 19158 $\mu\text{g.L}^{-1}$, respectively), perhaps reflecting their general tolerance of poor water quality. Those organisms with exoskeletons, the insects and crustaceans, were more tolerant of LAS, although there was a great deal of intra-species variation in LC50 values within these two groups of taxa (LC50s ranged from 3353 – 323292 $\mu\text{g.L}^{-1}$). The isopod *Asellus* sp., considered as pollution tolerant (Lewis and Suprenant 1983), recorded an exceptionally high LC50 of 323292 $\mu\text{g.L}^{-1}$ after a 48 hr exposure to LAS. The three gastropods — *Physa venustula*, *Heleobia cumingii* and *Melanooides tuberculata* — also recorded high LC50s (85505 – 241835 $\mu\text{g.L}^{-1}$) after 48 hr LAS exposure (Iannaccone and Alvarino 2002). These data should, however, be interpreted with caution as the gastropods have the ability to protect their soft body tissue from harmful chemicals in the water by withdrawing into their shells and sealing the entrance with an operculum. The short exposure period of 48 hr may have allowed these organisms to dramatically limit their exposure to the LAS present in the experimental medium. By comparison, the other mollusc listed in Table 3.2, the limpet *B. stenochoeris*, was considerably more sensitive to LAS (LC50 of 4559 $\mu\text{g.L}^{-1}$), perhaps reflecting a lessened ability to isolate itself from the LAS present in the exposure medium. Thus, those organisms with the greatest exposure of soft body tissues to the aquatic medium appear the most susceptible. This general trend, observed in the sensitivities of organisms listed in Table 3.2, appears to correlate with the accepted toxic mode of action of LAS i.e. causing narcosis, and ultimately death, through disruption of the functioning of cell membranes (Veith and Broderius 1990).

Table 3.2 Short-term (≤ 96 hr) lethality data for LAS toxicity to freshwater organisms. Data are normalised to C11.6 and multiple data per organism are presented as a geometric mean: n = number of toxicity trials.

Organism	Exposure period (hours)	LC50 ($\mu\text{g}\cdot\text{L}^{-1}$) and chain length	Geometric mean LC50 ($\mu\text{g}\cdot\text{L}^{-1}$) normalised to C11.6	n	Source
<i>Lepomis macrochirus</i> ¹	96	1670 (C11.8)	2000	1	A [#]
<i>Dero</i> sp. ²	48	1700 (C11.8)	2036	1	B
<i>Dugesia japonica</i> ³	96	1450 (C12)	2079	1	C
<i>Dugesia</i> sp. ³	48	1800 (C11.8)	2155	1	B
<i>Ictalurus punctatus</i> ¹	96	1700 (C12.0)	2437	1	D
<i>Pimephales promelas</i> ¹	96	4100 (C11.7)	3177	3	E
	96	1700 (C12.0)			D
	96	4600 (C11.1)			F [#]
<i>Leuctra</i> sp. ⁴	96	2800 (C11.8)	3353	1	G
<i>Gammarus</i> sp. ⁵	48	3300 (C11.8)	3951	1	B
<i>Hyalella azetec</i> ⁵	96	3100 (C12.0)	4445	1	D
<i>Burnupia stenochorias</i> ⁶	96	4559 (C11.6)	4559	1	H
<i>Ecdyonurus dispar</i> ⁴	96	3900 (C11.8)	4670	1	G
<i>Rhithrogena semicolorata</i> ⁴	96	4300 (C11.8)	5149	1	G
<i>Dugesia</i> sp. ³	96	5401 (C11.6)	5401	1	H
<i>Emphemerella ignita</i> ⁴	96	4900 (C11.8)	5867	1	G
<i>Daphnia magna</i> ⁵	48	18000 (C11.1)	6278	4	I
		3500 (C12.0)			J
		8100 (C12.0)			K
		1620 (C12.0)			L [#]
<i>Pimephales promelas</i> ¹	48	4700 (C12.0)	6738	1	J
<i>Gammarus pulex</i> ⁵	96	6310 (C11.7)	6905	1	M
<i>Caridina nilotica</i> ⁵	96	9196 (C11.6)	9196	1	H
<i>Erpobdella octoculata</i> ⁷	96	7800 (C11.8)	9340	1	G
<i>Daphnia pulex</i> ⁵	48	10383 (C11.6)	10383	1	H
<i>Ceriodaphnia dubia</i> ⁵	48	11840 (C12.0)	13787	2	N
		7810 (C12.0)			O
<i>Adenophlebia auriculata</i> ⁴	96	14982 (C11.6)	14982	1	H
<i>Rhyacophila dorsalis</i> ⁴	96	13500 (C11.8)	16165	1	G
<i>Rhadbitis</i> sp. ⁸	48	16000 (C11.8)	19158	1	B
<i>Ceriodaphnia silvestrii</i> ⁵	48	13520 (C12.0)	19384	1	N
<i>Daphnia similis</i> ⁵	48	14170 (C12.0)	20316	1	N
<i>Paratanytarsus parthenogenica</i> ⁴	48	23000 (C11.8)	27540	1	B
<i>Physa venustula</i> ⁶	48	71410 (C11.8)	85505	1	P
<i>Heleobia cumingi</i> ⁶	48	82930 (C11.8)	99299	1	P
<i>Melanoides tuberculata</i> ⁶	48	201970 (C11.8)	241835	1	P
<i>Asellus</i> sp. ⁵	48	270000 (C11.8)	323292	1	B

Notes: # Validated by OECD (2005); ¹ Fish; ² Oligochaete; ³ Turbellarian; ⁴ Insecta; ⁵ Crustacean; ⁶ Mollusc; ⁷ Hirudinea; ⁸ Nematode;

Sources: A) Lewis and Perry 1981; B) Lewis and Suprenant 1983; C) Li 2008; D) Versteeg and Rawlings 2003; E) Holman and Macek 1980; F) Swisher et al. 1978; G) Blake 2001; H) Current study; I) Canton and Slooff 1982; J) Kimerle and Swisher 1977; K) Verge et al. 2001; L) Hooftman and Van Drongelen-Sevenhuijsen 1990; M) McLoughlin et al. 2000; N) Da Silva Coelho and Rocha 2010; O) Warne and Shifko 1999; P) Iannaccone and Alvarino 2002.

3.4 Long-term (> 96 hr) lethal toxicity data

Comprehensive toxicity assessments of LAS undertaken by Feijtel and Van de Plassche (1995), Van de Plassche et al. (1999), HERA (2007) and the OECD (2005) included mortality as a 'chronic' stress response (chronic in this context meaning long-term) along with sub-lethal responses in their determinations of a safe environmental concentration for LAS. In the current study, however, long-term lethality data are not combined with sub-lethality data and are presented separately (Table 3.3). Data for two fish and two crustacean species could be sourced from the literature. In addition, a 240 hr exposure trial with *C. nilotica* juveniles exposed to LAS was available from the Unilever Centre for Environmental Water Quality Toxicity Database (UCEWQ 2011). In this trial *C. nilotica* was exposed to LAS concentrations of 0, 1200, 1900, 3200, 5400, 9000, 15000 and 25000 $\mu\text{g}\cdot\text{L}^{-1}$ on a static renewal basis, with the exposure medium replaced every 48 h. The quality of the data from the studies on the two fish species and *D. magna* was validated by the OECD (2005). The quality of the *H. azteca* and *C. nilotica* data was assessed using Hobbs et al. (2005) and deemed suitable. Data from the *C. nilotica* trial were available as both a point estimate of toxicity (i.e. LC50 – point estimates other than 50% could not be determined as only the Trimmed-Spearman Karber model would fit the data) and as a hypothesis-based determination (i.e. no observed effect concentration (NOEC)). The remaining long-term lethal data were a mixture of point estimates and NOECs. Both fish species were considerably more sensitive to LAS than the crustaceans (Table 3.3), although the fish experienced longer exposure periods. Fish were also more sensitive than crustaceans in the short-term lethality data (Table 3.2).

Table 3.3 Long-term (> 96 hr) freshwater lethality data for organisms exposed to LAS. Data are normalised to C11.6 and multiple data per organism are presented as geometric mean (n = number of toxicity trials).

Organism	Exposure period (days)	LC50 / NOEC ($\mu\text{g.L}^{-1}$) and chain length	Geometric mean ($\mu\text{g.L}^{-1}$) normalised to C11.6	n	Source
<i>Oncorhynchus mykiss</i> ¹	14	LC50 120 (C12.0)	LC50 172	1	A [#]
<i>Pimephales promelas</i> ¹	196	NOEC 620 (C12.0)	NOEC 646	4	B [#]
	30	NOEC 1000 (C11.1)			C [#]
	40	NOEC 480 (C11.7)			D [#]
	60	NOEC 106 (C13.5)			D [#]
<i>Hyalella azteca</i> ²	24	LC20 950 (C12.0)	LC20 1362	1	E
<i>Caridina nilotica</i> ²	10	NOEC 3200 (LC50 5239) (C11.6)	NOEC 3200 (LC50 5239)	1	F
<i>Daphnia magna</i> ²	7	NOEC 5000 (C11.8)	NOEC 5987	1	G [#]

Notes: [#] Validated by OECD (2005); ¹ Fish; ² Crustacean

Sources: A) Vailati et al. 1975 cited in OECD 2005; B) Pickering and Thatcher 1970 cited in OECD 2005; C) Swisher et al. 1978 cited in OECD 2005; D) Holman and Macek 1980; E) Versteeg and Rawlings 2003; F) UCEWQ Toxicity Database 2011; G) Mount and Norberg 1983 cited in OECD 2005.

3.5 Long-term (> 96 hr) sub-lethal toxicity data: whole organism and population level responses

The increased complexity involved in generating sub-lethal stress response data from prolonged exposure of organisms to a contaminant generally means that fewer long-term sub-lethal toxicity data are available, compared with short-term lethal data. This was certainly the case for LAS. The BKH (1993) database did include a quantity of long-term sub-lethal data, although many of the long-term exposure data measured mortality as the stress response. In addition to the BKH database, searches of the US EPA ECOTOX database and the general scientific literature were undertaken to obtain long-term sub-lethality data. As described previously, data were only included in the collation if the original literature source could be accessed and was considered of acceptable quality. Data assessed by the OECD (2005) and found to be of acceptable quality were also included.

A long-term sub-lethality test was also undertaken in the course of this research, exposing the indigenous crustacean *C. nilotica* to LAS over a 10-week period. The materials and methods employed for this toxicity test are described in Chapter 2. This toxicity test is described below, after which a table is presented collating all selected long-term sub-lethality data (Table 3.4).

3.5.1 Sub-lethal responses determined from long-term exposure of shrimp *Caridina nilotica* to LAS

Introduction

There have been recent calls to focus on using ecologically relevant and sensitive biological stress response measures in ecotoxicology (Chapman 2002; Emlen and Springman 2007; Landis and Kaminski 2007). This is a consequence of the perception that most environmental contaminant studies focus on the lethal responses of individual organisms and that these stress responses are generally not good predictors of population and community level impacts in the natural environment (Stark 2005). In addition, there is a realisation that early life stage toxicity testing (applied under the premise that the population will remain viable if the weakest link in an individual's life stages is protected) does not always guarantee population viability (Newman 2001). In terms of aquatic organisms, however, conducting population level toxicity experiments on longer-lived organisms can be expensive and of prohibitively long duration.

Suter et al. (2005) argue that stress responses from the organism level of biological organisation, for example growth or reproduction and even survival (if applied collectively) can be used as population level assessments as these attributes directly affect the population. Consequently, an experiment was designed to expose *C. nilotica* to LAS for an extended duration (70 days) to measure growth and reproductive responses. Growth rates of experimental shrimp were determined using the Von Bertalanffy growth model. The materials and methods used to undertake this toxicity test are described in Chapter 2.

Results

Mean (\pm standard deviation) water temperature ($22.95 \pm 0.70^\circ\text{C}$), pH (8.21 ± 0.18), electrical conductivity ($65.01 \pm 6.90 \text{ mS}\cdot\text{m}^{-1}$) and dissolved oxygen ($5.73 \pm 0.41 \text{ mg}\cdot\text{L}^{-1}$) did not differ significantly ($p \leq 0.05$) between treatments. The LAS concentrations measured within each tank over the period of the trial were variable, especially at higher concentrations (Figure 3.2). The variation of LAS concentration in replicated tanks of the same treatment were however of similar magnitude. Furthermore, LAS concentrations within tanks from one treatment did not overlap with tanks from other treatments, suggesting shrimp in replicates within the same treatment were exposed to similar LAS concentrations over the duration of the experiment. Average LAS concentrations for each treatment were determined to be 0, 65, 331 and $1225 \mu\text{g}\cdot\text{L}^{-1}$.

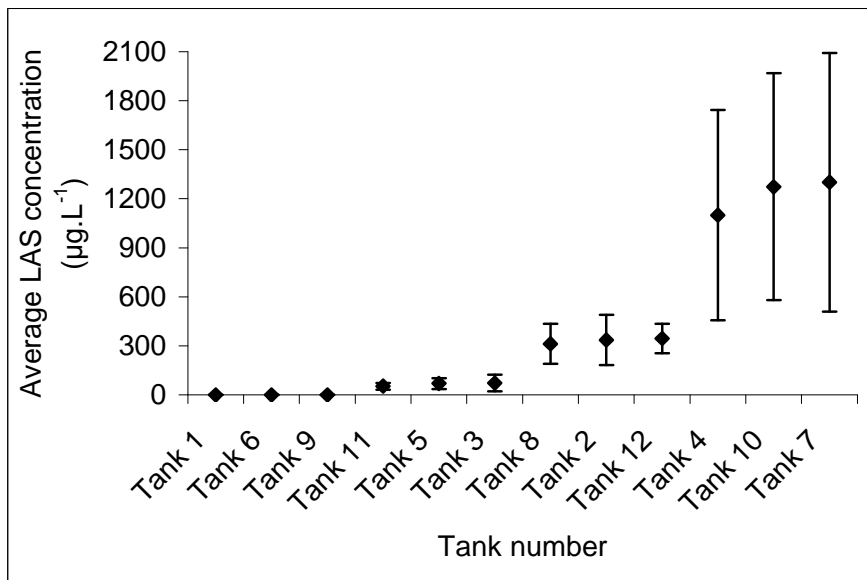


Figure 3.2 Average LAS concentration (\pm standard deviation) measured weekly in experimental vessels over the course of the experiment (70 days).

Shrimp mortality at the end of the 70-day exposure did not follow a dose response relationship (Figure 3.3). The average mortality of shrimp in the control treatment was 16%, a similar mortality to shrimp exposed to $331 \mu\text{g}\cdot\text{L}^{-1}$ LAS (19%). Shrimp exposed to $65 \mu\text{g}\cdot\text{L}^{-1}$ had the highest mortality at 39%, while the highest LAS exposure treatment, of $1225 \mu\text{g}\cdot\text{L}^{-1}$, resulted in a mortality of 31%.

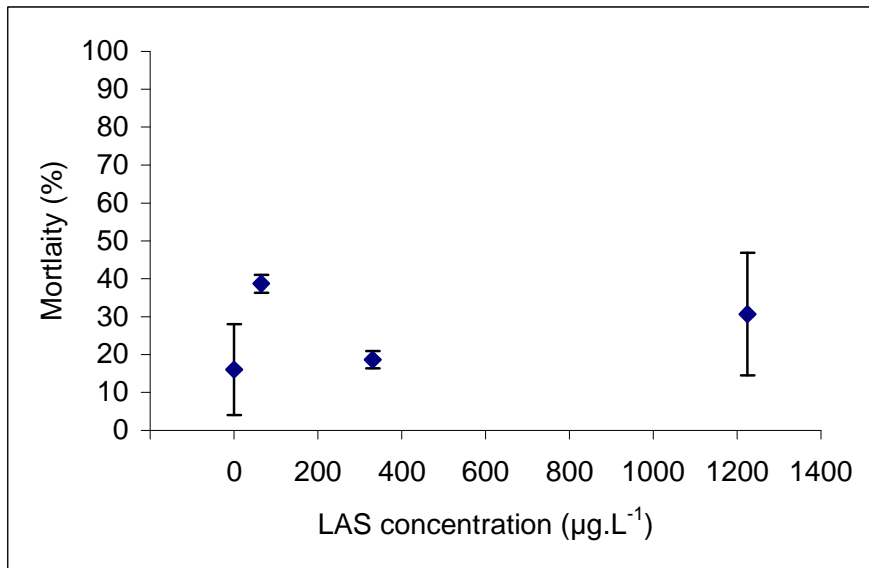


Figure 3.3 Average mortalities (\pm standard deviation) of *Caridina nilotica* after 70 days of exposure.

Growth rate of shrimp did not follow a dose response relationship (Figure 3.4) but rather a pattern similar to the average mortality recorded for each treatment (Figure 3.3). The growth rates of shrimp exposed to 65 $\mu\text{g.L}^{-1}$ and 1225 $\mu\text{g.L}^{-1}$ were significantly different from those exposed to 331 $\mu\text{g.L}^{-1}$ and the control treatment. During the first two weeks of exposure, shrimp from all treatments grew at a similar rate (initial $n = 75$ in all treatments). After four weeks of exposure, however, the fastest growth rate of shrimp was recorded from the highest exposure concentration (1225 $\mu\text{g.L}^{-1}$), with the control shrimp growing at the slowest rate. Large standard deviations associated with average growth rate at all exposure concentrations suggest variable growth rate in replicate treatments. Despite this, according to the likelihood ratio test, shrimp exposed to 65 $\mu\text{g.L}^{-1}$ ($n = 46$) and 1225 $\mu\text{g.L}^{-1}$ ($n = 52$) had significantly higher growth rates compared to shrimp exposed to 331 $\mu\text{g.L}^{-1}$ ($n = 61$) and shrimp from the control treatment ($n = 63$) (Figure 3.4).

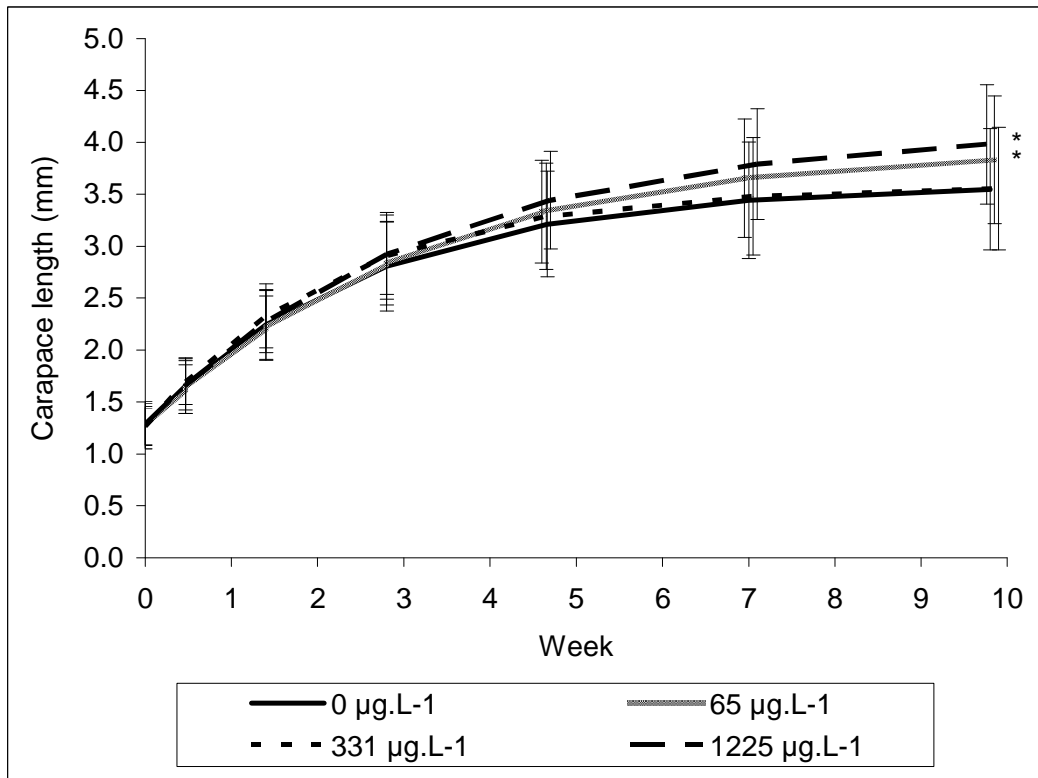


Figure 3.4 Von Bertalanffy growth rate curves determined for control ($0 \mu\text{g.L}^{-1}$) and LAS-exposed *Caridina nilotica*. Shrimp exposed to LAS concentrations of $65 \mu\text{g.L}^{-1}$ and $1225 \mu\text{g.L}^{-1}$ had significantly different growth rates to shrimp in the control treatment and those exposed to $331 \mu\text{g.L}^{-1}$ (* denotes statistically significant difference from the control).

Gravid females were observed in experimental tanks during the last two length measurements (Weeks 8 and 10). The total number of gravid females recorded for each treatment was averaged and comparisons between treatments undertaken (Figure 3.5). The average number of gravid females declined with increasing LAS concentration. The data were normally distributed and a Student's *t* test showed that the number of gravid females in the $1225 \mu\text{g.L}^{-1}$ LAS treatment was significantly lower than those in the control, resulting in a NOEC of $331 \mu\text{g.L}^{-1}$. The EC5, EC10 and EC15 values, with lower and upper 95% confidence limits, determined using the Probit model were considerably lower than the NOEC at 2.7 (0.2–9.5), 7.4 (1.0–20.5) and 14.7 (2.7–34.6) $\mu\text{g.L}^{-1}$, respectively. The EC20, determined through interpolation was $29.7 \mu\text{g.L}^{-1}$. The number of juveniles produced in each tank during the whole experimental period were summed and compared between treatments (Figure 3.6). Treatments had different variations

around the mean however, and consequently the data were log transformed. As the data were normally distributed, a Student's *t* test was undertaken, revealing significantly fewer juveniles produced by shrimp exposed to 1225 $\mu\text{g.L}^{-1}$ compared with control shrimp, resulting in a NOEC of 331 $\mu\text{g.L}^{-1}$ (Figure 3.6). The EC5, EC10 and EC15, with lower and upper 95% confidence limits, determined using the Probit model were 6.3 (2.0–12.5), 10.5 (4.0–18.9) and 14.8 (6.3–25.0) $\mu\text{g.L}^{-1}$, respectively. The EC20, determined through interpolation was 11.7 $\mu\text{g.L}^{-1}$. It was not possible to determine the ratio of gravid females to total number of females, or number of juveniles to reproductively capable females, as it was not possible to discern the sex of live adult shrimp (unless gravid) without physically harming them.

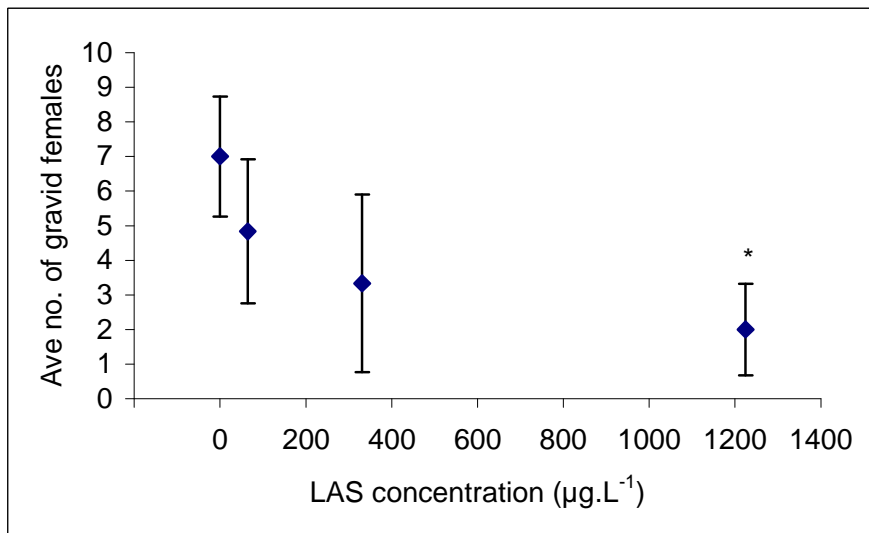


Figure 3.5 Average number of gravid female shrimp per treatment after 10 weeks exposure to increasing concentrations of LAS (* denotes statistically significant difference from the control).

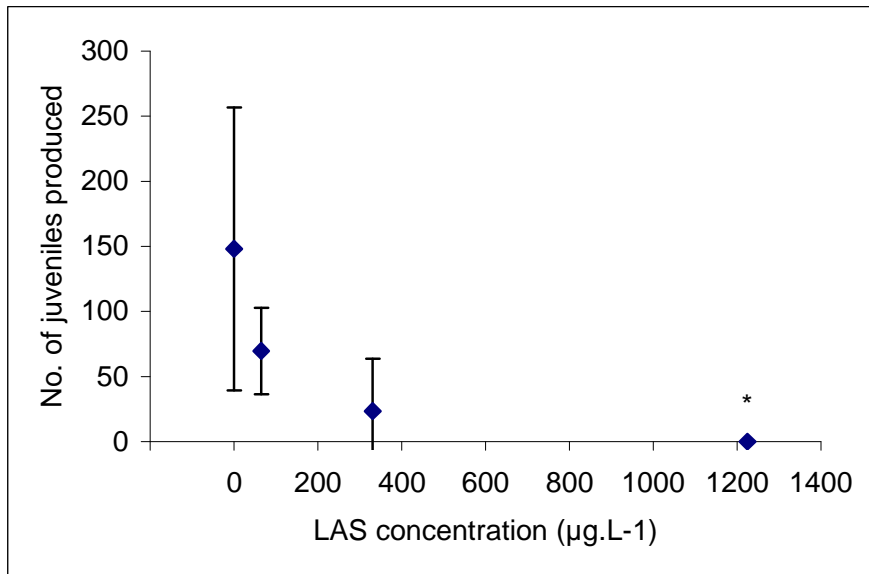


Figure 3.6 Average number of juveniles produced per treatment after 10 weeks exposure to increasing concentrations of LAS (* denotes statistically significant difference from the control).

Discussion

Shrimp growth (based on Von Bertalanffy growth curves determined using the MULTIFAN-CL approach) appears to reflect the number of shrimp within each tank rather than the LAS exposure concentration. Faster growth rates were recorded in treatments with higher mortalities (i.e. there were fewer shrimps in each tank) but also resulted in larger individuals at the end of the experiment (Figure 3.3 and Figure 3.4), suggesting a density-dependent relationship with growth. This despite shrimp from all treatments being fed fish food flakes to satiation. A possible explanation is that *C. nilotica* has the ability to feed on algae and bacteria growing on faecal pellets (Hart 1980) and other surfaces within the experimental vessel, resulting in potentially more nutritious natural food being available in experimental vessels with fewer individuals. Algae are relatively tolerant to LAS exposure, with Van de Plaasche et al. (1999) reporting NOEC values of 800–15000 µg.L⁻¹.

The differentiated allocation of energy reserves between growth and reproduction is also a possible explanation for the lower growth rate in shrimp from control treatments (where the highest number of gravid females were recorded) and for the higher growth rate in the highest LAS exposure treatment (where only 2.00 ± 1.32 gravid females were

recorded) (Figure 3.5). However, the intermediate exposure treatments of 65 $\mu\text{g.L}^{-1}$ and 331 $\mu\text{g.L}^{-1}$ did not follow this trend, suggesting that the number of individuals within each treatment possibly overrides other factors (such as energy allocation and LAS exposure concentration) in dictating growth pattern. The possibility of density dependent growth confounds the use of growth rate as a possible stress response measure for LAS exposure in this experiment.

The reproductive measures of 'average number of gravid females' and 'number of juveniles produced' followed a dose response relationship with LAS exposure. More gravid females (Figure 3.5) and considerably more juveniles (Figure 3.6) were observed in the control treatment compared to the next highest concentration of only 65 $\mu\text{g.L}^{-1}$. However, it was only at concentrations of 1225 $\mu\text{g.L}^{-1}$ that number of gravid females and juveniles produced were statistically lower than in the control treatment. Consequently, a NOEC of 331 $\mu\text{g.L}^{-1}$ for both reproductive stress responses could be determined, with respective EC5, EC10 and EC15 values being 2.7, 7.4 and 14.7 $\mu\text{g.L}^{-1}$ for the average number of gravid females and 6.3, 10.5 and 14.8 $\mu\text{g.L}^{-1}$ for the number juveniles produced. This NOEC corresponds well with the NOECs of 270 $\mu\text{g.L}^{-1}$ derived by Belanger et al. (2002) using a comprehensive model stream ecosystem study with a range of aquatic invertebrates, as well as the PNEC of 250 $\mu\text{g.L}^{-1}$ derived by Dyer et al. (2003) from a species-sensitivity distribution of LAS sub-lethal toxicity data. Although an EC20 is not produced by the Probit model, it would appear that this value for shrimp fecundity would be considerably lower than the EC20 of 387 $\mu\text{g.L}^{-1}$ (normalised to C11.6) for growth of the bivalve *Corbicula fluminea* (Versteeg and Rawlings 2003).

3.5.2 Collation of long-term sub-lethal toxicity data

As with the collation of short-term lethal (Section 3.3.2), and long-term lethal (Section 3.4) toxicity test results, long-term sub-lethal data available in Feijtel and Van de Plassche (1995) and Van de Plassche et al. (1999) were not used. Only data directly available from the literature, which met the criteria developed by Hobbs et al. (2005), or data already verified by the OECD (2005), were collated. Table 3.4 presents these validated single-species long-term sub-lethal data. Those long-term sub-lethal stress responses measured in mesocosms, reflecting multispecies or community effects, are incorporated in a separate table (Table 3.5).

The predominant long-term sub-lethal stress responses summarised in Table 3.4 are growth and fecundity, with representation from fish, crustaceans, insects, a mollusc, and a macrophyte. There was a large range in sensitivity to LAS exposure, with the lowest NOEC datum ($331 \mu\text{g.L}^{-1}$) being fecundity of *C. nilotica* over 70 days and the highest ($14377 \mu\text{g.L}^{-1}$) being growth of *Hydrocharis dubis* over 14 days (Table 3.4). The low NOEC for *C. nilotica* could be a consequence of the longer exposure period used to determine the fecundity response datum. Exposure periods for other NOECs presented in Table 3.4 are in most cases considerably shorter. From these data there appeared to be no particular biological stress that represented a more susceptible response to LAS. This could be due to the nonspecific narcosis mode of action of this chemical. It is interesting to note that, generally for fish, long-term lethality stress responses (Table 3.3) were more sensitive than the sub-lethal stress responses (Table 3.4). This was not the case for crustaceans. From the results of the long-term exposure of *C. nilotica* to LAS, as described above, it appears that the sub-lethal stress responses measured experimentally may not always reflect an accurate and direct response to toxicant exposure. For example, the growth of *C. nilotica* appeared to relate more to the density of organisms within the exposure vessel than to the concentration of LAS. Versteeg and Rawlings (2003) also suggest that the low EC_{20} for growth observed in *Corbicula fluminea* reflects an indirect effect of LAS exposure, since bivalves have been observed to siphon lower volumes of water when they detect the presence of toxicant and hence feed less and grow less. Nevertheless, both direct and indirect sub-lethal responses to LAS exposure were considered in this study.

Table 3.4 Long-term (> 96 hr) freshwater sub-lethal data for toxicity responses at organism and population levels of biological organisation. All NOECs from literature normalised to C11.6 LAS (n = number of toxicity trials).

Organism	Stress response and exposure period (days)	NOEC (or otherwise stated) ($\mu\text{g.L}^{-1}$) and chain length	NOEC (or otherwise stated) ($\mu\text{g.L}^{-1}$) normalised to C11.6	n	Source
<i>Caridina nilotica</i> ¹	Fecundity, 70d	331 (C11.6)	331	1	A
<i>Corbicula fluminea</i> ²	Growth, 32d	EC ₂₀ 270 (C12.0)	387	1	B
<i>Lepomis macrochirus</i> ³	Growth, 28d	1000 (C11.6)	1000	1	C [#]
<i>Daphnia magna</i> ¹	Fecundity, 21d	1180(C11.8)	1413	2	D [#]
<i>Ceriodaphnia dubia</i> ¹	Fecundity, 8d	1000 (C12.0)	1434	1	E
<i>Poecilia reticulata</i> ³	Behaviour, growth, 28d	3200 (C11.1)	2040	1	F
<i>Daphnia magna</i> ¹	Fecundity, 21d	1990 (C11.8)	2383	2	G [#]
<i>Chironomus riparius</i> ⁴	Emergence, 24d	2400 (C11.8)	2874	1	H [#]
<i>Pimephales promelas</i> ³	Fecundity, 40d	5100 (C11.2)	3557	1	I
<i>Ceriodaphnia silvestrii</i> ¹	Fecundity, 8d	2500 (C12.0)	3584	1	E
<i>Paratanytarsus parthenogenica</i> ⁴	Fecundity, 28d	3400 (C12.0)	4875	1	J [#]
<i>Hydrocharis dubis</i> ⁵	Growth, 14d	10000 (C12.0)	14377	1	K

Notes: [#] Validated by OECD (2005); ¹ Crustacean; ² Mollusc; ³ Fish; ⁴ Insecta; ⁵ Macrophyte

Sources: A) Current study; B) Versteeg and Rawlings 2003; C) Maki 1981 cited in OECD 2005; D) Maki 1979 cited in OECD 2005; E) Da Silva Coelho and Rocha 2010; F) Canton and Slooff 1982; G) Taylor 1985 cited in OECD 2005; H) Pittinger et al. 1989 cited in OECD 2005; I) Holman and Macek 1980; J) Maki 1981 cited in OECD 2005; K) Wu et al. 2010.

Multispecies, or community responses, to LAS exposure were reviewed and discussed in Van de Plaasche et al. (1999), Versteeg et al. (1999) and Belanger et al. (2002). Whether undertaken indoors or outdoors, the exposures could be broadly divided into lentic and lotic ecosystem models. In the studies reviewed by Belanger et al. (2002), the lentic studies had higher NOECs than the lotic studies, with mean NOECs \pm standard deviations of 5720 ± 7640 and $530 \pm 430 \mu\text{g.L}^{-1}$, respectively. The differences in NOECs are attributed to differences in the experimental design. The lentic studies were predominantly single-dose exposures resulting in reduced LAS concentration over time as a consequence of biodegradation and sorption of the LAS homologue. Lentic studies also focused on autotrophic organisms, which appeared to be more tolerant of LAS compared to fish and invertebrates (Van de Plassche et al. 1999). In contrast, lotic

studies were almost always flow-through systems with continuous LAS dosing. These tended to include a broader range of test organisms, particularly benthic organisms and fish, which generally appeared to be more sensitive to LAS (Belanger et al. 2002). As the lotic studies tended to reflect a more realistic scenario of LAS input to the aquatic environment and measured responses from a wider range of more sensitive organisms, it was decided for the purpose of the present thesis, to rely only on the lotic studies for an assessment of the multispecies or community responses to LAS.

For the lotic studies it was decided to exclude the study by Tattersfield et al. (1996) as it failed the validation process used by OCED (2005). The reasons cited for this failure were a restrictive test design and inconsistencies in the data. The original reference for Holt and Mitchell (1994) could not be obtained as it was published in the proceedings from a conference held in China and was not reviewed in OECD (2005). The LAS exposure concentrations reported in Takamura (1995), although measured, were reported as being 'mostly between 1 and 2 mg.L⁻¹'. For this reason, the results from this study were not considered, particularly as they were also conflicting, since no differences between the total number of emergent midges in the control and exposed treatments were indicated. Yet a significant difference was observed in both exposed treatments if the ratio of midges failing to emerge to the total number of trapped midges was considered. In neither case could a NOEC be determined.

The long-term multispecies or community responses to LAS exposure in lotic ecosystem models are presented in Table 3.5. The NOEC recorded by Lewis et al. (1993) was the highest (1441 µg.L⁻¹), probably reflecting the increased tolerance of autotrophic organisms to LAS. The NOEC (472 µg.L⁻¹) determined by Fairchild et al. (1993) should be interpreted with caution as it was the only concentration tested and consequently the NOEC could have been higher. Overall, the NOEC determined by Belanger et al. (2002) of 384 µg.L⁻¹ (normalised to C11.6) was considered by the OECD (2005) to be the most reliable, robust and defensible value for freshwater ecosystems. In Belanger et al. (2002), a wide range of responses from a variety of organisms (community structure and function of periphytic microbes and benthic invertebrates, drift of benthic invertebrates, and emergence of adult invertebrates) were measured over a 56-day exposure period. An integrated model ecosystem NOEC, calculated using principal response curve analysis of 293 µg.L⁻¹ (LAS C12), was determined and adjusted to 268 µg.L⁻¹ (LAS C12)

using an LAS bioavailability model determined for the model ecosystem (Belanger et al. 2002).

Table 3.5 Long-term (>96 hr) freshwater multispecies or community responses to LAS exposure in lotic ecosystem models. All NOECs from literature normalised to C11.6 LAS.

Communities assessed and exposure period (days)	Affected parameters	NOEC ($\mu\text{g.L}^{-1}$) and chain length	NOEC ($\mu\text{g.L}^{-1}$) normalised to C11.6	Source
Benthic microbial and invertebrate communities, 56d	Increased drift, reduced benthic invertebrate abundance	268 (C12)	384	A
Benthic microbial and invertebrate communities, 45d	No effects observed at only concentration measured (360 $\mu\text{g.L}^{-1}$)	360 (C11.9)	472	B [#]
Benthic algal community, 21d	Photosynthetic inhibition, number of taxa	1100 (C11.9)	1441	C

Notes: [#] Validated by OECD (2005)

Sources: A) Belanger et al. 2002; B) Fairchild et al. 1993; C) Lewis et al. 1993

3.6 Sub-lethal toxicity data: cellular and sub-cellular responses

There were two objectives to the study described in this section.

The first objective was to identify what cellular and sub-cellular response data to LAS exposure are available in the literature and to assess their quality in terms of application to WQG derivation using Hobbs et al. (2005).

The second objective was to identify the challenges of generating these data to the quality required for WQG derivation.

The second objective was investigated with particular reference to cellular and sub-cellular data because the exposure protocols and biochemical response assays used to generate these data are generally not as well standardised as toxicity tests used to generate lethal and sub-lethal reproductive and growth responses. Consequently, an opportunity existed to identify particular problems associated with the generation of

cellular and sub-cellular data that possibly contribute to the reluctance of many (Clark et al. 1999; Adams and Tremblay 2003; Moore et al. 2004; Tannenbaum 2005; Forbes et al. 2006) to use these data in water resources management. The discussion of the identified challenges is presented in Section 3.6.4.

In addressing the second objective, this study chose to investigate cholinesterase activity (ChE), lipid peroxidation (LPx) and stress protein responses to LAS exposure. All are indicators of impairment and damage to cellular and sub-cellular functioning within the organism. An inhibition of ChE leads to malfunctions of the nervous system and neurological malfunctions and has the potential to cause detrimental effects at higher levels of organisation (Eason and O'Halloran 2002). Lipid peroxidation is a widely used indicator of oxidative stress in organisms. Oxidative stress to fundamental biomolecules and antioxidant defences of organisms can result in severe impairment and can lead to disease (Livingstone 2001). Lastly, stress protein analysis has the potential to identify more specific protein damage (or proteotoxicity) in organisms exposed to xenobiotics.

3.6.1 Stress proteins

Introduction

In order to be biologically active, the majority of cellular proteins must fold into a unique three-dimensional structure (Radford 2000). Yet they are synthesized as linear chains of amino acids on ribosomes. Consequently, many of these proteins require assistance to fold. This is provided by 'helper proteins', collectively known as 'molecular chaperones'. Chaperones assist the folding process by taking part in the biosynthetic process and by capturing mis-folded states in the cytosol post-translationally, allowing renewed opportunities to refold (Radford 2000). Many of these molecular chaperones are stress-inducible and although they have housekeeping functions, under stressful physiological conditions some have important damage-control functions during, and following, stress (Ben-Zvi and Goloubinoff 2001).

The first stress-inducible proteins were described in cells from *Drosophila melanogaster*, and were induced during exposures to high temperatures (Ritossa 1962), so were termed 'heat shock proteins' (Hsp). Since then, a range of environmental stressors have been shown to induce Hsp (Lindquist 1986), and the term 'stress protein' has also been

used to describe them (Lewis et al. 1999). There are four major families of stress proteins, divided according to their molecular weight, measured in KiloDaltons (KDa). Nomenclature of these forms may vary, but generally those belonging to the 90 KDa family (with variants between 80 and 100 KDa) are referred to as Hsp90 or stress-90; 70 KDa stress proteins (with variants between 65 and 75 KDa) are referred to as Hsp70 or stress-70; and 60 KDa proteins as Hsp60 or chaperonin. Proteins with molecular weights between 16–40 KDa are referred to as Low Molecular Weight (LMW) proteins (in-depth reviews of the different stress proteins have been undertaken by Gething and Sambrook (1992) and Sanders (1993)). Each of the four major families of stress proteins comprises multiple isoforms, each of whose synthesis is independently regulated (Lindquist 1986; Bierkens 2000).

Besides playing an important role in physiological processes that involve rapid breakdown and reorganisation of tissues (such as larval settling, moulting and changes in life stage (Sanders 1993)) under adverse conditions — physical, chemical or biological — stress proteins are also induced to counter the resultant protein damage (or proteotoxicity) experienced by an organism (Bierkens 2000). The primary mechanism of toxicity caused by exposure to adverse conditions is protein denaturation, occurring as a consequence of weakening polar bonds and exposure of hydrophobic groups, resulting in unravelling and misfolding of the protein chain and subsequent protein aggregation (Sanders 1993). These damaged proteins trigger the activation of stress genes (Ananthan et al. 1986), which in turn leads to the activation of the stress proteins themselves (Bierkens 2000). The activated stress proteins are thought to counter these proteotoxic effects in two ways: by preventing denaturation of proteins by holding them in a state of folding or assembly to facilitate repair (Gething & Sambrook 1992); and by promoting the degradation of abnormal proteins or protein aggregations (Bierkens 2000). In addition to isoforms that are synthesised specifically in response to adverse conditions (termed inducible Hsp), some members of Hsp90, Hsp70 and Hsp60 are also synthesised under normal unstressed conditions. Collectively, they are called 'heat shock cognates' (Hscs) and play a critical role in regulating protein homeostasis, i.e. ensuring correct spatial and folding arrangements of cellular proteins (Lewis et al. 1999). When the organism is exposed to adverse physical and/or chemical conditions causing proteotoxicity, the synthesis of some Hscs is increased by two to three times (Pockley

2003) and the HsCs take on additional roles of repairing denatured proteins (Sanders 1993; Bierkens 2000; Ben-Zvi and Goloubinoff 2001).

As a polar narcotic, LAS acts by nonspecific disruption of various bioactive macromolecules, for example proteins (Nielsen et al. 2000). The induction of stress proteins following exposure to anionic surfactants has been investigated in only a few organisms. Pyza et al. (1997) determined Hsp70 synthesis in the centipede *Lithobius mutabilis* on exposure to LAS, but no dose-dependent relationship. The green algae *Raphidocelis subcapitata* and bacteria *Escherichia coli* have been exposed to sodium dodecyl sulphate (SDS: also a surfactant), with Hsp70 being produced in a dose-dependent manner by *R. subcapitata* (Bierkens et al. 1998a), and four unique proteins induced in *E. coli* determined by two-dimensional gel electrophoresis (Adamowicz et al. 1991). It appears (to the best of my knowledge) that freshwater aquatic invertebrates have not been investigated.

The use of stress proteins as sub-cellular-level response indicators, for estimating xenobiotics hazards, has been extensively assessed in reviews by Sanders (1993), Lewis et al. (1999) and Bierkens (2000). The induction of stress proteins has been shown to occur at toxicant concentrations lower than those causing traditional organism-population- or community-level effects (Sanders et al. 1991; Bierkens et al. 1998a; Kammenga et al. 1998). There are, however, a number of important factors that need to be considered when utilising stress proteins for tests associated with water resources management:

- Stress proteins are induced by a wide range of chemical stressors (Sanders 1993; Lewis et al. 1999; Bierkens 2000) and although certain stress proteins (for example, Hsp70) are central to the general stress response and are almost always induced (Gething and Sambrook 1992), there are times when different chemical stressors induce different families of stress proteins (Amiard et al. 2006). Consequently, a suite, or variety of stress proteins, is needed to assess contaminant exposure (De Pomerai 1996).
- As a consequence of adaption to naturally occurring fluctuations in the environment, stress proteins may be induced on a continuous basis and

subsequently mask the effects of any additional stress experienced (Sanders 1993). This is particularly the case with sessile organisms (Bierkens 2000).

- Stress proteins have been shown to exhibit seasonal (Fader et al. 1994; Pyza et al. 1997) and daily (Schill et al. 2002) synthesis cycles in relation to stress. Factors that have been shown to cause transient increases in stress protein synthesis include dietary influences (Sanders 1993; Wheelock et al. 2002; Rossi et al. 2006) and abiotic stressors such as pH, temperature, humic acids, nitrates and phosphates (Pyza et al. 1997; Bierkens et al. 1998b). Nevertheless, Sanders (1993) reports that stress proteins do not appear to be induced by reasonable sample handling.
- Although Bierkens et al. (1998b) suggest that stress proteins are activated early on in the cellular events following toxin exposure, other authors warn that induction of stress proteins can sometimes be a secondary consequence of the damage incurred by the primary receptor of a specific chemical assault (Sanders 1993). Consequently, stress protein responses might reflect total damage to proteins only, instead of more specific subtle toxicological effects. The stress protein would therefore be a less sensitive biomarker than assays based on the primary target (De Pomerai 1996).

In the pages that follow, a preliminary investigation into potential stress protein induction in response to LAS exposure in four South African indigenous aquatic invertebrates is presented. Four organisms were initially chosen: laboratory-reared flatworm *Dugesia* sp. and shrimp *C. nilotica*; wild-caught mayfly *A. auriculata* and Ancyliidae limpet *B. stenochorias*. However, the flatworm and mayfly stress protein analyses yielded poor resolution of protein profiles. Results from tests done on these organisms were therefore excluded from this thesis.

Methods

Shrimp and limpets that survived the 96 hr toxicity exposure trials, described in Section 3.3.1, were utilised for stress protein analysis. Full details of the experimental exposures and stress protein analysis procedure are included in Chapter 2. Shrimp were exposed to nominal LAS concentrations of 0, 1000, 2000, 4000, 8000 and 16000 $\mu\text{g}\cdot\text{L}^{-1}$ and limpets to 0, 500, 1000, 1500, 2000 and 4000 $\mu\text{g}\cdot\text{L}^{-1}$. Measured LAS concentrations were always within 20% of nominal concentrations and consequently results are

reported as nominal concentrations. General qualitative protein analysis was undertaken by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis to attempt to identify the specific stress proteins being induced (Chapter 2).

Results

Shrimp *Caridina nilotica*

The SDS-PAGE gel showed evidence of the presence of ≈ 90 and ≈ 70 kDa proteins in unexposed shrimp and shrimp exposed to all LAS concentrations (Figure 3.7: see data labels a and b). There appeared to be induction of a putative ≈ 70 kDa protein at the highest LAS concentration (data label c), and induction of putative ≈ 45 and ≈ 40 kDa proteins at concentrations of approximately $4000 \mu\text{g.L}^{-1}$ LAS and above (Figure 3.7: see data labels d and e).

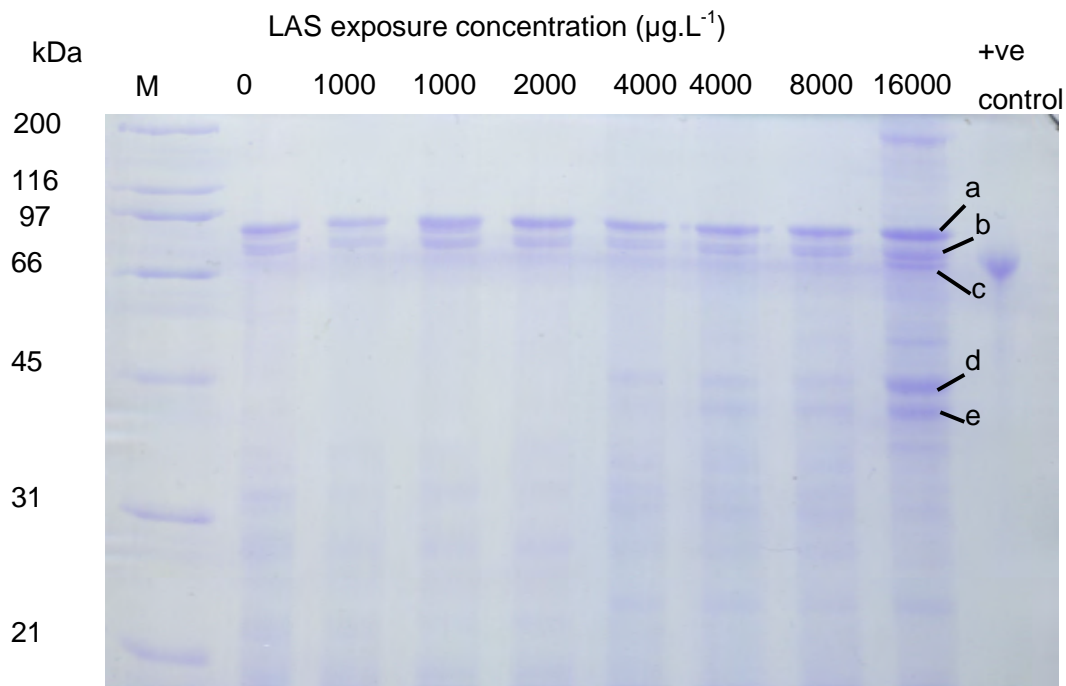


Figure 3.7 SDS-PAGE gel of *Caridina nilotica* exposed to increasing concentrations of LAS (protein content per well = $10.8 \mu\text{g}$).

Legend: M = molecular marker; +ve control = DNA K (Hsp70); a = ≈ 90 kDa protein; b = ≈ 70 kDa protein; c = induced putative ≈ 70 kDa protein; d = induced putative ≈ 45 kDa protein; e = induced putative ≈ 40 kDa protein.

Note: repeated lanes of $1000 \mu\text{g.L}^{-1}$ and $4000 \mu\text{g.L}^{-1}$ are replicate samples.

In Western blot analysis of *C. nilotica*, neither the stress-inducible Hsp70 (Hsp72) nor the Hsp40 proteins were detected when using antibodies specific for these proteins (Figure 3.8a and b). However, an Hsp70 was detected at the highest concentration of LAS exposure when using the antibody capable of detecting the inducible and constitutive Hsp70s (anti-Hsp72/Hsc73) (Figure 3.8c). Thus, the lower bands are possibly the inducible Hsp70 (Hsp72) and the upper band visible in all lanes (except the positive control which consists of purified Hsp72) possibly the constitutively produced Hsp70 (Hsp73) (Figure 3.8c).

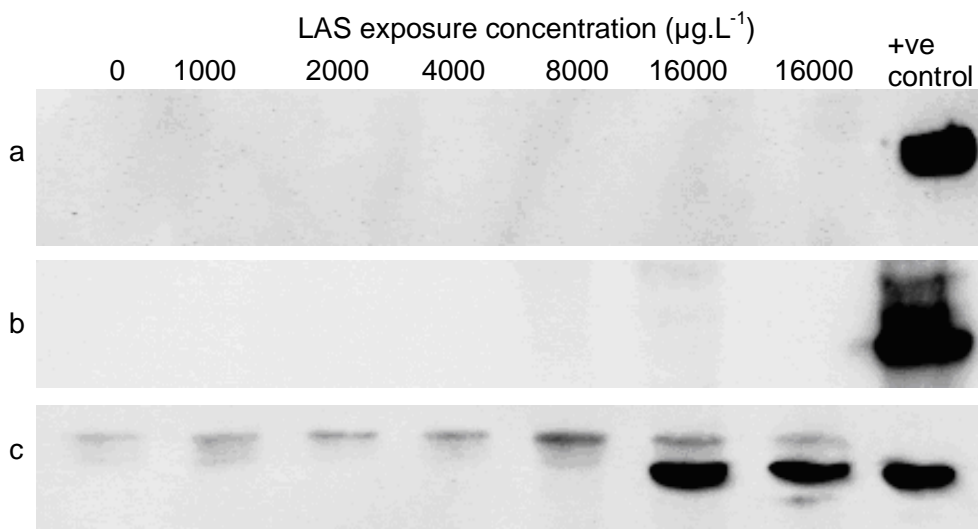


Figure 3.8a-c Western blots of SDS-PAGE gels of *Caridina nilotica* probed with (a) anti-Hsp72, (b) anti-Hsp40 and (c) anti-Hsp72/HSc73. No detection was made at any of the exposure concentrations when probed with anti-Hsp72 and anti-Hsp40 (Panels a and b). However, a Hsp70 protein was detected in the two replicates of *C. nilotica* exposed to 16000 $\mu\text{g.L}^{-1}$ LAS when probed with anti-Hsp72/Hsc73 (Panel c).

Legend: Panel a (image exposure time = 800sec; protein content per well = 6 μg ; positive control = purified human Hsp72); Panel b (image exposure time = 2 sec; protein content per well = 9.1 μg ; positive control = heat shocked baby hamster kidney (BHK-21) cells); Panel c (image exposure time = 250 sec; protein content per well = 18.7 μg ; positive control = purified human Hsp72).

To measure the induced protein banding observed at the ≈ 45 and ≈ 40 kDa levels in the SDS-PAGE gels (Figure 3.7), the increase in fold intensity was determined. Although this technique does not allow for complete identification of the protein being measured, it can be useful as a preliminary indication of the LAS concentration causing protein induction. The Kruskal Wallis Two Sample Test indicated that the average fold intensity,

of ≈ 45 kDa bands from *C. nilotica* exposed to $8000 \mu\text{g.L}^{-1}$ and $16000 \mu\text{g.L}^{-1}$ LAS, was significantly higher than *C. nilotica* exposed in the control ($p \leq 0.05$) (Figure 3.9). For the ≈ 40 kDa band, a significant difference was found for *C. nilotica* exposed to $16000 \mu\text{g.L}^{-1}$ LAS ($p \leq 0.05$).

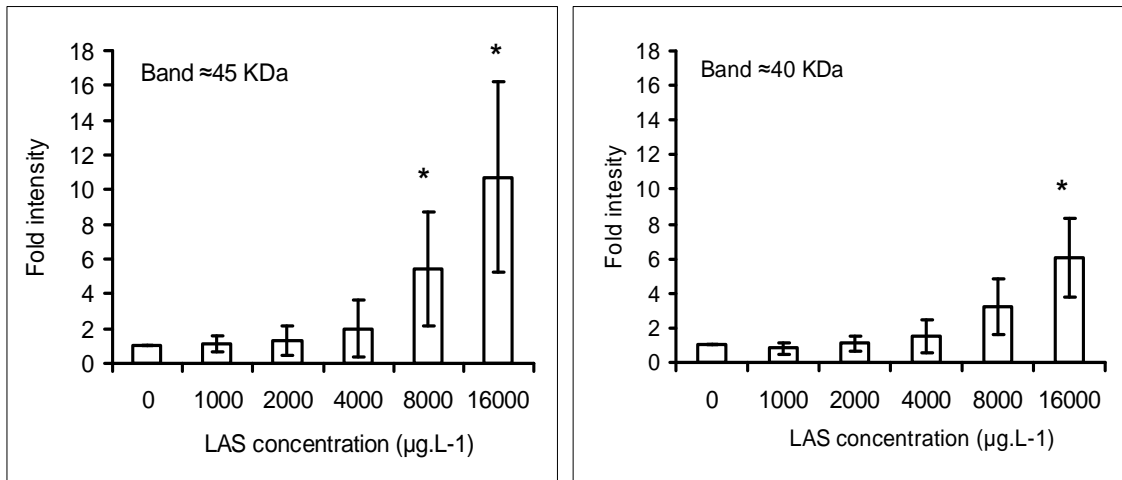


Figure 3.9 Fold intensity (with standard deviations) of induced proteins corresponding with ≈ 45 (left graph) and ≈ 40 kDa (right graph) on SDS-PAGE gels representing *Cardina nilotica* exposed to increasing concentrations of LAS for 96 hr (* represents statistical significance of treatment compared with control at $p \leq 0.05$).

The cumulative mortalities of *C. nilotica* exposed to LAS at the end of the 96 hr trial (from which surviving organisms were removed for stress protein analyses) are presented in Table 3.6. Mortality within the control was considered acceptable at 15% for a field-collected organism. At the first significant indication of ≈ 45 kDa stress protein induction ($8000 \mu\text{g.L}^{-1}$ LAS) mortality was already 57.4%. The induction of Hsp72 occurs at $16000 \mu\text{g.L}^{-1}$ and corresponded to a cumulative mortality of 62.7% (Table 3.6).

Table 3.6 Percentage cumulative mortality for *Caridina nilotica* exposed to increasing concentrations of LAS for 96 hr (mean \pm standard deviation).

LAS concentration ($\mu\text{g.L}^{-1}$)	Percentage cumulative mortality at 96 hr
0	14.5 \pm 0.8
1000	14.1 \pm 14.4
2000	24.7 \pm 5.7
4000	33.3 \pm 16.5
8000	57.4 \pm 7.9
16000	62.7 \pm 15.6

Limpet *Burnupia stenochorias*

Significant quantities of protein within the \approx 200, \approx 60, \approx 45 and \approx 40 KDa areas were evident from the SDS-PAGE gels undertaken, although there appeared to be no specific induction of stress proteins with increasing concentrations of LAS (Appendix 4; Figure A). Western blot analysis of SDS-PAGE gels of *B. stenochorias* using anti-Hsp72 could not detect the presence of this protein within *B. stenochorias* exposed to increasing concentrations of LAS (Figure 3.10a). However, anti-Hsp40 detected Hsp40 synthesis in the unexposed and exposed organisms (Figure 3.10b).

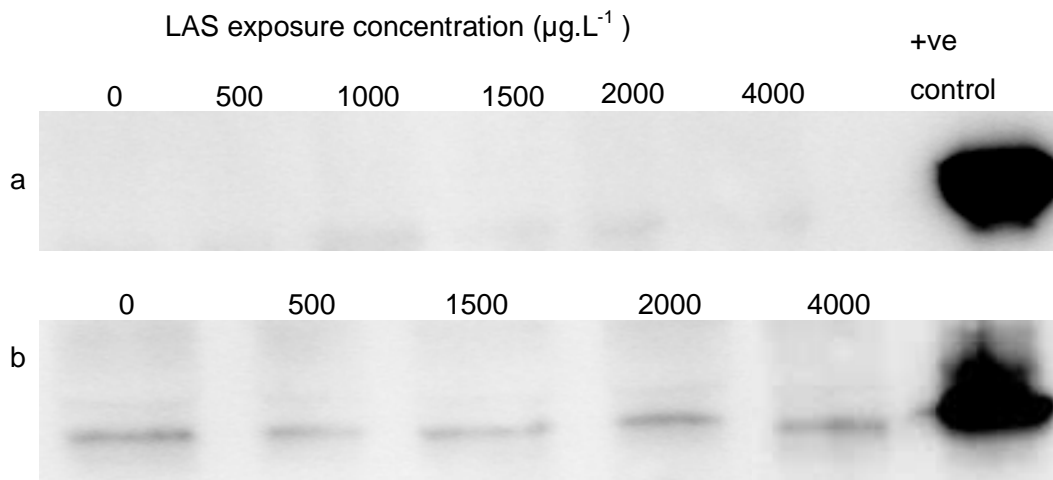


Figure 3.10a–b Western blots of *Burnupia stenochorias* probed with anti-Hsp72 (Panel a) and anti-Hsp40 (Panel b). No detection was made at any of the exposure concentrations when probed with anti-Hsp72 (Panel a). A Hsp40 protein was, however, detected within control and exposed organisms when probed with anti-Hsp40, although no apparent induction occurred (Panel b).

Legend: Panel a (image exposure time = 60 sec; protein content per well = 16 μg ; positive control = purified human Hsp72); Panel b (image exposure time = 2 sec; protein content per well = 9.5 μg ; positive control = heat shocked baby hamster kidney (BHK-21) cells).

Discussion

Not all of the antibodies used to probe the exposed organisms cross-reacted with the apparent induced stress proteins that could be observed in the SDS-PAGE gels. To fully explore the induction of stress proteins within these organisms, antibodies would have to be specifically generated for the purpose. Although this option was beyond the scope of this study, the responses observed in *C. nilotica* and *B. stenochorias* allow for some deductions.

The SDS-PAGE gels of *C. nilotica* exposed to increasing concentrations of LAS suggested induction of ≈ 70 KDa, ≈ 45 KDa, ≈ 40 KDa stress proteins. Probing with the anti-Hsp72 antibody yielded no detection of the ≈ 70 KDa protein within the exposed organisms (Figure 3.8a). Nevertheless, it is possible that the antibody could not cross-react with the particular Hsp isoforms produced by *C. nilotica*. This theory is supported by the results obtained when a third antibody, anti-Hsp72/Hsc73, appeared to cross-react with a Hsp70 in all treatments (possibly the constitutively-produced Hsp73) and

with an inducible Hsp70 in samples exposed to 16000 $\mu\text{g.L}^{-1}$ LAS (possibly Hsp72) (Figure 3.8c). Although both Hsp70 antibodies probe the inducible Hsp72, they are generated toward two different epitopes on the full-length protein.

Analysis of the SDS-PAGE gels of LAS exposed *C. nilotica* indicated a dose dependant induction of ≈ 45 KDa and ≈ 40 KDa proteins (Figure 3.9). Dose dependant relationships for stress protein synthesis have been reported in a number of studies: increasing Hsp induction with increasing toxicant concentration was observed between Hsp70 and anionic surfactant sodium dodecyl sulphate (SDS) in green algae *R. subcapitata* (Bierkens et al. 1998a) and in Hsp60 synthesis and copper concentration in mussel *Mytilus edulis* (Sanders et al. 1991). However, Pyza et al. (1997) reported no dose response relationship when *L. mutabilis* was exposed to LAS. Furthermore, dose dependent relationships can be confounded by the existence of a species-specific threshold level (concentration) above which the cellular metabolism may be too damaged to respond or act, resulting in an observed decrease in the response indicator: examples include the rotifer *Brachionus plicatis* (Cochrane et al. 1991), the isopod *Oniscus asellus* (Eckwert et al. 1997), the centipede *L. mutabilis* (Pyza et al. 1997) and the marine ascidian *Pseudodistoma crucigaster* (Agell et al. 2004). Unfortunately, in the present study, the Hsp40 antibody did not detect the induction of the ≈ 40 kDa proteins observed in the SDS-PAGE gel; thus, the protein could not be identified as Hsp40 (Figure 3.8b). The Hsp40 antibody did however weakly detect Hsp40 protein in heat-shocked *C. nilotica*, suggesting there may be a problem with the efficacy of the antibody when probing this species (Appendix 5: Figure A). According to the technical specifications for the Hsp40 antibody (Stressgen Bioreagents, USA), the specificity of this antibody has not been tested against a crustacean but has been detected in two molluscs.

Hsp40 was detected in *B. stenochorias* from all treatments (both unexposed and those exposed to LAS) (Figure 3.10b) with no evidence of induction. This suggests that either this stress protein was being continually induced within *B. stenochorias* (possibly an adaption allowing existence within a harsh environment: Bierkens 2000) or that the handling of these organisms during experiments caused excessive stress. Although handling of all the organisms was kept to a minimum, this potential effect cannot be ruled out, despite Sanders (1993) reporting that stress proteins appear to not be induced

by reasonable organism handling. The alternative explanation is based on observations that stress proteins can be produced at high levels on a continual basis as an adaptation to naturally occurring fluctuations in the environment (Sanders 1993; Bierkens 2000). Pyza et al. (1997) found no clearly correlated relationship between Hsp70 content and metal concentration gradient in field-exposed *L. mutabilis* and attributed this to the induction of Hsp70 in resident centipedes in areas that had not been impacted by abiotic stressors.

Hsp72 was induced in *C. nilotica* at 16000 $\mu\text{g.L}^{-1}$, correlating well with the concentration that resulted in statistically significant higher band densities of ≈ 45 KDa- and ≈ 40 KDa-induced proteins in this organism. These concentrations are much higher than the generally-accepted NOEC for LAS, of 384 $\mu\text{g.L}^{-1}$ (normalised to C11.6) (Belanger et al. 2002) and the Australian and New Zealand WQG for LAS of 280 $\mu\text{g.L}^{-1}$ (ANZECC and ARMCANZ 2000), although the exposure duration of the Hsp trial was considerably shorter than that used in the Belanger et al. (2002) multiple species test and in experiments undertaken to derive the Australian and New Zealand WQG. Cellular and sub-cellular responses are however meant to provide early warning signs of potential organism harm; thus, the stress proteins probed in this present study cannot be considered as sensitive indicators of LAS exposure in the two South African invertebrates. Mortalities in *C. nilotica* were 33.3% when the first stress response was observed in the SDS-PAGE gels at ≈ 45 KDa and ≈ 40 KDa; when the Hsp72 stress protein was induced at 16000 $\mu\text{g.L}^{-1}$, mortality was 62.7%. In contrast, Bierkens et al. (1998a) found that Hsp70 synthesis in *R. subcapitata*, after exposure to SDS, was at concentrations lower than the classical cytotoxicity-stress-response measures of growth inhibition and lethality. With respect to responses to other toxicants, Sanders et al. (1991) reported that Hsp60 was significantly elevated at a copper concentration one order of magnitude lower than that affecting growth in *M. edulis*. Kammenga et al. (1998), in a study exposing *Plectus acuminatus* nematodes to copper, demonstrated that Hsp60 was three orders of magnitude more sensitive than the EC20 for reproduction. When a similar experiment was conducted with cadmium, Hsp60 induction was one order of magnitude more sensitive. In these cases, stress proteins proved to be sensitive early warning response indicators. In the current study, the induction of the stress proteins in *C. nilotica* did not appear to have a direct relationship with the mechanism of toxicity of LAS. They may, however, reflect exposure to LAS and, consequently, the total

protein damage (De Pomerai 1996). The use of specific antibodies for *C. nilotica* and *B. stenochorias* in future stress protein analysis may be more successful for detecting toxic effects at lower concentrations of LAS exposure.

Conclusion

In this study, the Hsp72 stress protein was induced at 16000 $\mu\text{g.L}^{-1}$, resulting in a NOEC of 8000 $\mu\text{g.L}^{-1}$. No point estimate of toxicity could be made as Hsp72 was not induced at concentrations lower than 16000 $\mu\text{g.L}^{-1}$. Although a specific antibody could not be identified for the induced ≈ 45 KDa and ≈ 40 KDa proteins, fold intensities suggest NOECs similar to that of Hsp72. Since the stress protein NOEC value of 8000 $\mu\text{g.L}^{-1}$ determined for *C. nilotica* is much higher than the long-term lethality NOEC of 3200 $\mu\text{g.L}^{-1}$ (Table 3.3) and the fecundity NOEC of 331 $\mu\text{g.L}^{-1}$ (Table 3.4) determined for this species, the conclusion is drawn that this specific sub-cellular stress response is not an effective early warning indicator of LAS induced stress.

3.6.2 Cholinesterase assay

Introduction

Neurons of the central nervous system communicate with each other or effector organs (most often muscles) by triggering neurotransmitters from the axon of one neuron across the synaptic cleft to influence the dendrite of another cell (Chapman 1998). One such neurotransmitter is acetylcholine. Once the acetylcholine (ACh) has been triggered, another reaction is necessary to remove the ACh from the synaptic cleft in preparation for the next ACh transmission. This process is undertaken by the enzyme acetylcholinesterase (AChE), which catalyzes ACh into choline and acetic acid, thereby regulating the concentration of the transmitter at the synapse (Soreq and Seidman 2001). Acetylcholine can also be catalysed by related, but less specific, enzymes (e.g. butyrylcholinesterase – BuChE). However, AChE is most often the dominant enzyme (Soreq and Seidman 2001). The most popular method for determining AChE (Ellman et al. 1961) also detects BuChE and thus some authors refer to the enzyme activity, measured as cholinesterase (ChE) activity, and do not attempt to distinguish between the different enzyme types, while other authors just assume that AChE is the dominant enzyme being measured. Although AChE is said to be the dominant enzyme in the insect central nervous system (Charpentier et al. 2000), this thesis will refer to enzyme

activity measured as ChE activity, despite the likelihood that it is indeed AChE being measured.

Inhibition of AChE results in a build up of acetylcholine, which causes continuous and excessive stimulation of the nerves and/or muscle fibers, leading to tetany, paralysis and eventual death (Forget et al. 2003). This biochemical reaction has been exploited to produce organophosphorous and carbamate pesticides, which inhibit AChE (Charpentier et al. 2000), and the measurement of AChE inhibition has been used extensively to assess the exposure of humans (Hsieh et al. 2001) and a wide range of organisms in the field to pesticides (estuarine organisms reviewed by Fulton and Key (2001)).

An increasing number of studies have recently shown that AChE inhibition may be caused by chemicals other than pesticides. In field studies, AChE and/or ChE activities have been affected in a variety of marine and freshwater organisms exposed to sewage discharges and various industrial effluents (Payne et al. 1996; Gagne and Blaise 2004; Perez et al. 2004; Ait Alla et al. 2006; Gaitonde et al. 2006; Magni et al. 2006; Monteiro et al. 2007; Tejada-Vera et al. 2007). While in the laboratory, copper exposure caused significant AChE inhibition in the marine crab *Carcinus maenas*, a significant increase of AChE in the marine limpet *Patella vulgate*, and no effect in the marine mussel *Mytilus edulis* (Brown et al. 2004). In the marine mussel *M. galloprovincialis*, chromium (IV) significantly inhibited AChE while molybdenum and barium did not (Guilhermino et al. 1998). McLoughlin et al. (2000) showed that zinc also had no effect on AChE in *Gammarus pulex*. With regard to the salt copper sulphate, a significant inhibition of ChE in freshwater guppy *Poecilia reticulata* was observed (Garcia et al. 2000). Detergent surfactants have also been shown to affect AChE activity. Marine fish *Lateolabrax japonicus* exposed to 1000 µg.L⁻¹ sodium dodecylbenzene sulfonate (SDBS) (an LAS homologue) exhibited increased AChE activity while a different anionic surfactant, sodium dodecyl sulphate (SDS), did not affect AChE activity at 1000 µg.L⁻¹ (Jifa et al. 2005). In *M. galloprovincialis* both SDS and dodecyl benzyl sulphonate (DBS: another LAS homologue) inhibited AChE activity (Guilhermino et al. 1998). Hampel et al. (2001) report non-specific esterase inhibition in four marine microalgae exposed to LAS. In *P. reticulata*, DBS significantly inhibited ChE activity (Garcia et al. 2000), a similar result to that obtained when *Daphnia magna* was exposed to DBS and SDS (Guilhermino et al. 2000). In contrast, McLoughlin et al. (2000) found that *G. pulex*, exposed to increasing

concentrations of LAS, showed no difference in AChE activity compared to that of unexposed individuals.

Abiotic factors have been shown to affect AChE activity and have to be considered as confounding influences in biomonitoring or laboratory toxicity testing. Sea urchin *Paracentrotus lividus* coelomocytes exposed to cold stress showed increasing AChE activity with increasing exposure time (Angelini et al. 2003). Cailleaud et al. (2007) also report higher AChE activities in the marine copepod *Eurytemora affinis* exposed to low temperatures, as opposed to individuals exposed to higher temperatures. Low temperatures were also correlated with higher AChE activities in field collected freshwater mussel *Anodonta cygnea* (Robillard et al. 2003). Temperature was, however, positively correlated with AChE activity in marine mussel *Mytilus sp.* and estuarine shrimp *Crangon crangon* (Menezes et al. 2006). Robillard et al. (2003) report lower AChE activities in *A. cygnea* as pH increases or decreases from a pH of approximately 7.9.

There can be large natural variability in AChE activity too, unrelated to abiotic effects. Olsen et al. (2001) analysed freshwater *Chironomus riparius* larvae exposed at 13 uncontaminated sites for 48 hr and found two-fold variations in activity between sites, with no correlation with physical or chemical characteristics at each site. In addition, changes in total protein content of organisms have been shown to affect AChE activity (Printes and Callaghan 2003). Where the whole organism, instead of specific tissues, is used for the enzyme analysis, the total protein quantified can include structures not associated with the enzyme (e.g. eggs and embryos). This results in an underestimation of the amount of enzyme per unit of protein (Printes and Callaghan 2003).

In this study I undertook to determine if exposing four selected indigenous aquatic invertebrates (flaworm *Dugesia sp.*; shrimp *C. nilotica*; mayfly *A. auriculata*; and, limpet *B. stenochorias*) to increasing concentrations of LAS would be reflected in changes in ChE activity and, ultimately, whether this response could provide a sensitive sub-lethal indicator of stress for use in estimating the hazard of LAS to aquatic macroinvertebrates.

Methods

Shrimp, mayflies, flatworms and limpets surviving the 96 hr toxicity exposure trials, outlined in Section 3.3.1, were utilised for ChE activity analysis. Full details of the experimental exposures and ChE analysis procedure are given in Chapter 2. Shrimp and mayflies were exposed to nominal LAS concentrations of 0, 1000, 2000, 4000, 8000 and 16000 $\mu\text{g.L}^{-1}$, and flatworms and limpets to 0, 500, 1000, 1500, 2000 and 4000 $\mu\text{g.L}^{-1}$. Measured LAS concentrations were always within 20% of nominal concentrations; consequently, the results are reported as nominal concentrations. The method for determining ChE activity in the above organisms was based on the method developed by Ellman et al. (1961) and later refined by McLoughlin et al. (2000) (Chapter 2).

Results

Within-treatment variability in ChE activity, measured in the four organisms exposed to LAS, was often high. This variability was also sometimes considerably different between the different LAS exposure concentrations and consequently the data for all four organisms were log transformed to ensure homogeneity of variance before statistical analysis. Data sets from all organisms were found to be normally distributed (the Kolmogorov-Smirnov D statistic was never significant at $p \leq 0.05$) and consequently a Student's *t* test was undertaken in which ChE activity of organisms from each LAS exposure treatment was compared with ChE activities from organisms in the unexposed treatment. Limpets (*B. stenochorias*) exposed to increasing concentrations of LAS exhibited a dose-dependent increase in ChE activity, with significantly different activities being recorded at 2000 and 4000 $\mu\text{g.L}^{-1}$ (Figure 3.11a). Consequently, the NOEC for ChE activity in *B. stenochorias* exposed to LAS was estimated as 1500 $\mu\text{g.L}^{-1}$. A point estimate of toxicity could not be determined by either the Probit or Trimmed Spearman-Kärber models as the toxicity data did not fit these models. Flatworms (*Dugesia* sp.) exposed to increasing concentrations of LAS exhibited dose-dependent inhibition of ChE activity, with significantly different activities being recorded in organisms exposed to 2000 and 4000 $\mu\text{g.L}^{-1}$ (Figure 3.11b). Consequently, the NOEC for ChE activity in *Dugesia* sp. exposed to LAS is also 1500 $\mu\text{g.L}^{-1}$. The non-monotonic relationship between *Dugesia* sp. ChE activity and LAS concentration precluded a point estimation of toxicity. Shrimps (*C. nilotica*) and mayflies (*A. auriculata*) exposed to LAS exhibited no statistically significant difference in ChE activity, compared to unexposed organisms (Figure 3.11c and Figure 3.11d).

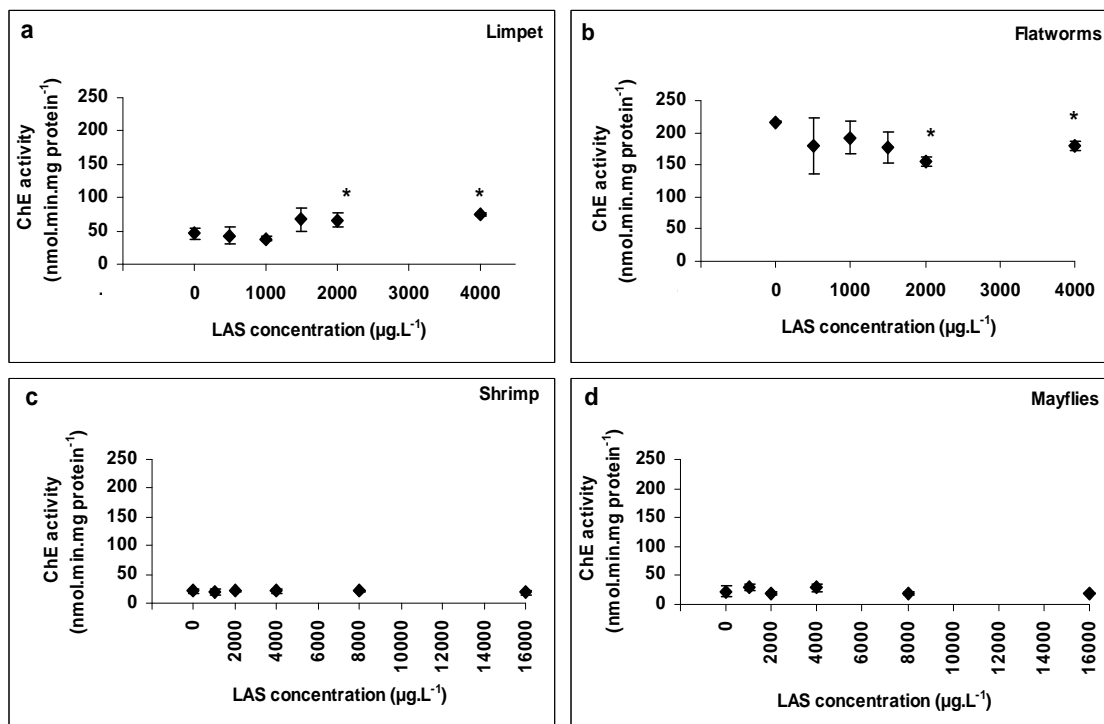


Figure 3.11 ChE activities recorded in limpet *Burnupia stenochorias*, flatworm *Dugesia* sp., shrimp *Caridina nilotica* and mayfly *Adenophlebia auriculata* exposed to LAS (* denotes significant difference at $p \leq 0.05$).

The change in ChE activity in exposed *B. stenochorias*, relative to unexposed specimens, showed a slight inhibition at low LAS concentrations, after which activity increased by approximately 45% in organisms exposed to 1500 and 2000 µg.L⁻¹ LAS, and approximately 63% in those exposed to 4000 µg.L⁻¹ (Table 3.7). At 4000 µg.L⁻¹ an increase in ChE activity of 63% correlated with 70% mortality, while at lower LAS concentrations the cumulative mortality, which varied between 15–20%, showed no discernible correlation with changed ChE activity in specimens exposed to LAS at concentrations between 0–2000 µg.L⁻¹ (Table 3.7). In *Dugesia* sp., the pattern of inhibition in ChE activity relative to unexposed individuals with increasing LAS concentration could not be correlated with cumulative mortality (Table 3.7). There were no discernible trends in ChE activity change in exposed *C. nilotica* and *A. auriculata* relative to unexposed individuals, and no correlation with cumulative mortality was measured at any LAS concentration (Table 3.7).

Table 3.7 Changes in ChE activity in limpet *Burnupia stenochorias*, flatworm *Dugesia* sp., shrimp *Caridina nilotica* and mayfly *Adenophlebia auriculata* exposed to increasing concentrations of LAS relative to unexposed organisms, and associated percentage cumulative mortality at each concentration.

Limpet			Flatworm		
LAS conc. ($\mu\text{g.L}^{-1}$)	ChE activity change relative to control (%)	Cumulative mortality (%)	LAS conc. ($\mu\text{g.L}^{-1}$)	ChE activity change relative to control (%)	Cumulative mortality (%)
0	-	8.5	0	-	0
500	-5.8	17.5	500	-17.6	0
1000	-17.2	16.7	1000	-11.1	6.7
1500	+45.4	20.0	1500	-18.0	0
2000	+44.6 *	15.0	2000	-28.3 *	0
4000	+63.4 *	70.0	4000	-17.2 *	0
8000	-	100.0	8000	-	100.0
Shrimp			Mayflies		
LAS conc ($\mu\text{g.L}^{-1}$)	ChE activity change relative to control (%)	Cumulative mortality (%)	LAS conc ($\mu\text{g.L}^{-1}$)	ChE activity change relative to control (%)	Cumulative mortality (%)
0	-	10.1	0	-	0
1000	-4.1	14.1	1000	+34.5	0
2000	+0.6	24.7	2000	-16.3	0
4000	+1.6	33.3	4000	+27.3	3.2
8000	+4.6	39.5	8000	-11.2	32.8
16000	-11.8	62.7	16000	-16.2	51.7

Note * denotes significant difference in log transformed ChE activity compared with control organisms ($p \leq 0.05$)

Discussion

The high variability in ChE activities measured in organisms from replicates within the same exposure treatment limited the power of the statistical analyses undertaken and limited the likelihood of determining significant differences between treatments. Although some organisms show large natural variability in the AChE (Olsen et al. 2001), this variability may be related to the assay protocol used. It has been suggested that the use of whole body homogenates for ChE activity analysis can lead to over- or under-estimates of the amount of enzyme per unit protein (Printes and Callaghan 2003). The use of small invertebrates, however, makes it difficult to differentiate specific tissues on which to conduct the assay. The issue of high variability in results of biochemical assays is one of the arguments used to limit their use in water resources management. With continued refinement of the assay protocol it may, however, be possible to reduce the laboratory-associated variability.

Although LAS-exposed *Dugesia* sp. exhibited inhibition of ChE, a significant increase in ChE activity was noted in exposed *B. stenochorias*. Increased ChE activity after exposure to SDBS has been observed in marine fish *L. japonicus* (Jifa et al. 2005; Jifa et al. 2006). These workers have postulated that this increase is caused by an electrical attraction between the anionic surfactant and the enzyme, which causes a change in the enzyme conformation that results in an enhanced affinity between enzyme and substrate. Brown et al. (2004), who reported an increase in AChE activity in the haemolymph of marine limpet *P. vulgate* exposed to copper, offer a different explanation, postulating that the haemolymph AChE enzyme may function in ways unrelated to cholinergic neurotransmission, and instead may have a physiological role. One of the issues — related to applying cellular and sub-cellular stress responses in general, and ChE activity specifically, to water resource management — is whether to only use data exhibiting the behaviour generally expected during a stress response. (In the case of ChE activity, inhibition is generally accepted as having an impact on an organism's neurological functions.) Jifa et al. (2005) state, however, that any abnormal content of ACh will lead to alterations in the physiology, biochemistry and even growth and reproduction, of fish. In the present study it was decided to use any deviation from normal as an indication of a sub-lethal stress response to LAS exposure.

No significant change in ChE activity was observed in *C. nilotica* and *A. auriculata* exposed to LAS, a similar result to the one reported by McLoughlin et al. (2001) in which *G. pulex* exposed to LAS showed no change in ChE activity. A significant increase in ChE activity was, however, measured in *B. stenochorias* exposed to LAS concentrations of 2000 $\mu\text{g.L}^{-1}$ and higher, while a significant inhibition in ChE activity was measured in *Dugesia* sp. flatworms exposed to LAS concentrations of 2000 $\mu\text{g.L}^{-1}$ and higher. The next lowest concentration (1500 $\mu\text{g.L}^{-1}$) was taken as the NOEC. In studies, available in the international literature, reporting the exposure of aquatic organisms to surfactants, some tolerance data are available. Li (2008) report ChE inhibition in flatworm *Dugesia japonica* at 500 and 1000 $\mu\text{g.L}^{-1}$, although this was not statistically significant. Jifa et al. (2005) report a significant difference in AChE activity in *L. japonicus* exposed to 1000 $\mu\text{g.L}^{-1}$ SDBS. As this was the only concentration tested, a NOEC could not be determined. In marine mussel *M. galloprovincialis*, only the lowest DBS concentration showed inhibition and thus a NOEC could not be determined (Guilhermino et al. 1998).

The same situation exists for *D. magna* exposed to DBS, where AChE inhibition was observed at 12500 µg.L⁻¹, the lowest concentration measured (Guilhermino et al. 2000). Lastly, *P. reticulata* exposed to DBS exhibited significant ChE inhibition at 19900 µg.L⁻¹ with a NOEC of 10100 µg.L⁻¹ (Garcia et al. 2000).

To investigate the links between biochemical responses and effects at higher levels of biological organisation, attempts were made to identify the level at which AChE activity inhibition indicates significant exposure to a chemical, and the level that will correspond to significant mortality of exposed organisms. Varo et al. (2003), citing Mayer and Ellersieck (1986), state that 20% AChE inhibition or greater is an accepted indicator of organophosphorus exposure in birds, fish and invertebrates. Fulton and Key (2001) report that most studies of estuarine fish generally indicate that brain AChE inhibition in excess of 70% of the level in control organisms is well correlated with imminent mortality. This relationship does not, however, hold true for all organisms and they identify a need to establish the specific relationship between AChE inhibition and lethality for the specific species being considered as a bioindicator (Fulton and Key 2001). In contrast, the relationship between AChE inhibition and mortality in invertebrates is less well established. In invertebrates exposed to organophosphorus, mortality is often observed in association with low levels of inhibition (Fulton and Key 2001). Fulton and Key (2001) postulate that this may be due to the use of whole body homogenates in invertebrates instead of specific neurological tissue homogenates. Frasco et al. (2006) provide another explanation, citing Bocquene et al. (1997) who describe an experiment in which two different cholinesterases were measured within the gills of oyster *Crassostrea gigas*. One of the cholinesterases was highly sensitive and the other almost insensitive to organophosphorus and carbamate pesticides. Consequently, in a case like this, the use of ChE inhibition should only be considered if the two enzymes can be separately assessed (Frasco et al. 2006). As Frasco et al. (2006) point out, however, other studies (Varo et al. 2002; Diamantino et al. 2003; Cunha et al. 2005) have described ChEs that have atypical properties, such as overlapping substrate preferences and atypical behaviour toward selective inhibitors. Classifying the activity as being due to AChE or BchE enzymes may thus be unfeasible.

Conclusions

Two of the organisms tested showed significant changes in ChE activity in exposed individuals in comparison to unexposed individuals. The NOEC derived for *B. stenochorias* and *Dugesia* sp. exposed to LAS (based on ChE activity) was 1500 $\mu\text{g}\cdot\text{L}^{-1}$. A point estimate of toxicity could not be determined for either organism. No significant trend could be observed for *C. nilotica* and *A. auriculata* exposed to increasing LAS concentrations. Further optimization of the assay protocol may improve sensitivity and precision by increasing the number of replicates per treatment, with fewer organisms per replicate allowing for better statistical analysis. Furthermore, in some organisms, where feasible, it would be advantageous to conduct analysis in specific tissues or parts of the body (e.g. just head or tail in shrimp) instead of whole body homogenates. Lastly, a greater emphasis should be placed on ensuring that organisms analysed are of similar age, size and physiological condition as possible, so as to limit variability of enzyme activity when related to protein content of the organism.

Although ChE activity appears a more sensitive indicator of LAS exposure when compared to stress protein induction, there are a number of concerns regarding its reliability as an indicator of sub-cellular stress. Only two of the four organisms exposed to LAS exhibited changes in ChE activity with increasing LAS concentration. Furthermore, the ChE activity measured in those two organisms exhibited contrasting trends, with ChE inhibition in *Dugesia* sp. and ChE increase in *B. stenochorias*.

3.6.3 Lipid peroxidation assay

Introduction

Oxygen metabolism within any aerobic organism results in the production of variously-termed pro-oxidants (e.g. free radicals, oxyradicals or reactive oxygen species (ROS)), primarily as unwanted byproducts (Livingstone 2001). These free radicals have a potent oxidative potential and interfere with cellular components, causing damage to the lipids of biological membranes, enzymes, and DNA, ultimately resulting in various diseases (Livingston 2001; Valavanidis et al. 2006). Consequently, free radicals have to be regulated by antioxidant mechanisms, either enzymatic (catalyse, superoxide dismutase and various peroxidases) or nonenzymatic (extracellular low-molecular-weight free radical scavengers) (Livingstone 2001). If the balance between free radical production

and antioxidant neutralization is disturbed, then the organism experiences oxidative stress (Valavanidis et al. 2006).

Lipid peroxidation (LPx) (the oxidation of polyunsaturated fatty acids) is an important consequence of oxidative stress (Van der Oost et al. 2003) as a number of the products formed (lipid hydroperoxides) can be toxic and highly reactive, thus augmenting free radical proliferation and oxidative stress (Esterbauer et al. 1991). One of the products formed during LPx is malondialdehyde (MDA), which can be used in the thiobarbituric acid (TBA) reactive substances test to infer the level of oxidative stress being experienced by an organism (Janero 1990). This assay needs to be used with caution, however, as MDA sometimes reacts with molecules other than TBA, neither is it a substance generated exclusively through LPx, and lastly, other lipid peroxide-derived decomposition products are also TBA positive (Janero 1990; Lykkesfeldt 2007). Despite the above constraints, the TBA test has been widely used as an indicator of lipid peroxidation caused by oxidative stress (Valavanidis et al. 2006).

In addition to endogenous factors, there are exogenous factors that generate pro-oxidants and/or inhibit antioxidant enzymes, thus disrupting the balance between pro-oxidant production and antioxidant neutralization (Valavanidis et al. 2006). These exogenous factors include certain environmental contaminants, as well as various other biotic or abiotic factors (Livingstone 2001). Laboratory and field-identified natural and anthropogenic compounds that cause oxidative stress include the following: various organic compounds such as redox-cycling compounds (quinones, nitroaromatics, nitroamines, bipyridyl herbicides), polycyclic aromatic hydrocarbons (PAHs) (benzene, PAH oxidation products), halogenated hydrocarbons (bromobenzene, dibromoethane, polychlorinated biphenyls, lindane), dioxins and pentachlorophenol, and inorganic contaminants (aluminium, arsenic, cadmium, copper, chromium, mercury, nickel, vanadium and zinc) (Winston and Giulio 1991; Livingstone 2001; Valavanidis et al. 2006). Surfactants have also been shown to cause oxidative stress. Sirisattha et al. (2004) found that LAS and SDS caused oxidative stress in yeast cells, most probably because of the damage inflicted on membrane structures or the disruption of carbon metabolism by these detergents. Bindu and Babu (2001) and Bindu et al. (2005) demonstrated that three surfactants (anionic SDS, cationic cetyl trimethyl ammonium bromide - CTAB, and nonionic Triton X-100) caused severe oxidative stress in

freshwater tilapia *Oreochromis mossambicus* when assessed by measuring antioxidant and lysosomal enzymes. They postulate that this stress is induced as a consequence of the surfactants' interactions with cell membrane lipids and proteins, resulting in lipid peroxidation. A similar conclusion was also reached by Yamaguchi et al. (2006) who investigated the effects of sodium dodecylbenzenesulfonate (DCBS) (a homologue of LAS) on rat thymocytes. Regarding aquatic organisms, Li (2008) exposed flatworm *D. japonica* to an LAS concentration range of 0–1000 $\mu\text{g.L}^{-1}$, but reported no significant LPx activity as a result. In contrast, Wu et al. (2010) recorded increased LPx in the leaves of the aquatic macrophyte *Hydrocharis dubis* exposed to 100–500 $\mu\text{g.L}^{-1}$ LAS, but this was followed by a decrease in LPx at higher LAS exposure concentrations.

Some constraints need to be considered when utilising LPx as a response indicator. Various biotic factors can also affect LPx: for example, hypoxia and hyperoxia (Livingstone 2001); diet (Van der Oost et al. 2003; Manduzio et al. 2004); and reproductive state (De Almeida et al. 2007). The environmental influence of temperature should also be noted (Van der Oost et al. 2003; Verlecar et al. 2007), although Malek et al. (2004) found that decreased temperatures did not suppress LPx potential in zebra fish *Danio rerio* despite upregulation of oxidative stress response genes. Lastly, as with the ChE assay, changes in protein concentration within the organism can affect the measured LPx activity (Lau et al. 2004).

In this study, I undertook to investigate the oxidative responses of four indigenous aquatic invertebrates (flatworm *Dugesia sp.*; shrimp *C. nilotica*; mayfly *A. auriculata*, and limpet *B. stenochorias*) to LAS exposure.

Methods

Shrimp, mayflies, flatworms and limpets surviving the 96 hr toxicity exposure trials, outlined in Section 3.3.1, were utilised for LPx analysis. Full details of the experimental exposures and LPx analysis procedure are given in Chapter 2. Briefly, shrimp and mayflies were exposed to nominal LAS concentrations of 0, 1000, 2000, 4000, 8000 and 16000 $\mu\text{g.L}^{-1}$, and flatworms and limpets to 0, 500, 1000, 1500, 2000 and 4000 $\mu\text{g.L}^{-1}$. Measured LAS concentrations were always within 20% of nominal concentrations and, consequently, results are reported as nominal concentrations. The LPx assay used here

was adapted from the method described by Ringwood et al. (2003) for estuarine species. Lipid peroxidation is reported as the MDA activity.

Results

The MDA activity in shrimps from some of the LAS exposure concentrations showed high within-treatment variability when compared to other treatments; consequently, the data were log transformed prior to statistical analysis to ensure homogeneity of variance. Differences in variability of different treatments were not as great for the remaining three invertebrate species and thus their data were not log transformed for statistical analysis. Data sets for all organisms were found to be normally distributed (Kolmogorov-Smirnov D statistic was never significant at $p \leq 0.05$) and a Student's t test was undertaken, comparing MDA activity of organisms exposed to each of the LAS treatments with the MDA activity from organisms in the unexposed treatment. The dose response in all four invertebrates to increasing LAS concentrations was of a non-monotonic nature, with an initial increase in MDA activity at the lowest LAS concentration tested but a subsequent decrease in MDA activity with increasing LAS exposure concentrations (Figure 3.12a-d). *Dugesia* sp. showed a statistically significant MDA activity increase at 500 and 1000 $\mu\text{g.L}^{-1}$ (Figure 3.12b). *Caridina nilotica* had significantly higher MDA activity at 1000 $\mu\text{g.L}^{-1}$ and a significant decrease at 16000 $\mu\text{g.L}^{-1}$ LAS (Figure 3.12c). MDA activity in *B. stenochorias* was little changed with increasing LAS concentrations (Figure 3.12a), while *A. auriculata* MDA activity increased at 1000 and 2000 $\mu\text{g.L}^{-1}$, but not significantly (Figure 3.12d).

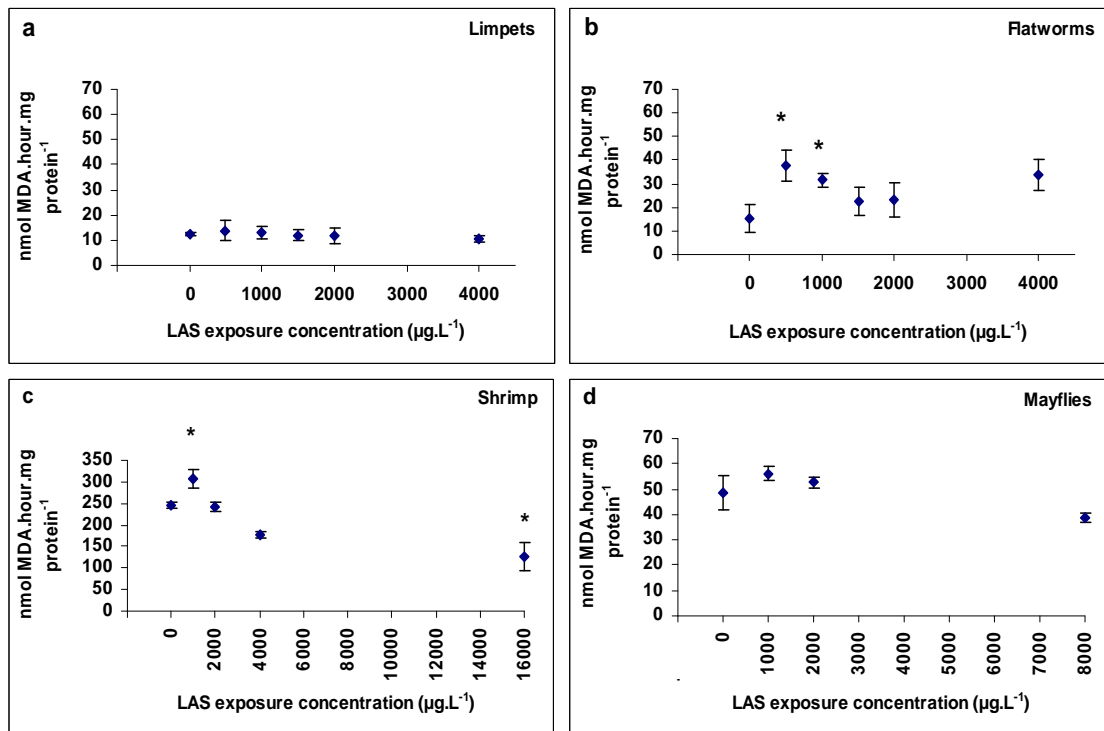


Figure 3.12a–d Malondialdehyde activities recorded in limpet *Burnupia stenochorias*, flatworm *Dugesia* sp., shrimp *Caridina nilotica* and mayfly *Adenophlebia auriculata* exposed to LAS (* denotes significant difference from unexposed organisms at $p \leq 0.05$).

The percentage increase and inhibition in MDA activity varied greatly among the species tested (Table 3.8), with *Dugesia* sp. showing a greater increase in MDA activity in exposed individuals (up to 93% increase), whereas the other species showed only moderate increases of between 12% and 25% compared to those of unexposed organisms. Despite the high increase and then decrease in percentage MDA activity observed in flatworms, mortality in these organisms was low, whereas the other organisms experienced significant mortalities but little change in percentage MDA activity (Table 3.8).

Table 3.8 Changes in malondialdehyde activity in limpet *Burnupia stenochorias*, flatworm *Dugesia* sp., shrimp *Caridina nilotica* and mayfly *Adenophlebia auriculata* exposed to increasing concentrations of LAS relative to unexposed organisms, and associated percentage cumulative mortality of individuals at each concentration.

Limpet			Flatworm		
LAS conc ($\mu\text{g.L}^{-1}$)	MDA activity change relative to control (%)	Cumulative mortality (%)	LAS conc ($\mu\text{g.L}^{-1}$)	MDA activity change relative to control (%)	Cumulative mortality (%)
0	-	8.5	0	-	0
500	+12.2	17.5	500	+93.4	0
1000	+5.9	16.7	1000	+63.5	6.7
1500	-3.4	20.0	1500	+14.2	0
2000	-4.4	15.0	2000	+18.3	0
4000	-14.9	70.0	4000	+72.9	0
Shrimp			Mayflies		
LAS conc ($\mu\text{g.L}^{-1}$)	MDA activity change relative to control (%)	Cumulative mortality (%)	LAS conc ($\mu\text{g.L}^{-1}$)	MDA activity change relative to control (%)	Cumulative mortality (%)
0	-	10.1	0	-	0
1000	+25.0	14.1	1000	+15.5	0
2000	-0.9	24.7	2000	+8.5	0
4000	-27.4	33.3	4000	-	3.2
8000	-	39.5	8000	-20.6	32.8
16000	-48.3	62.7	16000	-	51.7

Discussion

The response in MDA activity to increasing LAS exposure in all four invertebrates was an initial increase at low LAS concentrations and subsequent decrease at higher concentrations. This is a similar pattern to that reported by Wu et al. (2010) where MDA in the leaves of the aquatic macrophyte *Hydrocharis dubis* increased in plants exposed to 100 and 500 $\mu\text{g.L}^{-1}$ LAS, but then steadily decreased at concentrations up to 50000 $\mu\text{g.L}^{-1}$. Wu et al. (2010) suggest that, at lower LAS concentrations, the plant's antioxidant enzyme system had not yet been activated and hence could not protect cells from membrane damage and LPx. At higher LAS concentrations, however, they measured increased activity of peroxidase, catalase and superoxide dismutase in *H. dubis* and suggested that these enzymes were possibly responsible for reducing oxidative stress, and thus lipid peroxidation. Similarly, Sirisattha et al. (2004) report that LAS induced the pleiotropic drug-resistance network in yeast cells, resulting in the activation of drug-extrusion pumps. Thus, considering the responses of the four indigenous invertebrates exposed to LAS as part of this thesis, it is possible that

antioxidant enzyme systems were initiated in these organisms at concentrations higher than 500-1000 $\mu\text{g.L}^{-1}$. It is further possible that the internal concentration of LAS in these organisms may have been reduced through activation of drug-extrusion pumps, reducing lipid damage and thus decreasing MDA activity. In contrast, at concentrations below 500-1000 $\mu\text{g.L}^{-1}$ (depending on the species), the extrusion pumps and antioxidant enzymes were not activated and LPx occurred.

The MDA activity of unexposed shrimp (244 nmol of MDA.hour.mg protein⁻¹) was considerably higher than that of unexposed mayflies (49 nmol of MDA.hour.mg protein⁻¹), flatworms (20 nmol of MDA.hour.mg protein⁻¹) and limpets (12 nmol of MDA.hour.mg protein⁻¹). Although antioxidant levels can vary between invertebrates and vertebrates (Livingstone 2001), no mention is made of any significance regarding species-specific differences in MDA activity in unexposed organisms.

Although flatworms experienced only 6.7% mortality in the 1000 $\mu\text{g.L}^{-1}$ treatment, and no mortality at any of the other concentrations tested, the remaining invertebrates suffered up to 70% mortality at the highest concentrations when, according to the data, little LPx occurred (Table 3.8). No reports on attempts to link % MDA activity increase with an effect at a higher level of biological organisation could be found in the literature. The results from this study show no correlation either, but further investigation into effects of low LAS concentrations may enhance understanding. Regarding other xenobiotics, there is evidence that oxidative damage can be associated with some aspect of impaired cellular or higher biological function, including disease (Livingston 2001). However, differences in responses between phylogenetic groups, and the complexity of interactions between pro- and antioxidant systems and the endogenous and exogenous factors that influence them, need further investigation, to improve understanding of the links between oxidative stress at cellular levels and decreased animal fitness and associated population effects.

Conclusions

An LPx NOEC could not be determined for any of the invertebrates exposed to LAS. LOECs of 1000 $\mu\text{g.L}^{-1}$ and 500 $\mu\text{g.L}^{-1}$ could however be determined for *C. nilotica* and *Dugesia* sp. Point estimates of toxicity could not be generated due to the non-monotonic

dose response. Greater understanding of LPx in these indigenous invertebrates may be possible if further toxicity tests at lower LAS concentrations were undertaken.

The low number of replicates per treatment limited the power of the statistical tests undertaken and confidence in results obtained. It is therefore recommended that the number of replicates per treatment be increased when undertaking future studies into this topic. The number of organisms exposed in each replicate should provide enough biological material to allow for re-analysis, should the initial sample fail to provide a result. Although the within-treatment variability was lower than variability in the ChE assay, variability is still an issue of concern and, as in the ChE assay, probably relates to the use of whole body homogenates (Printes and Callaghan 2003). The reason for high variability may however be due to the MDA assay being affected by other lipid peroxide-derived products (Janero 1990; Lykkesfeldt 2007).

3.6.4 Difficulties identified in producing cellular and sub-cellular response data

In the process of determining ChE, LPx and stress protein responses for the four indigenous organisms exposed to LAS, a number of technical issues were identified. These could potentially limit the production of good-quality cellular and sub-cellular response data.

- Within-treatment variability was particularly high in the ChE assay and to a lesser extent in the LPx assay. High natural variation in AChE has been reported (Olsen et al. 2001), but this may be attributable to the assay methodology, i.e. the use of whole body homogenates of test organisms. In future research into effects on small macroinvertebrates, consideration should be given to refining these assays for use of particular body parts.
- The low number of replicate exposure vessels and high within-treatment variability limited the ability of statistical tests to determine significant differences between treatments. In the toxicity tests undertaken for ChE, LPx and stress protein response detection, three replicates per treatment were used with multiple organisms per replicate. However, for the statistical analysis employed, only one response value per replicate could be used. Thus, increasing the number of exposure vessels per treatment (i.e. replicates) with fewer organisms per vessel would improve the power of the statistical test. In a static renewal

experiment, this approach does not require a lot of extra effort. In the case of LAS, where a flow-through system is required, increasing the number of exposure vessels by any worthwhile amount does however become much more difficult.

- Inconsistent responses among organisms exposed to LAS were observed. Whereas flatworm and limpet ChE activity responded to LAS exposure, in shrimp and mayflies there appeared to be no effect on ChE activity. Similarly, there appeared to be no LPx occurring in limpets, with remaining organisms exhibiting significant MDA production. Lastly, only shrimp exhibited Hsp72 stress protein induction due to LAS exposure.
- Complicated dose-dependant responses were often observed. ChE activity in flatworms decreased with increasing LAS concentration, while in limpets ChE activity increased with increasing LAS concentrations. These results raise the issue of whether any deviation from control values is acceptable as a legitimate stress response, or do we rely on our current knowledge of cellular and sub-cellular stress response behaviour, whereby it is generally accepted that it is inhibition of ChE that has negative consequences for organisms? In flatworms, limpets and mayflies LPX, determined by measuring MDA activity, initially increased with increasing LAS concentrations, but then decreased at the higher exposure concentrations. The issue needing attention in this case is whether this type of response should be considered as representing a negative consequence of toxicity or just a compensatory mechanism to cope with the toxic exposure. This is especially the case because there was no correlation between MDA activity and mortality.

The above issues are difficult to resolve because of a relatively limited understanding of the significance of biochemical responses in organisms to toxic exposure, especially in terms of the resultant impact at the population and community levels of biological organisation. This is further complicated, in the case of LAS, by a non-specific narcotic mode of action. Consequently, there are difficulties in generating reliable biochemical data from toxicity assays and in interpreting the consequences of measured cellular and sub-cellular stress responses to the overall health of the organism, especially in terms of proving ecological relevance. These factors, together with the low numbers of biochemical data of suitable quality available in the general literature (at least for LAS –

to be discussed in section 3.6.5), limits their application in water resource management tools such as WQG derivation.

3.6.5 Assessment of sub-lethal biochemical stress response data available in the scientific literature

Eighteen publications reporting cellular and sub-cellular responses to LAS exposure were located in the literature (Table 3.9). They included responses from five types of organisms: eight species of fish, seven aquatic macrophytes, two crustaceans and one algal and turbellarian species. A wide range of stress responses were measured, including those relating to various antioxidant enzymes, cholinesterases, lipid peroxidation, and various drug metabolizing enzymes. No studies with histopathology as a stress response were found. Unfortunately, in four of the publications only one concentration of LAS was used as an exposure treatment, and in two of the publications LAS was injected into the body of the organism. Furthermore, only seven references possessed biological stress response endpoints (NOECs, LOECs or EC50s). Lastly, only five studies provided an indication of the LAS chain length. An overall assessment of the scientific rigor of toxicity testing reported in the 18 literature sources, determined using criteria proposed by Hobbs et al. (2005), considered one source to be of high quality, 15 of acceptable quality and two of unacceptable quality (Table 3.9). Of the 15 acceptable sources, eight scored 50–60%, and provided little useful information regarding cellular and sub-cellular responses to LAS. Generally, the more recent articles had higher scores as their testing methodology included the determination of a concentration response relationship, with adequate controls and replication. Consequently, much of the older data available in the literature are of little use in hazard and risk assessments. Ultimately, only six references provided potentially useful cellular and sub-cellular response data of acceptable quality (Table 3.10). The cellular and sub-cellular responses, determined when exposing indigenous invertebrates to LAS in this thesis, are also included in Table 3.10. When considering the superoxide dismutase (SOD), catalase (CAT) and LPx stress responses it appears that LAS could be causing oxidative damage at concentrations between 262 and 1000 $\mu\text{g}\cdot\text{L}^{-1}$. An effect on the nervous system through alterations in ChE activity is also observed, at concentrations above 1500 $\mu\text{g}\cdot\text{L}^{-1}$.

Table 3.9 Available literature reporting cellular and sub-cellular stress responses in freshwater aquatic organisms exposed to LAS. Quality score and class were determined according to Hobbs et al. (2005).

Organism	Biological stress response and exposure period (days unless otherwise stated)	Endpoint determined ($\mu\text{g.L}^{-1}$ unless otherwise stated) (in vivo unless otherwise stated)	LAS chain length	Source quality score (%) #	Source
<i>Hydrocharis dubis</i> ¹	Superoxide dismutase (SOD), Catalase (CAT)	NOEC = 500	C12	81	A
	Lipid peroxidation (LPx), Peroxidase (POD)	No statistically significant effect, although LPx and POD increased in a dose dependent manner			
<i>Daphnia magna</i> ²	Acetylcholinesterase (AChE), 2d	In vitro NOEC \leq 12500, IC ₅₀ = 6600. In vivo NOEC \leq 2000, EC ₅₀ = 11400	No details	79	B
<i>Gammarus pulex</i> ²	Cholinesterase (ChE) and glutathione-S-transferase (GST), 2d	No statistically significant effect	C11.9	77	C
<i>Poecilia reticulata</i> ³	ChE, 0.5hr	In vitro LOEC = 19900, NOEC = 10100, IC ₅₀ = 14600	No details	76	D
<i>Oncorhynchus mykiss</i> ³	Phagocytotic activity, 54d	NOEC = 200	C11-12	74	E
	Cellular respiratory burst activity, plasma lysosyme activity 54d	No statistically significant effect			
<i>Dugesia japonica</i> ⁴	CAT, 2d	NOEC = 100	C11.9	74	F
	SOD, LPx, ChE, 2d	No statistically significant effect, although ChE in LAS treatments decreased \pm 40% compared to average control levels			
<i>Ictalurus sp.</i> ³	Adenosine 3',5'-cyclic monophosphate (cAMP), 15d	NOEC \leq 3 ppm	No details	71	G
<i>Daphnia magna</i> ²	General enzyme inhibition, 1hr	Only EC ₅₀ of 117000 reported.	C11.2	66	H
<i>Heteropneustes fossilis</i> ³	Phosphate activity, 4d	Initial changes in activity return to control levels after 4d	No details	60	I
<i>Cirrhina mringala</i> ³	Tissue glycogen, lactic acid and sialic acid, 4d	Significant differences at only exposure concentration (0.005 ppm)	No details	60	J
<i>Puntius sophore</i> ³					
<i>Scenedesmus quardicauda</i> ⁵	DNA synthesis, 15d	Large differences from control observed at 0.05% (v/v), data presented graphically	No details	59	K

Table 3.9 (continued) Available literature reporting cellular and sub-cellular stress responses in freshwater aquatic organisms exposed to LAS. Quality score and class were determined according to Hobbs et al. (2005).

Organism	Biological stress response and exposure period (days unless otherwise stated)	Endpoint determined ($\mu\text{g.L}^{-1}$ unless otherwise stated) (in vivo unless otherwise stated)	LAS chain length	Source quality score (%) [#]	Source
<i>Channa punctatus</i> ³	Mg++ATPase, acid and alkaline phosphatase, glucose-6-phosphatase, 5-nucleotidase, 45d	Significant differences in activity of all enzymes at only exposure concentration (5mg/dm ⁻³)	No details	58	L
<i>Cyprinus carpio</i> ³	Tissue glycogen, lactic acid and sialic acid, acid and alkaline phosphatase 4d	Significant differences at only exposure concentration (0.005 ppm)	No details	57	M
	Metallothioneins and heme oxygenase, 3d	LAS injected directly into body	No details	57	N
<i>Cirrhina mringala</i> ³	Lactic acid dehydrogenase enzyme activity and lactic acid content, 30d	Significant differences at only exposure concentration (0.015 ppm)	No details	57	O
<i>Cyprinus carpio</i> ³	Various drug metabolizing enzymes and cytochrome P-450, 3d	LAS injected directly into body	No details	55	P
<i>Salvia molesta</i> ¹	Total chlorophyll-a	Decreases from control levels observed from 0.005% (v/v), data presented graphically	No details	44	Q
<i>Hydrilla verticillata</i> ¹					
<i>Ceratophyllum demersum</i> ¹					
<i>Lemna minor</i> ¹					
<i>Spirodella polyrhiza</i> ¹					
<i>Pista stratiotes</i> ¹					
<i>Scenedesmus quardicauda</i> ⁵	SOD, LPx	Differences from control levels observed at 0.02% (v/v), the lowest concentration measured	No details	42	R

Notes: [#] Literature source quality class: High quality (Hq) \geq 80%, Acceptable quality (Aq) 51-79%, Unacceptable (U) \leq 50%.

¹ Macrophyte, ² Crustacean; ³ Fish; ⁴ Turbellarian; ⁵ Alga.

Sources: A) Wu et al. 2010; B) Guilhermino et al. 2000; C) McLoughlin et al. 2000; D) Garcia et al. 2000; E) Bakirel et al. 2005; F) Li 2008; G) Pareschi et al. 1997; H) Janssen et al. 1993; I) Trivedi et al. 2001; J) Misra et al. 1990; K) Chawla et al. 1986; L) Mathur et al. 1990; M) Misra et al. 1991; N) Ariyoshi et al. 1990^a; O) Lal et al. 1984; P) Ariyoshi et al. 1990^b; Q) Chawla et al. 1989; R) Kakkar et al. 1989.

Table 3.10 Cellular and sub-cellular stress responses to LAS exposure of acceptable quality and potential use in water resource management applications. Exposure duration of response data from the literature is summarised in Table 3.9. Exposure duration of response data was determined from toxicity tests undertaken in this thesis is 96 hr.

Stress response	Endpoint measure ($\mu\text{g.L}^{-1}$) and LAS chain length	Endpoint normalised to C11.6 if chain length known ($\mu\text{g.L}^{-1}$)	Organism	Source
Phagocytotic activity	NOEC 200, C11-12	NOEC 200	<i>Oncorhynchus mykiss</i> ¹	A
Catalase (CAT)	NOEC 200, C11.9	NOEC 262	<i>Dugesia japonica</i> ²	B
Superoxide dismutase (SOD)	NOEC 500, C12	NOEC 717	<i>Hydrocharis dubis</i> ³	C
Catalase (CAT)				
Cholinesterase (CHE)	NOEC 1500, C11.6	NOEC 1500	<i>Burnupea stenochorias</i> ⁴ <i>Dugesia sp.</i> ²	D
Hsp72	NOEC 8000, C11.6	NOEC 8000	<i>Caridina nilotica</i> ⁵	
Lipid peroxidation (LPx)	LOEC 500, C11.6	LOEC 500	<i>Dugesia sp.</i> ²	
	LOEC 1000, C11.6	LOEC 1000	<i>Caridina nilotica</i> ⁵	
Acetylcholinesterase (ACHE)	LOEC 2000, C?	LOEC 2000	<i>Daphnia magna</i> ⁵	E
Adenosine 3',5'-cyclic monophosphate (cAMP)	LOEC 3000, C?	LOEC 3000	<i>Ictalurus sp.</i> ¹	F
Acetylcholinesterase (ACHE)	EC50 11400	EC ₅₀ 11400	<i>Daphnia magna</i> ⁵	E
General enzyme inhibition	EC50 117000, C11.2	EC ₅₀ 81606		G

Notes: ¹ Fish, ² Turbellarian; ³ Macrophyte; ⁴ Mollusc; ⁵ Crustacean

Sources: A) Bakirel et al. 2005; B) Li 2008; C) Wu et al. 2010; D) Current study; E) Guilhermino et al. 2000; F) Pareschi et al. 1997; G) Janssen et al. 1993.

3.7 Overall chapter summary

Toxicity test data on stress responses of freshwater organisms to LAS exposure were sourced from ecotoxicology databases, the scientific literature, and also generated through tests involving the exposure of indigenous macroinvertebrates to LAS. The suitability of these data for use in water resources management was determined using established quality-assessment schemes. Data of satisfactory quality were collated according to the duration of exposure and type of biological stress response measured.

Four groupings resulted: short-term lethality data (≤ 96 hr exposure), long-term lethality data (> 96 hr exposure), long-term sub-lethal data (> 96 hr exposure including organism level, population and community responses), and cellular and sub-cellular responses over any exposure period. In the next chapter the application of these data to the derivation of a WQG for LAS, specifically for South African freshwaters, is examined.

Since little is known about the type, quantity and quality of cellular and sub-cellular response data for LAS available in the literature, a particular assessment of these data was undertaken. Of the 18 publications identified, only six provided potentially useful data of acceptable quality. Furthermore, in order to identify the difficulties of producing cellular and sub-cellular toxicity data of an acceptable quality, indigenous macroinvertebrates were exposed to LAS and values for LPx, ChE and stress protein induction were determined. A confounding factor was the high within-treatment variability of measured responses. Although attributable, to some extent, to natural variation among individuals, the use of whole body homogenates was also identified as a potential cause of such variation. The use of cellular and sub-cellular assays utilising particular body parts from organisms of similar age or length, and of the same sex, should be considered as an alternative to tests involving the use of whole-body homogenates. Applying this approach to macroinvertebrates, which are usually of a small size and indeterminate sex, will require considerable technical skill development.

Other factors confounding the production of cellular and sub-cellular responses of suitable quality as water resource management tools include inconsistent responses by some taxa to LAS exposure. In some incidences, a particular cellular and sub-cellular stress response was measured in one taxon but not in other taxa. Furthermore, in some cases the response measured increased while in other taxa it decreased, relative to controls. Such inconsistencies in cellular and sub-cellular response data to LAS exposure create uncertainty regarding the implication of observed responses to the fitness of the individual, let alone the implication for higher levels of biological organisation.

Chapter 4 Deriving a water quality guideline for LAS for South African freshwaters

4.1 Introduction

Environmental water quality guidelines (WQGs) (chemical specific concentrations below which undesirable effects are not expected to occur) are utilised by water resource managers as a tool to assess, and then either maintain or improve, the water quality of a resource. The aim of WQGs is to protect the aquatic ecosystem; they are thus generally focused on protecting ecosystem structure (community composition). It is generally assumed that ecosystem function would therefore also be protected (Matthiessen et al. 2010). As a holistic approach, WQGs enable identification of chemical/s that may lead to, or have already caused, ecosystem deterioration, help in quantifying the potential severity that the presence of the chemical may have, and help to identify the potential source of the chemical (Matthiessen et al. 2010).

Different political jurisdictions have used various terms as an alternative to WQG (e.g. 'standard', 'limit', 'benchmark', 'criterion', 'threshold' or 'trigger value') with no consensus on definition and use. In this thesis the term 'guideline' is used in preference to the terms 'criteria', 'limit' or 'standard' as the latter three have sometimes been used as a legal obligation (Farmer et al. 2010). The LAS WQG to be derived in this thesis is not intended to be legally binding.

Water quality guidelines for a range of potential contaminants were derived for South Africa in 1996 (DWAF 1996). These were extended to include WQGs for inorganic salts using a similar derivation method and adapted to the water resource classification system introduced in South Africa at the time (Jooste and Rossouw et al. 2002). In 2005, additional WQGs were proposed for selected organic toxicants, but this time the derivation approach was based on that used by Australia and New Zealand (Warne et al. 2005). A guideline for LAS, however, was not among these. To date the only political jurisdictions with a WQG for LAS are Australia and New Zealand (ANZECC and ARMCANZ 2000), although a number of predicted no-effect concentrations (PNEC) have been developed by scientists for independent risk assessments (Van de Plaasche

et al. 1999; Dyer et al. 2003) (Table 4.1). Water quality guidelines developed for a particular jurisdiction are not always applicable to other jurisdictions. Guidelines need to take account of the social and economic context of the specific jurisdiction in which they are to be implemented. This is in order to balance environmental protection with economic costs and social implications of meeting the WQG. The derivation of a specific LAS WQG for South Africa also provides the opportunity to include toxicological data produced more recently (including those associated with species indigenous to South Africa) than those utilised in the derivation of the WQG for Australia and New Zealand.

Table 4.1 Water quality guideline and predicted no-effect concentrations (PNECs) determined for LAS.

Jurisdiction	WQG or PNEC ($\mu\text{g}\cdot\text{L}^{-1}$) and chain length	WQG or PNEC normalised to C11.6	Reference
Australia and New Zealand	280 (C11.6)	280	ANZECC and ARMCANZ 2000
Independent risk assessment	250 (C11.6)	250	Van de Plaasche et al. 1999
Independent risk assessment	245 (C12)	351	Dyer et al. 2003

Note: The WQG and PNECs were derived using species sensitivity distributions and defined at protective concentrations of 95% (i.e. PC95).

South Africa is planning a revision of the 1996 WQGs (DWA 2008b). It is envisaged that the derivation process will be in the form of a probabilistic risk assessment framework based on a Bayesian network (Jooste pers. com. 2011). Unfortunately, the methodology of this new approach is yet to be developed. Consequently, in order to develop an interim WQG for LAS for South African fresh waters, available international WQG derivation methodology will be assessed and, utilising the most appropriate aspects of the various theories, a guideline for LAS will be developed that will specifically align with the water resource management approach in South Africa. The above process is outlined in this chapter.

4.2 Issues to consider in the derivation of water quality guidelines

There is no internationally-accepted approach to deriving WQGs. Approaches differ in terms of the mathematical or statistical method used to derive the guideline from available toxicological response data. Furthermore, derivation approaches vary in the

type of information required: the type of biological measure (e.g. lethal or sub-lethal), the period of exposure (short-term or long-term), amount of toxicity data needed, and specification in terms of which organisms these data must represent. The level or type of protection the WQG affords the ecosystem also varies, depending on the water resource management requirements of the specific jurisdiction. The above-mentioned issues are discussed in greater detail in the sections below, with examples from current WQGs from various countries. Some other issues that are usually important considerations in WQG derivation, but that are not of direct relevance in the exercise of deriving a guideline for LAS, are also briefly listed.

4.2.1 Derivation methods

The derivation method is the process of utilising available toxicological response data to determine a concentration of a particular chemical that will provide a specific level of protection to the aquatic ecosystem. Derivation methods usually employ an assessment factor (AF) or a species sensitivity distribution (SSD) approach, or some combination of both of these. These derivation methods differ in statistical complexity and their consideration of risk. Confidence in the values they produce is largely dependent on the amount of data available.

An AF is a numerical value (e.g. 1, 10, 100, 1000) that, in the process of deriving a WQG, is used to divide the most sensitive toxicological datum, or result from an SSD, in order to take account of various sources of uncertainty. These include uncertainties associated with epistemological factors (incomplete understanding of ecological systems), methodological factors (data derived from only a small fraction of species present within the ecosystem being used to derive the WQG), and technological factors (possible data unreliability due to inter- and intra-laboratory differences and uncertainty of extrapolating from laboratory to the field) (Merrington et al. 2010). The greater the degree of uncertainty, the larger the AF applied. For example, deriving a guideline from few data will attract a higher AF than deriving a guideline from many response data. Similarly, a guideline derived from lethal response data attracts a higher AF than one derived using sub-lethal data as there is more confidence that the guideline derived using sub-lethal data will be sufficiently protective of the aquatic ecosystem. The advantage of AFs is that even when limited toxicological response data exist, these can

still be applied to derive a WQG (although the confidence in this guideline will be lower). Criticisms of the AF approach, discussed in Chapman et al. (1998) and Warne (1998), include issues such as the following:

- AFs are arbitrary values with no theoretical scientific basis;
- there is no way to estimate the margin of error associated with WQGs derived using the AF approach;
- the AF is usually applied to the lowest toxicological datum (i.e. most sensitive) and ignores all other data.

Overall, the AF approach is considered at odds with the risk-based philosophy of WQG derivation currently undertaken (Warne 1998).

An SSD is a statistical extrapolation method whereby a statistical distribution is fitted to toxicological data representing a range of species or some other taxonomic grouping (e.g. genus or family) (Posthuma et al. 2002). From this distribution of toxicological data (wherein one datum represents one taxon grouping), a theoretical extrapolation of the concentration that should be protective of x% of species in the environment can be determined. The determination of a definitive no-effect level using SSDs, i.e. 0% species affected, is not possible as the extreme tails of the typical normal or logistic statistical distribution are extended to plus and minus infinity (the same is true for determining an absolute effect level, i.e. 100% species affected) (Van Straalen 2002). Consequently, there is a need to choose an arbitrary cut-off point in the distribution to express the risk of the chemical to the environment. The 5th percentile is the most commonly chosen value. It represents the concentration that theoretically protects 95% of species from the effect that the toxicological data represent and is termed the PC95 (PC = protective concentration). The toxicological effect on 5% of species not protected by the PC95 is considered acceptably small. The advantage of the SSD approach is that a measure of associated uncertainty of the chosen PC can be determined (Warne 1998). Although Warne (1998) recommended using the 95% confidence level, subsequent statistical investigations have suggested that this would compound uncertainties associated with estimating the PC, and that the 50% confidence level should be used instead (i.e. 'PC95 50%' is the concentration that protects 95% of species with 50% certainty) (Fox 1999). Consequently, the use of SSDs allows for debate on the level of protection that a community may require and the certainty with which that level of protection can be delivered (ANZECC and ARMCANZ 2000). From the time when the SSD approach was

first suggested as an alternative to the AF approach, there have been recommendations to adopt a cautionary approach regarding its use (Forbes and Forbes 1993). Forbes and Calow (2002) have warned against ignoring the assumptions (Table 4.2) of distribution functions and of being deluded by their appearance of having more sophisticated statistical and risk attributes (compared to the AF approach). The SSD approach has therefore been criticised for lack of adequate proof of their improved ability to estimate environmentally-realistic no-effect concentrations. Despite the fact that, for most toxicant SSDs, the assumptions of this method are not met (Kefford et al. 2005a), the SSD approach is now generally acknowledged as a more desirable method than the AF approach for undertaking WQG derivation (Warne 1998; Lepper 2005; Matthiessen et al. 2010). The approach by various jurisdictions to the use of SSDs varies however, with no consensus on the minimum or optimum number of data points required, or even on the most appropriate statistical distribution to be employed. In the case of many contaminants, the quantity and type of data required for use in the SSD approach do not exist, and consequently the AF method is often relied upon for the derivation of the WQG.

Table 4.2 Assumptions of statistical extrapolation models used in SSDs.

No.	Assumption	Implication
1	The toxicological data subjected to the SSD are well modeled by the selected distribution	Has implications for the number of data points and type of statistical distribution utilised
2	The sensitivities of species used in laboratory toxicity tests approximate the sensitivities of species in the ecosystem of concern	Has implications for the type of taxa represented in the SSD
3	The sample of species used to produce toxicity data are a random, or at least a representative, sample of the species in the ecosystem of concern	Has implications for the type of taxa represented in the SSD
4	The chosen level of protection, statistically derived from an SSD of toxicity data (i.e. PC95), provides the desired protection in the ecosystem of concern	The implication is that if laboratory-generated toxicity tests (usually based on single species) are to be used to establish guideline concentrations that provide a desired level of ecosystem protection, then the biological responses measured during these tests need to be ecologically relevant
5	Chosen confidence limits around the protection level are appropriate	The chosen confidence limits need to be justified

Relevant references: Forbes and Forbes (1993); Warne (1998); Versteeg et al. (1999); Forbes and Calow (2002); Kefford et al. (2005a).

The derivation and structure of the South African national WQGs for toxic substances (DWAF 1996) follow those developed for the United States Environmental Protection Agency (US EPA) (Stephan et al. 1985). Guidelines were derived for both short-term exposures (e.g. chemical spills) and long-term exposures (the continuous presence of a chemical in the water resource). The statistical extrapolation chosen for the derivation process is a log-triangular distribution (Stephan et al. 1985; Roux et al. 1996) with AFs used when there are insufficient biological-effects data available for the SSD (Table 4.3). Use of the log-triangular distribution has been criticised by Warne (1998), ANZECC and ARMCANZ (2000) and Warne et al. (2005). In support of this criticism, these authors cite the critical comments put forward by a number of workers in this field: Pedersen et al. (1994) questioned the scientific merit of this distribution; the OECD (1992) stated that there is no biological basis for the triangular approximation; work by Okkerman et al. (1991) questioned the assumption that there are no toxic effects at concentrations below the determined chronic effect value (the long-term exposure guideline) irrespective of duration of exposure, and work by Maltby et al. (2003) showed that this distribution fitted only half the toxicity datasets for chemicals tested by the authors. Indeed, the US EPA acknowledged the limitations of this procedure for deriving WQGs and the need for revision (Delos 1995).

In Canada the SSD approach is preferred. However, if the taxon requirements are not met then a safety factor of 10 can be applied to the most sensitive toxicological datum from a specific list of required taxa (CCME 2007) (Table 4.3). In the European Union (EU) provision is made for the use of a SSD (a log-normal distribution), but the data requirements are onerous (at least ten species from specific taxa) and most often the AF method is applied (Table 4.3) (Lepper 2005). There has also been criticism of the log-normal distribution used by the EU, with Newman et al. (2000) reporting that 15 of the 30 published datasets tested failed a formal test of conformity to a log-normal distribution.

In Australia and New Zealand the SSD method is the preferred approach, but only in certain circumstances (usually in cases where data are insufficient) the AF approach can be used (Table 4.3) (Warne 1998; ANZECC and ARMCANZ 2000; Chapman 2001; Warne et al. 2005). The SSD method was initially based on the work of Aldenberg and Slob (1993) in which a log-logistic distribution was used. Shao (2000), however, suggested the use of the Burr Type III statistical distribution method (a family of

distributions incorporating the log-logistic distribution) since the larger range of distributions within the Burr Type III family would increase the likelihood of a closer fit of the toxicological data and, consequently, greater confidence in the derived PC value.

4.2.2 Toxicological data utilised in water quality guideline derivation

The quality, quantity and type of toxicological data used in the derivation process have important implications for the accuracy of the resultant WQG, the confidence placed in them and the level of protection they provide (Wheeler et al. 2002; Matthiessen et al. 2010). In terms of the type of toxicological data employed in the derivation process, there are probably five key characteristics relevant to the derivation of WQG (some related to the assumptions of the SSD extrapolation models) (Warne 1998; ANECC and ARMCANZ 2000). These can be summarised as follows:

- The choice of which taxa will best represent the potential harm of a chemical on the ecosystem;
- The ecological relevance of the experimental system (i.e. single species laboratory tests versus field-based multiple species tests);
- The duration of exposure to the toxicant (i.e. short-term being less than 96 hr and long-term greater than 96 hr);
- Ecological relevance of the biological stress response measured in the toxicity test (i.e. the level of biological organisation at which the toxic effect is observed)
- The statistical measure describing the biological effect (i.e. point estimates such as L(E)Cx versus hypothesis based estimates such as no observed effect concentrations - NOECs).

Implications associated with the above-mentioned issues are discussed below.

Data quality

Data assessment schemes are designed to assess the scientific rigor of the toxicity test reported in a scientific paper or report, and thereby the quality of data. The US EPA originally developed an assessment scheme for the ECOTOX database. A modification of this scheme (Warne 1998) was used for the Australian Ecotoxicity database and the development of the WQGs for Australia and New Zealand. Subsequently, there has been more refinement of this approach by Hobbs et al. (2005). These schemes use a scoring system in which points are awarded based on a series of questions relating to

exposure duration, biological stress response measured, characteristics of the test organism, test methodology (including statistical analyses) and type of data reported. Other assessment schemes include one by the Dutch (RIVM 1995), Klimisch et al. (1997) for use in OECD hazard and risk assessments and one by Wheeler et al. (2002) for the ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) database. As the method refined by Hobbs et al. (2005) is the most recently developed approach, and appeared the most objective, it was used for assessing the quality of toxicity data from LAS exposure tests (Chapter 3).

Data quantity

In theory, a large quantity of data is desirable when deriving WQGs as, especially in the case of SSDs, uncertainty decreases as n increases (Matthiessen et al. 2010). Forbes and Calow (2002) show, however, that this is only the case if the species included in the SSD are indeed a random unbiased sample of taxa present in the ecosystem of concern (i.e. the number of species is a proportional representation of trophic levels and taxonomic groups found in the ecosystem of interest). They found that if additional species, at specific trophic levels or functional groups, were chosen in proportions greater than they would naturally represent in the ecosystem, then the margin of error of the SSD increased with increasing n . It is thus clear that the taxon representation is an important consideration when using this method. A detailed discussion on this aspect is included in the next section.

There is no consensus on the minimum amount of data required for use in an SSD. Newman et al. (2000) report that sample sizes producing PC95 estimates with minimal variance required toxicity data on 15-55 species for the SSD (median number of species data was 30). On the other hand, Wheeler et al. (2002) showed that, for both log-normal and log-logistic distributions, a minimum of 10 data points were required to generate reliable estimates. The number of toxicological data points available for the chemical of concern is however limited and thus WQGs in most jurisdictions have had to compromise on this issue in order for WQGs to be derived. Decisions by various jurisdictions concerning the required number of data for use in the derivation process are therefore often arbitrary (Kefford et al. 2005a), ranging from five in Australia and New Zealand to ten in the European Union (as outlined in Table 4.3, along with required taxon representations).

Table 4.3 Quantity and taxon specificity of toxicological data required for water quality guideline derivation in different jurisdictions (adapted from Matthiessen et al. 2010).

WQG type	Australia	Canada	European Union *	USA and South Africa [§]
Short-term guideline (derived from L(E)C50 data)	None derived	SSD method preferred. At least six species: 3 fish (at least 1 salmonid and 1 non-salmonid) 3 invertebrates (at least 1 planktonic crustacean) If taxon requirements for SSD not met, a safety factor of 10 is applied to the lowest appropriate toxicity endpoint of at least: 2 fish (1 salmonid and 1 non-salmonid) 2 invertebrates (at least 1 planktonic crustacean)	SSD method preferred At least ten species, including at least: 1 fish 1 other chordate 1 crustacean 1 insect 1 Rotifera, Annelida or Mollusca representative 1 other insect or family in any phylum not already represented 1 algae 1 higher plant AF method, at least: 1 fish; Daphnia; 1 algae	SSD method preferred At least eight species including 1 cold-water fish 1 warm water fish 1 other chordate 1 planktonic crustacean 1 benthic crustacean 1 insect 1 Rotifera or Annelida or Mollusca representative 1 other insect or mollusc. If taxon requirements not met, various safety factors are applied depending on the amount of missing data (see Roux et al. 1996)
Long-term guideline (usually derived from NOEC data unless otherwise noted)	SSD method preferred At least 5 different species from at least 4 taxonomic groups AF method At least 5 data points representing at least 1 fish 1 alga or aquatic plant 2 invertebrates	SSD method preferred. ECx endpoints preferred. At least seven species: 3 fish (as above) 3 invertebrates (as above) 1 aquatic plant If taxon requirements for SSD not met, a safety factor of 10 is applied to the lowest appropriate ECx toxicity endpoint. Taxon requirements as above	As above, but using sub-lethal data	SSD method preferred At least three species, provided at least 1 is a fish 1 is an invertebrate If taxa requirements not met, various safety factors are applied depending on the amount of missing data (see Roux et al. 1996)
References	ANZECC and ARMCANZ (2000)	CCME (2007)	Lepper (2005)	Stephan et al. (1985); Roux et al. 1996)

Notes: * France had developed WQGs independently of the EU, but utilised only the AF approach stipulated by the EU (Babut et al. 2003).

[§] Warne et al. (2005) proposed a different derivation method for the determination of WQG for organic contaminants in South Africa. Remaining toxics are derived using the Roux et al. (1996) method.

Taxon representation

It is generally accepted that an organism's sensitivity will vary with exposure to different chemicals (i.e. there is no one 'most sensitive' species) (Cairns 1986); consequently, a variety of taxa should be used to assess the toxicity of chemicals (Matthiessen et al. 2010). The types of taxa chosen to represent all those in the ecosystem of interest can, however, affect the resultant value determined from an SSD or AF. Versteeg et al. (1999) investigated the effect of taxon inclusion on the behaviour of various distribution functions subjected to ammonium, cadmium and copper datasets. They reported that the distribution slope was indeed affected by the species composition of the dataset. As taxon representation clearly matters, Forbes and Calow (2002) suggest that the taxa utilised be representative of the target ecosystem, in terms of: trophic and taxonomic groupings. Representation by indigenous organisms, rather than toxicity data from species that do not occur in that geographic location, was also recommended. In terms of addressing the first point, one of the assumptions of the SSD method is that the data utilised should represent a random sample of species from the environment (Aldenberg and Slob 1993). Yet Forbes and Calow (2002) found that the composition of taxa used for most SSDs indicated an under-representation of primary producers and an over-representation of predators, relative to taxa composition in typical aquatic ecosystems. When adjustments were made to the relative proportions of species in the SSD dataset (so as to be more representative of trophic levels in the ecosystem of concern) the PC95 was materially affected (Forbes and Calow 2002; Duboudin et al. 2004). Furthermore, Kefford et al. (2005a) argued for the inclusion of toxicity data from rare species. In almost all cases, rare species are not represented in toxicity datasets as such rare individuals can never be collected in large enough numbers to satisfy standard toxicity test methodology. Yet consideration of rare species is important as they make up a relatively large proportion of the total number of species present in an ecosystem, even if — in terms of total number of individual organisms — they represent a small fraction of the species assemblage in a particular area.

Tests on the sensitivity of organisms from different geographical regions to various stressors have produced confounding results. Kefford et al. (2005b) report differing PC95s for salinity determined from SSDs of organisms indigenous to South Africa and South Australia. However, most taxa of the same order had similar tolerances in both locations; thus, it was the differences in crustacean, odonate, and non-arthropod relative richness that affected SSD results. Dyer et al. (1997) compared SSDs for six compounds (carbaryl, DDT, lindane, malathion, PCP and phenol) derived from

separate datasets of temperate, coldwater and tropical dwelling fish species. These authors showed that, with the exception of DDT, geographical origin of the fish species utilised did not influence sensitivity to these compounds. Maltby et al. (2005) found no significant difference in sensitivity of the temperate and tropical arthropods to insecticides chlorpyrifos, fenitrothion and carbofuran. In a recent comprehensive study, Kwok et al. (2007) constructed separate SSDs for temperate and tropical aquatic species for 18 chemicals. Results were conflicting, with temperate species and tropical species having different sensitivities to different kinds of chemicals. Daam and Van den Brink (2010) report results from a number of studies that compared the sensitivity of multiple species to pesticide and insecticide exposure from different geographical regions. They concluded that, generally, sensitivity among regions was similar. Thus, although it is recommended that the taxon representation in SSDs reflect the specific biotic community in the ecosystem of concern (Forbes and Calow 2002), the use of taxa from other geographic regions can be taken into account if needed, with consideration of relevant trophic level representation (Warne 1998; Kefford et al. 2005b). Kwok et al. (2007) recommend the application of an AF in such circumstances in order to take account of the uncertainties.

In most cases, the lack of available toxicity data requires that jurisdictions deriving WQGs compromise on the requirements of taxon representation recommended in the academic studies cited above. Since the SSD method used for WQG derivation in Australian and New Zealand is based in the Aldenberg and Slob approach, the data requirement is for at least four different taxonomic groups to be utilised, but the groups are not specified (Warne 1998). When the AF method is used, however, the data have to come from specified taxon groupings (ANZECC and ARMCANZ 2000) (Table 4.3). The taxa required are based on recommendations by the OECD (1981), which specifies that at least the basic trophic levels should be represented (e.g. fish, invertebrate and plant). The EU AF method also follows this approach, requiring data from a fish, a *Daphnia* sp. and an alga. The requirements for the SSD approach in the EU are however far more stringent than those of Australian and New Zealand, in which 10 taxa from specified groups are required (Table 4.3). Both the AF and SSD approaches used in Canada, USA and South Africa also require complicated taxon-specific data (Table 4.3).

Single species versus multiple species toxicity test data

Warne (1998) presents an extensive summary of arguments regarding the use of single species versus multispecies (concurrently exposed) toxicity test data in WQG derivation. Essentially, multispecies tests are more environmentally realistic, taking account of species interactions. These tests are, however, an expensive and time consuming method for producing toxicity response data. Consequently, there are often very few of these types of data available. In contrast, far more single species toxicity data exist, and because these data can allow for some prediction of effects at higher levels of biological organisation (Suter et al. 2005), they can be used in WQ derivation (Warne 1998). In the case of LAS there is a relatively large body of toxicity test data available, with three multispecies toxicity tests (Table 3.5), 11 sub-lethal (Table 3.4) and 29 short-term lethal (Table 3.2) single species data points that meet the data quality requirements.

Duration of exposure to the toxicant

It should be noted, in the context of the discussion that follows, that 'short-term' is defined as being less than 96 hr and 'long-term' as being greater than 96 hr.

Data used in the derivation of WQGs for Australian and New Zealand were categorized into 'acute' (data from test durations of less than 96 hr for multi-celled organisms and less than 72 hr for single celled organisms) and 'chronic' (greater than the durations specified for the above acute tests for the respective organisms). Consequently, according to the Australia and New Zealand approach, 'chronic' data could include both sub-lethal and lethal (i.e. mortality) stress responses (ANZECC and ARMCANZ 2000). The South African WQGs (DWAF 1996) incorporated both duration and type of stress response into the definitions of 'acute' and 'chronic', with the type of stress response overriding the duration (i.e. a lethal response from a toxicity test of greater than 96 hr was still classified as 'acute') (Roux et al. 1996). In the derivation of WQGs for inorganic salts in South Africa, Jooste and Rossouw (2002) actually projected LC50 values determined from 96 and 48 hr lethality tests to LC50 values expected from 14 day exposures (LC50: 336 hr) for use as 'acute' data. Termed the 'threshold' or 'incipient' LC50 (Rand 1995), this value takes account of results indicating that observed mortalities tend to stabilise after approximately 10 days of exposure (Heming et al. 1989; Jooste and Rossouw 2002). As described in Chapter 3, this thesis attempts to avoid the confusion that 'acute' and 'chronic' terminology generate by using the terms 'short-term lethal' (\leq 96 hr exposure), 'long-term lethal' ($>$ 96 hr exposure) and 'long-term sub-lethal'. Continuing with the approach used in South Africa for previous WQG derivation, lethal responses are

never considered in the same dataset as sub-lethal responses, regardless of exposure length. It is however interesting to note that for LAS, the long-term lethal NOEC for *Pimephales promelas* was lower than the long-term sub-lethal NOEC for fecundity (Table 3.3 and Table 3.4), although the LAS exposure period for the lethal NOEC was longer than that of the sub-lethal NOEC. For *Daphnia magna*, the long-term lethal NOEC was somewhat higher than long-term sub-lethal NOECs for fecundity (Table 3.3 and Table 3.4), which was again correlated to the length of exposure.

Ecological relevance of biological stress responses measured in toxicity tests

As discussed in Chapter 1, stress responses at each level of biological organisation have positive and negative attributes associated with them. Although biological responses at community level are considered highly reliable predictors of toxic effects on ecosystems, there are few of these data available for use in WQG derivation. Data on population and organism level responses (growth and reproduction) are more available, these having been measured from single-species toxicity tests. Their ecological relevance is, however, of less importance than information on community level responses measured in multiple-species toxicity tests. However, there are often insufficient data on sub-lethal population and organism level stress responses for many chemicals, which results in a greater reliance on short-term lethality data from single species toxicity tests. When PC95s are generated using short-term lethality data (LC50s), it has been found that AFs of 5 (Maltby et al. 2005), and between 2-10 (Brock et al. 2006) are required to ensure protection of the aquatic ecosystem. However, when PC95s derived using single species sub-lethal toxicity data have been compared to NOECs determined from mesocosm or field studies the PC95s were found to adequately protect aquatic ecosystems (Versteeg et al. 1999; Van den Brink et al. 2006; Staples et al. 2008). Further to this, Van Wijngaarden et al. (2010) report that a PC95 derived for fungicide fluazinam from EC50 data (lethality and immobility) was slightly higher than the NOEC determined from multiple species field tests, but that the EC10 data fell below the multiple species NOEC. Consequently, when WQGs in Australian and New Zealand are derived from short-term lethality data, an AF of 10 is applied, but when single species sub-lethal toxicity data are available no AF is required (ANZECC and ARMICANZ 2000).

Stress responses at cellular and sub-cellular levels can be sensitive, but the implications of these responses on aquatic ecosystem structure and function are, in

most cases, unknown (Clements 2000). Matthiessen et al. (2010) state that at present, the use of cellular and sub-cellular stress response data in derivation of WQGs is not considered appropriate unless their relevance at population level has been clearly demonstrated. As future derivation methodology in South Africa is likely to be in the form of a probabilistic risk assessment framework based on a Bayesian network, the inclusion of cellular and sub-cellular responses is potentially provided for (Jooste pers. com. 2011). Although this new approach is yet to be developed, the response characterisation of organisms to LAS exposure, undertaken in Chapter 3, presented the opportunity to assess various attributes of these data for use in WQG derivation. The difficulties involved in generating good quality cellular and sub-cellular data in terms of the toxicity exposure system, accuracy of assays used to measure the desired stress response, and statistical challenges in analyzing data are discussed in Chapter 3 with reference to the toxicity tests exposing indigenous organisms to LAS. Further investigation on how to handle some of the peculiarities of these data when applying them to WQG derivation is, however, required.

In the case of traditional toxicity test stress responses (such as lethality, growth or reproduction), increasing toxicant exposure ultimately results in an observed effect on all organisms, even if there is some variation among organisms with respect to their tolerances to the toxicant. In terms of cellular and sub-cellular responses measured after LAS exposure, however, some organisms responded whereas others did not, even at concentrations high enough to cause lethality (e.g. see Section 3.6.4). If the WQG is being developed for a site-specific situation and the responding taxa are important components of that ecosystem, then their inclusion in the derivation process may be warranted. If the guideline is a generic one, however, then inclusion of responses from these particular taxa can only increase the uncertainty of the derived WQG.

Complicated dose response relationships were also observed. Many sub-cellular stress responses represent some form of compromise to an organism's molecular machinery: for example, a decrease in cholinesterase (ChE) activity is a good indicator of impairment to neurotransmitters, and an increase in lipid peroxidation (LPx) indicates oxidative damage. However, in the LAS exposure trials, ChE activity in the flatworm *Dugesia* sp decreased with increasing LAS concentration, while in the limpet *Burnupea stenochorias* ChE activity increased with increasing LAS concentration. These cases raise questions relating to whether any deviation from control levels can be acceptable as a legitimate stress response. Alternatively,

should the focus be confined to those responses concurring with currently-accepted biochemical stress response behaviour? Furthermore, transitory responses were also recorded. In *Dugesia* sp., *B. stenochorias* and mayfly *Adenophlebia auriculata*, LPX determined by measuring MDA activity initially increased with increasing LAS concentrations, but began decreasing at the higher exposure concentrations. Should this type of response be seen as representing a negative toxic effect, or just a compensatory mechanism to cope with the toxic exposure?

Given the above uncertainties, the dilemma arises as to whether different cellular and sub-cellular responses for a particular toxicant can be combined, perhaps as part of an SSD. Although sub-lethal effects like growth and reproduction are often combined, despite being different biological stress responses, they do represent an actual effect, whereas some of the cellular and sub-cellular measures may only be transitory responses and represent exposure to the toxicant only and not necessarily a toxic effect. Furthermore, some of the cellular and sub-cellular responses measured might not be related to the toxic mode of action of the chemical concerned, but may rather reflect the body's response to damage that has already occurred (for example, see the discussion on stress protein responses in Section 3.6.1). In this case, using these data might underestimate the toxicity of the chemical. An alternative is therefore to utilise the most sensitive cellular or sub-cellular response datum (or the mean data from more than one species, if available). In terms of current WQG methodology, this value could be used for comparison against the derived WQG, to ensure it is suitably protective.

It is not currently possible to envisage the role of cellular and sub-cellular stress response data in the proposed future WQG derivation approach in which probabilistic risk assessment and Bayesian network theory are utilised. Discussion outlined in the above paragraphs has however identified troublesome issues with the use of these data in this context. The resolution of these issues is hampered by the relatively limited understanding of the significance of these responses in exposed organisms and also in terms of the resultant impact at the population and community levels of biological organisation. Thus, for generic WQGs, the use of these data continues to appear limited. Nevertheless, in terms of generating more site specific guidelines, these data can play a significant role, provided that adequate acknowledgement is given to the issues discussed in the above paragraphs.

The measure describing the biological effect (i.e. L(E)Cx versus NOEC)

Chapman et al. (1996) provide a number of arguments against the use of NOECs for regulatory purposes. They provide evidence that the agreement of toxicity test results between laboratories is much less for NOECs (difference factors 2.2-9.0) than for EC50s (difference factors of 1.2-2.2), leading to increased variability in the data. This is because the determination of the NOEC is heavily dependent on the range of concentrations tested, but also due to differences in the power of the statistical test employed. Consequently, NOEC data generated from different tests and laboratories are not readily comparable, causing large variability in the assessment of effects of a particular chemical. For these reasons, Chapman et al. (1996) argue that although the NOEC is commonly used as an approximation of the no effect concentration (NEC), it is not a good estimate of the concentration at which the no effect actually occurs. Instead, they suggest the use of low effect point estimates (e.g. EC20 or EC10) as alternatives to the NOEC. Although low effect point estimates ECx do not actually determine the no effect concentration either, they do have a number of advantages. ECx estimates utilise all the data generated from the toxicity test to produce a point estimate, resulting in considerably less variability between tests. This type of estimate also provides error limits around the point estimate, allowing comparisons between the data generated from different tests, thus improving confidence in the effect measured. Fox (2008) points out, however, that the error limits around low point estimates (which would be required if this were to replace NOECs) increase dramatically, compared to the error limits observed at EC50, thus adding to the uncertainty associated with the effect measured.

Warne and Van Dam (2008) and Fox (2008) support the arguments of Chapman et al. (1996) against the use of NOECs in regulatory use, and lament the apparent reluctance of regulators to use point estimate data in preference to NOEC data. They conclude that the problem is that no jurisdiction has taken a definite decision that only point estimate data should be produced from that point forward, and that regulatory use of NOEC data should be phased out as ECx data become available. Van Straalen (2002) provides some insight into the reluctance of regulators to dispense with NOEC data stating that, "*Due to the strong legal base of the 'no adverse effect' principle, it is hardly possible to replace the NOEC as an endpoint by an effect concentration with a small effect (e.g. EC10), despite the fact that NOEC is considered a statistical dragon*".

Warne and Van Dam (2008) conclude their discussion by suggesting the use of EC10 or EC5 as an alternative to the NOEC. In the case of sub-lethal exposure data for LAS, identified as suitable for this thesis, only one datum of 12 was not a NOEC estimate. It is likely that, for many other chemicals, there is more NOEC than ECx data, highlighting the difficulty of replacing NOEC data for future WQG derivation.

4.3 Dealing with levels of protection

The derivation of WQGs initially focused on the determination of a concentration that would have no effect on the environment. However, in South Africa (National Water Act No. 36 of 1998) and other jurisdictions, there was a realization of the need to balance water resource use and protection. In order to manage this balance, water resources needed to be categorized into classes with associated water quality (and biological) guidelines for each class. Consequently, WQGs needed to be derived at different levels of protection in order to correlate with the specific resource class. In Table 4.4 the resource classes or levels of ecosystem protection for South Africa, Australia and New Zealand, the EU, and France are presented together with a short description of the methodologies employed in deriving these class-specific guidelines. The WQGs for the USA (Stephan et al. 1985) protect one resource class, derived from the 5th percentile of an SSD of relevant toxicological data, and are not presented in Table 4.4. The number of resource classes ranges from three for Australia and New Zealand to six for South Africa, and are classified according to narrative descriptions (Table 4.4). Methods used to determine the protection level for the WQG associated with each class vary with jurisdiction. The method utilised in South Africa is discussed in greater detail in Section 4.5, but is summarized here in order to compare with methods from other jurisdictions (Table 4.4). The threshold between Class A (natural) and B (slightly modified) is the 5th percentile of an SSD of sub-lethal toxicity data, and the threshold between Class D (largely modified) and E (seriously modified and unacceptable) is the 5th percentile of an SSD of lethal toxicity data (Jooste and Rossouw 2002). Remaining thresholds are interpolations of these two values (Table 4.4). In Australia and New Zealand, levels of protection are also determined using the SSD approach but from only one data set, preferably sub-lethal data only or, if insufficient data exist, then lethal data with an AF of 10. For the High Ecological Value class the level of protection is determined by measuring physicochemical concentrations against background concentrations. If, however, the background levels have not been determined, then the level of protection used is the PC99 from the SSD of available toxicity data.

Table 4.4 Levels of protection provided by water quality guidelines in different jurisdictions.

Jurisdiction	Water resource class / level of protection	Narrative description (Kleynhans and Louw 2008)	Determination of threshold limits or protection level indicators for South African Water Quality Guidelines for toxicants (Jooste and Rossouw 2002) *	Suggested threshold limits or protection level indicators for organic toxicants in South African (Warne et al. 2005)
South Africa	A	Unmodified, natural	Threshold between levels A and B: Sub-lethal benchmark – analogous to a long-term sub-lethal WQG	Threshold between levels A and B: PC95
	B	Largely natural with few modifications. Small change in biota have taken place but ecosystem functions are essentially unchanged	Threshold between levels B and C: Determine the difference in value between the lethal and sub-lethal benchmark concentrations, divide this value by 3, and add this one third to the sub-lethal benchmark	Threshold PCx values between levels B and C, and levels C and D were not stated
	C	Moderately modified. Loss of biota has occurred but basic ecosystem function predominantly unchanged	Threshold between levels C and D: Using the calculation above, add two thirds of the value determined to the sub-lethal benchmark	
	D	Largely modified. A large loss of biota and basic ecosystem function has occurred	Threshold between levels D and E: Lethal benchmark – analogous to a short-term lethal WQG	
	E	Seriously modified. Loss of biota and basic ecosystem function is extensive	Threshold between levels E and F: Add the one third value determine above to the lethal benchmark	Threshold between levels E and F: not stated
	F	Critically/extremely modified. The system has been modified completely with an almost complete loss biota and ecosystem function		

Notes: Data requirements for derivation of WQGs are presented in Table 4.3.

Terminology used for each jurisdiction's guideline is retained in the above table.

* Method to determine thresholds for major inorganic salts was the same as that for toxicants except that the threshold between levels D and E is determined from LC50 data extrapolated to 336 hr exposure time (Jooste and Rossouw 2002).

Table 4.4 (continued) Levels of protection provided by water quality guidelines in different jurisdictions.

Jurisdiction	Water resource class / level of protection	Narrative description	Determination of threshold limits or protection level indicator	Reference
Australia and New Zealand	High conservation / ecological value	Ecological integrity is intact. No change in biodiversity beyond natural variability	For anthropogenic toxicants: concentrations zero or below detection limit. For natural toxicants: background concentrations shouldn't be exceeded. If background concentrations not yet determined, measured toxicant concentration should be < PC99	ANZECC and ARMCANZ (2000)
	Slightly to moderately disturbed systems	Biological diversity may be adversely impacted to a small but measurable degree by human activity. Ecosystem integrity is largely retained	Generally, toxicant concentrations < PC95. Although, for some chemicals (e.g. mercury etc) < PC99 recommended	
	Highly disturbed systems	Measurably degraded ecosystems	Recommended that the same guideline as for slightly to moderately disturbed systems applied. However, lower protection levels (PC90 and PC80) can be applied if accepted by stakeholders	

Notes: Data requirements for derivation of WQGs are presented in Table 4.3.

Terminology used for each jurisdiction's guideline is retained in the above table.

Table 4.4 (continued)

Levels of protection provided by water quality guidelines in different jurisdictions.

Jurisdiction	Water resource class / level of protection	Narrative description	Determination of threshold limits or protection level indicator	Reference
European Union	High status	There are no, or minor, anthropogenic alterations to the values of physico-chemical elements from those normally associated with undisturbed condition. Biological components reflect those normally associated with undisturbed conditions	For synthetic contaminants: Concentrations close to zero and at least below limits of detection of the most advanced analytical techniques in general use For non-synthetic contaminants: Concentrations remain within the range normally associated with undisturbed conditions	EC 2000
	Good status	Biological components show low levels of distortion resulting from anthropogenic activity, but deviate only slightly from those normally associated with undisturbed conditions	Concentration does not exceed the environmental quality standard (i.e. WQG)	
	Moderate status	Due to anthropogenic activities, biological components show moderate deviation from those normally associated with undisturbed conditions, and are significantly more disturbed than under conditions of good status	Use Moderate status specifications for biological components detailed in EC (2000), e.g. for benthic invertebrates: the composition and abundance differ moderately from reference conditions; taxonomic groups representative of reference community are absent; the ratio of disturbance-sensitive taxa to insensitive taxa, and the level of diversity, are substantially lower than the reference condition (high status), and significantly lower than for good status	
	Poor status	Due to anthropogenic activities, biological components show major alterations from those normally associated with undisturbed conditions	Use Poor status specifications for biological components detailed in EC (2000)	
	Bad status	Due to anthropogenic activities, biological communities normally associated with undisturbed conditions are absent	Use Bad Status specifications for biological components detailed in EC (2000)	

Notes: Data requirements for derivation of WQGs are presented in Table 4.3.

Biological components refer to phytoplankton, macrophytes and phytobenthos, benthic invertebrate fauna, and fish fauna. Descriptions of taxon composition and abundance for various protection levels is provided in EC (2000).

Terminology used for each jurisdiction's guideline is retained in the above table.

Table 4.4 (continued)

Levels of protection provided by water quality guidelines in different jurisdictions.

Jurisdiction	Water resource class / level of protection	Narrative description	Determination of threshold limits or protection level indicator	References
France	1 (blue)	Negligible risk of adverse effects on all species	Threshold between levels 1 and 2: Lowest reliable chronic NOEC divided by an AF of 10 or the lowest reliable acute EC or LC50 divided by an AF of 1000	Babut et al. 2003
	2 (green)	Potential risk of chronic sub-lethal effects on sensitive species; may induce a decrease in recruitment in affected populations, and possibly a decrease in abundance or diversity		
	3 (yellow)	Probable effect of chronic sub-lethal effects on sensitive species; may induce a decrease in recruitment in affected populations, and possibly a decrease in abundance or diversity; possible increase in the proportion of tolerant species	Threshold between levels 2 and 3: Either the lowest reliable chronic NOEC (no AF applied) or the lowest reliable acute EC or LC50 divided by an AF of 100	
	4 (orange)	Significant risk of lethal effects for the most sensitive species, leading to a reduction in species diversity and a decrease in abundance; tolerant species are dominant	Threshold between levels 3 and 4: Lowest reliable acute EC or LC50 (no AF applied)	
	5 (red)	High risk of lethal effects for many species; large reduction in abundance and diversity; loss of many species, at least the sensitive ones	Threshold between levels 4 and 5: Geometric mean of the lowest EC50 or LC50 datum from each trophic level	

Notes: Data requirements for derivation of WQGs are presented in Table 4.3.

Terminology used for each jurisdiction's guideline is retained in the above table.

For the 'Slightly to Moderately Disturbed' class the PC95 value is most often used. For the 'Highly Disturbed Systems' class, PC90 or PC80 values can be utilised, depending on negotiations with stakeholders (Table 4.4). In the EU, a WQG is only used to describe the level of protection for the 'Good Status' class. The 'High Status' class is described in terms of background concentrations, and lower classes rely only on biological components (Table 4.4). Lastly, the approach utilised in France, before full implementation of the EU Water Framework Directive, utilises a range of AFs on either lethal or sub-lethal data to derive guidelines to correlate with the required level of protection of the resource class (Table 4.4).

The decision of what level of protection, derived from the toxicity data, best represents the narrative goals/descriptions of the resource classes appears to have little scientific basis. Perhaps the exception is the use of the PC95, which is often used to represent the threshold between natural and slightly modified resource classes (or to represent a concentration considered safe to the ecosystem in risk assessments). This issue has been the subject of scientific investigation. Versteeg et al. (1999) compared model ecosystem NOEC concentrations for 11 substances (including C12 LAS) to the results of SSDs, derived from single species toxicity test data for each chemical, to determine the percentage of affected species to which the model ecosystem NOEC related. The correlation of the NOEC result to percentage species affected ranged from 9.6% for lindane to 52% affected species for atrazine, with the C12 LAS model ecosystem NOEC relating to 15% species affected. In addition, Selck et al. (2002) derived 5th percentile protective level concentrations for LAS (chain length not reported) and tributyltin (TBT) in marine waters using both AF and SSD methods, and compared these to NOECs measured in multispecies field studies. The AF and SSD methods (both using sub-lethal data) produced similar protection level concentrations and in both cases were more conservative than multispecies field studies. These results indicate that realistic model ecosystem endpoints (assumed to be more environmentally realistic than SSDs of single species toxicity data) were less conservative than typically-used 5th percentile species protection level (i.e. PC95) estimates from the SSDs, suggesting that, for the particular chemicals investigated, the PC95 could be considered suitably precautionary. Nevertheless, the correlation of the NOEC value with high percentiles of species affected in some chemicals (e.g. atrazine) — determined by Versteeg et al. (1999) — does question the accuracy and environmental realism of low PCx estimates, such as PC90 or PC80, as WQGs for lower-resource-class protection levels representing moderately- or seriously-modified aquatic ecosystems (it is

however likely that the determined NOECs were not good estimates of actual no-effect concentrations). There appears to be little scientific justification for choosing specific low PCx estimates, or specific AFs in the case of the French approach, to represent the narrative goals of certain management classes.

A recent study by Mebane (2010), however, attempted to address this issue by comparing the protection levels determined from an SSD of sub-lethal cadmium toxicity data with NOEC responses at various levels of biological organisation in a variety of biota from 11 multiple-species field studies (Figure 4.1). The PC95 was found to roughly correspond to the lowest NOECs measured in the field studies, suggesting the PC95 was protective of the effects of cadmium on the ecosystems measured. Mebane (2010) goes further, and demonstrates that the magnitudes of adverse effects measured in the 11 field studies were roughly proportional to the laboratory-based fraction of species with adverse effects in the SSD (Figure 4.1). Further research into the applicability of low point estimates as credible protection levels for lower resource classes is warranted in order to reduce the uncertainty associated with their use.

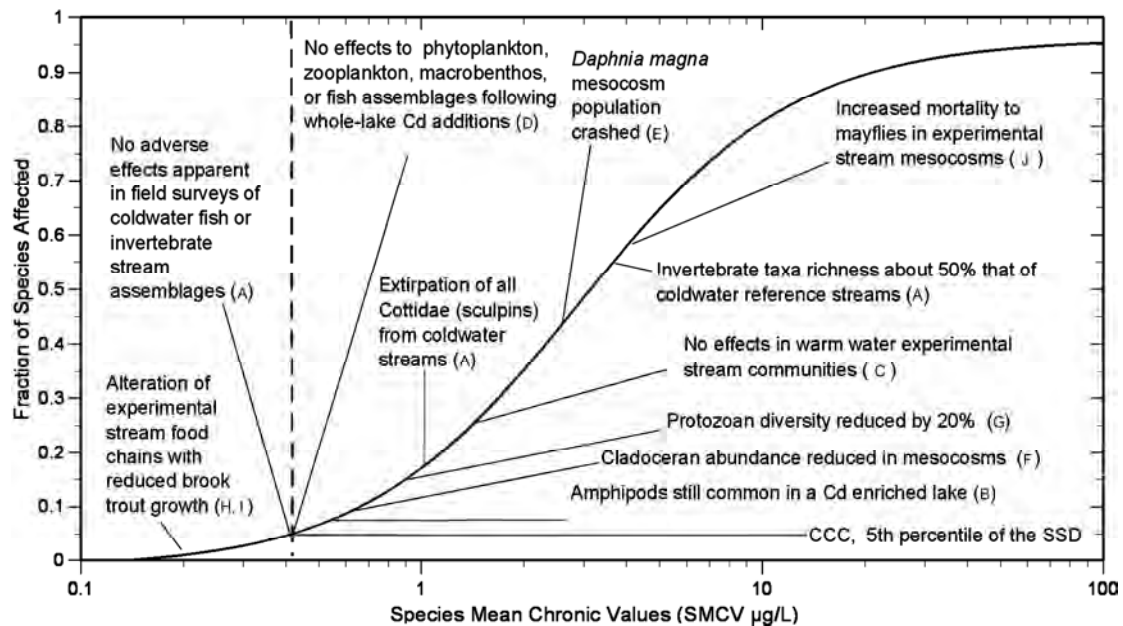


Figure 4.1 Comparison of cadmium effect concentrations estimated from ecosystem field studies or field assessments to proportions of adversely affected species based on a species sensitivity distribution (SSD) of laboratory-derived species mean chronic values (SMCVs). The solid curved line illustrates a SSD fitted from SMCVs using logistic regression. All values were adjusted to a hardness of 50 mg/L CaCO₃. The CCC refers to the chronic criterion concentration. Reproduced (with some adaptations) from Mebane (2010).

Sources: A) Kent et al. 2003; B) Stephan 2002; C. Aldenberg and Jaworska 2000; D) Suter et al. 2002; E) Cairns 1986; F) Forbes and Forbes 1993; G) Smith and Cairns 1993; H) Hopkin 1993; I) Underwood 1995; J) Power and McCarty 1997.

4.4 Water quality guideline derivation issues not considered in the determination of a WQG for LAS

There are a number of other issues that should be considered in the development of WQGs (Farmer et al. 2010; Matthiessen et al. 2010) but are not applicable to this study, and thus not discussed in more detail:

- selection and prioritization of substances/chemicals for inclusion in the WQGs (in this case the only chemical being considered is LAS);
- background contamination by naturally occurring substances (LAS is not a naturally occurring chemical);
- consideration of carcinogenicity, mutagenicity and endocrine disruption (the toxic mode of action of LAS is not considered to elicit these biological responses to any significant level);

- the use of quantitative structure-activity relationships (QSARs) to infer toxicity of a chemical (there are sufficient toxicity test data available for LAS);
- the issue of deriving guidelines that take account of interactions between chemicals and not simply the effects of the single substance alone requires further investigation and research;
- wide stakeholder participation in the derivation process incorporating scientific, social and economic factors: the derivation of a guideline in this thesis is an academic exercise investigating specific issues of the derivation process. For the development of an official WQ derivation methodology for South Africa, dedicated workshops of relevant scientific experts and social and political stakeholders should be undertaken.

4.5 Current South African water quality guidelines

4.5.1 National water quality guidelines for toxic substances

The derivation and structure of the South African WQGs (DWA 1996) follow those developed for the US EPA (Stephan et al. 1985). The national WQGs are expressed as two numerical values: the acute effect value (AEV) and the chronic effect value (CEV) (Roux et al. 1996). The AEV represents a concentration that, if exceeded, could result in a statistically significant chance of mortality. The AEV was designed for use in transient events such as toxic spills (Roux et al. 1996) and could also apply to areas in close proximity of pesticide runoff (Matthiessen et al. 2010). The AEV is defined as half the final acute value (FAV), which in turn is derived using log-triangular SSD from the short-term lethal response data of at least eight species so that the following taxonomic groups are at least represented once: cold-water fish; warm water fish; planktonic Crustacea; benthic Crustacea; insects, and arthropods or chordates (Roux et al. 1996) (Table 4.3). If the above data requirements are not met then specific safety factors (analogous to AFs) are applied to the FAV result, depending on the number of data available (even if only one biological response datum is available, it can be used once a safety factor of 100 has been applied). The rules governing the application of safety factors can be found in Roux et al. (1996).

The CEV represents a concentration which is statistically unlikely to affect the aquatic ecosystem even during continuous exposure. The CEV is the lowest value of the final chronic value (FCV) or final plant value (FPV). The FCV is derived using a log-triangular SSD of long-term sub-lethality data from at least three species of animal,

provided one is a fish and one is an invertebrate (Table 4.3). The FPV is the lowest 96 hr toxicity test on an alga or long-term sub-lethal response on a vascular plant (Roux et al. 1996). Safety factors are applied to the FCV if the above data requirements are not met. If only one biological response datum is available for the FCV, or only one plant datum available, then a safety factor of 1000 can be applied to generate the CEV (Roux et al. 1996). Although it is possible that the sub-lethal effects measured could be reversible, leading to an overprotective guideline value, this was not considered in the derivation process as the WQGs were intended to be precautionary (Roux et al. 1996).

4.5.2 Adapting the national water quality guidelines for use in the ecological Reserve

Two years after the development of the national WQGs in 1996, the National Water Act (No. 36 of 1998) was promulgated. One of the major changes introduced was a classification system for water resources (a six-class system: A-F) and the consequent need to manage water resources according to the specified class. The Ecological Reserve is the process used to determine the class of a particular water resource and also to assign the particular ecological specifications (ecospecs) associated with that class. Water resources can only be managed as one of the Classes A-D, with Classes E and F considered unacceptable. Any resource classed as E or F must be managed as a D class in order to improve it. Water quality guidelines form part of a suite of ecospecs along with biological responses (for example, of fish, macroinvertebrates and riparian vegetation). Consequently, there was a need to produce WQGs according to the six-class system. As an interim measure it was decided to adapt the AEVs and CEVs produced for toxics using the 1996 derivation procedure to the A-F classification system (Jooste and Rossouw 2002). The AEV was used as the threshold between Classes D and E, and the CEV as the threshold between Classes A and B. Thresholds for the remaining classes were interpolated from these two data points as described in Table 4.4. In addition, Jooste and Rossouw (2002) derived ecospecs for major inorganic salts. The derivation procedure used for the salts was similar to that used for the toxics in 1996 with the some exceptions as outlined below:

- The terms AEV and CEV were changed to lethality and sub-lethality benchmark respectively.

- The lethality benchmark was calculated differently, with LC50 data being extrapolated to 336 hr, using the method described in Jooste and Rossouw (2002).
- As before, the 5th percentile of the log-triangular distribution of LC50 data was used to generate the lethality benchmark, although this time a safety factor of 2 was not applied.

The sub-lethality benchmark was derived in the same manner as the CEV.

The derivation of WQGs for a range of protection levels or classes representing ecological effect is not only an important concept for South Africa, but has been developed in Australia and New Zealand (ANZECC and ARMCANZ 2000) and in France (Babut et al. 2003). As this concept will be an important component of future WQG derivation in South Africa, it is discussed in greater detail in Section 4.6.

4.5.3 Critical assessment of the current South African water quality guidelines

In an assessment of the derivation approach used by the US EPA (Stephan et al. 1985) and South Africa (Roux et al. 1996) a number of criticisms were identified (Warne 1998; ANZECC and ARMCANZ 2000; Warne et al. 2005). The large amount of data required for the above approach has been criticised as being too stringent, resulting in many of the WQGs being derived using the default AF method. This has implications for the environmental realism of the WQGs and associated confidence in the final value. Furthermore, as discussed earlier, use of the log-triangular distribution as a derivation method has also been questioned. Lastly, the use of the AEV (determined from short-term lethality data) as the threshold limit between Classes D and E for toxic substances is inappropriate. The AEV reflects lethality responses in toxicity tests of 96 hr and less, yet the eventual purpose of developing WQG is to protect organisms from long-term exposure. Since LC50s of taxa generally decrease with increasing exposure time to a toxicant, the derived WQG is likely to be 'under-protective'. In the subsequent derivation of major inorganic salts for South Africa, Jooste and Rossouw (2002) addressed this problem by extrapolating short-term exposure LC50 data to reflect theoretical LC50s at 336 hr. These data were used as the lethality benchmark for deriving the threshold guideline values between Classes D and E.

4.5.4 Proposed water quality guidelines for organic toxicants in South Africa

In 2005, Warne et al. (2005) proposed a different procedure for the derivation of organic toxicants in South Africa, utilising the approach adopted in Australia and New Zealand (ANZECC and ARMCANZ 2000). This approach was recommended because at the time it was the most recently developed set of guidelines and as such integrated the latest thinking in ecotoxicology, statistics and risk assessment. The philosophy behind this approach and the procedural intricacies are extensively detailed in Warne (1998), ANZECC and ARMCANZ (2000), Chapman (2001) and Warne et al. (2005). As discussed in Section 4.2.1, there are two methods used to derive WQGs, an SSD method being the preferred approach, but in certain circumstances (usually when there are insufficient sub-lethal data) the AF approach can be used. In this approach short-term guidelines (e.g. AEV or lethality benchmarks) are not derived, only long-term guidelines (Table 4.3). A complex hierarchical system has been developed to guide the derivation process and is driven by the quantity and types of toxicological response data available (ANZECC and ARMCANZ 2000). The advantage of the hierarchical derivation system is that, although WQGs can be derived for chemicals with only few biological effects data, the system is explicitly designed as an interim guideline, with associated lower confidence. In contrast, a **high reliability (HR)** guideline would require assessment in terms of one of the following four options (in order of preference):

- At least five multiple species (model ecosystem) tests which would then be subjected to the Burr Type III SSD;

OR

- At least three multiple species tests of which an AF of 10 would be applied to the lowest NOEC of these tests;

OR

- Single-species sub-lethal toxicity data from at least five species belonging to at least four separate taxon groupings, including at least one of the following: fish; invertebrate; or plant. These data would then be subjected to the Burr Type III SSD;

OR

- Although the SSD approach is preferred, provision is made for the use of the AF approach. In this case, requirements are single species sub-lethal toxicity data from at least five species, specifically: one fish; two taxonomically

different invertebrates; one plant; and any other one species). The lowest NOEC derived from these data would then be subjected to an AF of 10.

If the available toxicity data do not meet the requirements for derivation of a high reliability guideline, then a **moderate reliability (MR)** guideline could be produced providing the following data were available:

- Lethal toxicity data (LC/EC50s) from at least five species belonging to at least four separate taxon groupings including at least one of the following: fish; invertebrate; and plant. These data would then be subjected to the Burr Type III SSD and the result subjected to an AF of 10 or an ACR;

OR

- Although the SSD approach is preferred, provision is made for the use of the AF approach. In this case, lethal toxicity data (LC/EC50s) from at least five species, specifically: one fish; two taxonomically different invertebrates; one plant; and any other one species are required. The lowest LC/EC50 data would then be subjected to an AF of 100 or 10xACR (acute to chronic ratio).

As can be seen from the above, there is little difference in the data requirements for the SSD and AF approaches when deriving HR and MR guidelines; thus, in most circumstances, the SSD approach should be used. However, if there are still insufficient toxicity data available for derivation of a WQG, it may be possible to utilise an AF approach (requiring less stringent data requirements) to derive a **low reliability (LR)** guideline, that is derived according to one of the following options (in order of preference):

- The lowest of three single species NOEC data is divided by an AF of 20;

OR

- The lowest of three LC/EC50 data is divided by an AF of 100 or 10xACR;

OR

- A sub-lethal NOEC datum (if it is lower than any available lethal data) is divided by an AF of 200;

OR

- Any toxicity datum is divided by an AF of 1000.

Although there is little confidence in the accuracy of the LR guideline, it is considered preferable to have a tentative guideline rather than none at all (ANZECC and ARMCANZ 2000). The LR guideline should, however, be viewed as an interim figure

and the necessary toxicity data generated in order to derive a guideline of greater reliability.

The above explanation for the LR derivation procedure is for non-narcotic chemicals. For chemicals with a narcotic mode of action an AF of 10 is applied to the result of an SSD of quantitative structure-activity relationships of 19 species (ANZECC and ARMCANZ 2000; Warne et al. 2005). The necessity of deriving a LR guideline for LAS is unlikely as there is a relatively large amount of toxicity data available for this chemical.

4.6 Derivation approach adopted for this thesis.

The derivation approach adopted in this thesis was mainly based on those used for Australia and New Zealand. This approach was deemed more suitable for a number of reasons:

- The SSD approach is preferred when deriving WQGs. Despite some criticism of the use of SSDs for WQG derivation, the approach satisfies the statistical and risk requirements of WQGs to a greater extent than the AF approach. However, there is also acknowledgement of the advantage of the AF approach when only few data are available and that this approach is applied when that situation occurs.
- The requirements for data quantity and taxon representation when applying the SSD are not so onerous as to prevent the derivation of WQGs using this method, necessitating reliance on the AF method as is often the case for the EU approach. Yet the requirements are more rigorous than the USA and South African Toxics WQG approaches, which only require three data points when deriving long-term guidelines. Pedersen et al. (1994) determined that if the data requirements for the Aldenberg and Slob (1993) SSD method were reduced from five to four the method became unreliable, suggesting that the jurisdictions utilising less than five toxicity data in an SSD were increasing the uncertainty in the resultant guideline.
- An important attribute of the Australia and New Zealand approach is the use of a hierarchical derivation, whereby guidelines can be determined at various levels of confidence, depending on available toxicity data. High reliability guidelines are associated with a higher confidence in their ability to meet the required levels of protection desired for the aquatic ecosystem, compared to

moderate- or low-reliability guidelines. The high-reliability guidelines require more environmentally realistic toxicity test data in higher quantities so as to improve biological and statistical attributes. The production of lower reliability guidelines is preferable to not having any guidelines at all, although the uncertainty inherent in these guidelines should be explicitly stated.

Adaption of the Australia and New Zealand approach to the particular requirements of the South African context is required in terms of the alignment of protection levels for WQGs to the six-class resource classification system in South Africa. The WQ guidelines in Australia and New Zealand designate three water resource levels (Table 4.4) and stipulate the use of the PC95 as the level of protection for the middle class 'slightly to moderately disturbed systems' (although for chemicals that tend to bioaccumulate the PC99 is recommended). For the highest resource class, background concentrations or the PC99 are used as protection levels. For the lowest class (the 'highly disturbed systems') it is considered preferable to use the PC95 protection level as the management class, but allowance is made to use PC90 or P80 if agreed by relevant stakeholders. This three-class system is not applicable in South Africa. Thus when Warne et al. (2005) developed guidelines for organic toxicants in South Africa, using the Australia and New Zealand approach, they recommended the use of the PC95 as the threshold between Classes A and B and the PC80 as the threshold between Classes D and E. Protection levels or thresholds for the other classes were not stipulated (Table 4.4), although a possible solution is to arbitrarily assign a protection level to each resource class (Table 4.5). The use of the PC95 as the threshold between Classes A and B is a similar approach to that used by Jooste and Rossouw (2002) to derive a value for this threshold. However, different approaches were used to derive the D/E class threshold. These different approaches are assessed further to determine which is the most appropriate.

Table 4.5 Potential alignment of water quality guideline protection level and South African water resource classification system.

Water resource class		Level of protection (PCx) threshold
A		
B	←	PC95
C	←	PC90
D	←	PC85
E	←	PC80
F	←	PC70

The Jooste and Rossouw (2002) approach of designating protection levels for major inorganic salts in South Africa utilises long-term lethality data to determine the protection level, separating management Class D from the unacceptable Classes of E and F (Table 4.4). The attraction of this approach is that the biological effect being protected at the threshold of Classes D and E is easily defined i.e. protecting 95% of species from chemical concentrations that could result in 50% mortality (LC50). In effect, this results in the protection of most organisms present in the water resource from excessive mortality (at least theoretically). The ecological implications of exceeding this WQG protection level are much easier to comprehend. When using the Australia and New Zealand approach for defining the threshold between Classes D and E/F, an arbitrary level of protection (PC80) is chosen from along a gradient of unspecified sub-lethal effects, or — if insufficient sub-lethal data exist — from lethal effects subjected to an AF or ACR. As the biological effect associated with the PC80 value is less accurately defined, the ecological implications of the PC80 as the threshold value between Classes D and E/F is less clear than the use of the long-term lethality data approach of Jooste and Rossouw (2002). There is thus some potential for increased uncertainty regarding the ecological implications of the PC80 WQG.

The data requirements of the Jooste and Rossouw (2002) method for determining the protection level guideline between Classes D and E are more demanding than those needed to determine the PC80 using the Australia and New Zealand approach. The PC80 is the 20th percentile of an SSD of sub-lethal data (for HR guideline) or lethal data subjected to AF or ACR (for MR guideline) data. This contrasts with the Jooste and Rossouw (2002) approach, which requires long-term lethality data (336 hr

exposures) or — in the absence of these data — the extrapolation of short-term LC50 data to LC50 values that could be expected to occur after 336 hr of exposure. In the case of LAS, there are insufficient long-term lethality data to derive this guideline using an SSD (Table 3.3) and this is likely to be the case for almost all chemicals. The extrapolation of LC50(336 hr) values from short-term LC50 data can be undertaken using an exponential model as described in Jooste and Rossouw (2002) and is investigated further here using available LAS toxicity data. Two inputs are required for the model: the slope and the intercept value from a trend line between LC50 data from two different exposure periods. In the case of LAS, the following data were available: the median 48 hr LC50 and median 96 hr LC50 from international short-term lethality data presented in Table 3.2 (data based on tests on three gastropods: *Physa venustula*; *Heleobia cumingii*; and *Melanooides tuberculata*, which recorded high 48 hr LC₅₀s, were excluded due to the possibility of specimens withdrawing into their shells to avoid the LAS over the short exposure time). The median 48 hr LC50 and median 96 hr LC50 produced the trend line depicted in Figure 4.2. The extrapolated 336 hr LC50s derived using this trend line were, however, low (between 10⁻¹⁴ and 10⁻¹⁷ µg.L⁻¹). The few experimental long-term lethal data available for LAS (Table 3.3) list LC50s of 120 and 5239 µg.L⁻¹, suggesting an inability of the exponential model to extrapolate LAS short-term lethality data to a reasonable approximation of long-term lethality response. A possible explanation is the rather dramatic increase in lethality of LAS between 48 and 96hr in some organisms (Figure 4.2). Mayfly *A. auriculata* and, to a lesser extent the limpet *B. stenochorias*, are considerably less susceptible to the toxic mode of action of LAS over shorter exposures (48 hr) compared to 96 hr exposures, whereas the toxic mode of action of LAS appears more constant over time in flatworm *Dugesia* sp. (Figure 4.2). A 48 hr LC50 could not be determined for shrimp *C. nilotica*. An assumption of this type of exponential model is that all organisms respond in a similar way to the toxicant over time (Mayer et al. 1994), so that even if some species are more tolerant than others, the linearised slope of their LC50 values over time should be similar. This does not appear to be the case for LAS during the first 96 hr of exposure and this may be due to its narcotic mode of action, whereby lethality is a delayed response to exposure. More consistent responses among organisms may occur after longer periods of exposure and, unfortunately, not enough long-term lethal LC50 data exist to investigate this (Table 3.2). It was possible, however, to determine a trend line between 144 hr and 240 hr LC50s for *C. nilotica* and use the resultant slope and intercept values within the exponential model to predict LC50s at 336 hr for the available short-term lethal data in Table 3.2. The resultant extrapolated

336 hr LC50s had a median value of $15 \mu\text{g.L}^{-1}$ (and a mean of $18 \mu\text{g.L}^{-1}$) suggesting that, when based on longer term toxicity responses of *C. nilotica* to LAS, the model was still inappropriate for estimating long-term lethality for organisms in general. Consequently, it appears that the most reliable approach for determination of the protection level between Classes D and E/F is that of Australia and New Zealand with the production of a PC80 from available sub-lethal data. As discussed earlier, the Australia and New Zealand approach and the Jooste and Rossouw (2002) approach are similar with regard to deriving the A/B class threshold. Consequently, in the interest of using the same derivation approach for all class thresholds, it makes sense to use the Australia and New Zealand method for determinations of all resource class threshold values.

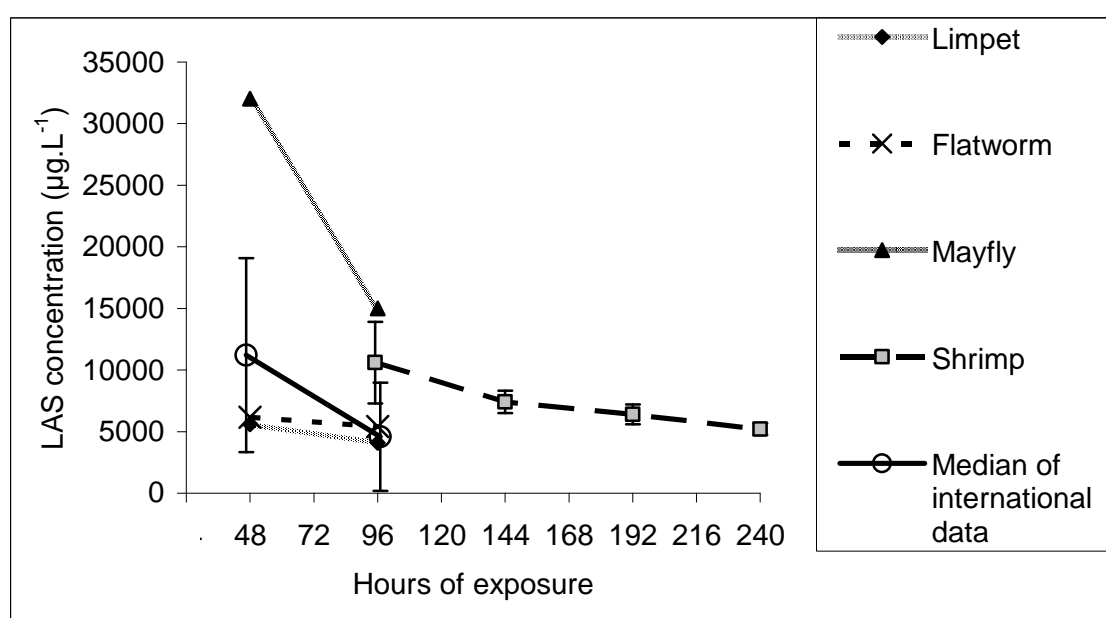


Figure 4.2 LC50 values at 48 and 96 hr of LAS exposure for limpet *Burnupia stenochorias*, flatworm *Dugesia* sp. and mayfly *Adenophlebia auriculata*. Median LC50 values (with standard deviations) for available international LAS lethality data from Table 3.2. Differences in the slopes of trend lines between the LC50 values determined at 48 and 96 hr amongst the three indigenous organisms suggest they respond differently to the toxic effects of LAS. The steepness of the trend line between the median of all available international 48 and 96 hr LC50 values summarised in Table 3.2 (with certain gastropod data excluded: see text) suggests a rapid change in the toxic effect of LAS, in this case over a 48 hr period. After longer exposures of up to 240 hr, however, the incipient LC50 had still not been attained in shrimp *Caridina nilotica* (means with standard deviations).

4.7 Deriving a water quality guideline for LAS for South African freshwaters

Following the Australian and New Zealand approach, it was possible to derive a HR WQG of $38 \mu\text{g.L}^{-1}$ from the multiple species toxicity test data (Table 3.5) using the AF approach for the boundary between water resource management Classes A and B (there were not enough data for the use of an SSD of multiple species data). In this case, the application of the AF of 10 to the lowest multiple species NOEC is perhaps overprotective, as the study on which this NOEC determination is based (Belanger et al. 2002) was a comprehensive investigation of many benthic microbial and invertebrate community-level stress responses and is probably a reasonable approximation of actual no-effect concentration. The value of $38 \mu\text{g.L}^{-1}$ is also considerably lower than peak LAS concentration of $122 \mu\text{g.L}^{-1}$ measured in a Philippines river where biological monitoring revealed no significant effects on macroinvertebrate communities (Dyer et al. 2003). Compared to international sub-lethal data (Table 3.4) and experimentally-determined cellular and sub-cellular stress responses (Table 3.10), this derived guideline appears low. Although it is possible that a more sensitive cellular and sub-cellular stress response was not measured or that the non-specific narcotic mode of action of LAS means that cellular or sub-cellular stress responses are not good indicators of LAS toxicity. Regarding application to water resource management in South Africa, the AF approach does not provide guidelines for levels of protection other than the threshold between Class A and B, and appears over protective in the case of LAS, which could have negative consequences for socio and/or economic development. This approach is thus not advantageous for use in South Africa.

A HR guideline for LAS can also be derived using long-term sub-lethal single species data (Table 3.4). The resultant protective concentrations (PCs) at a variety of percentages are presented in Table 4.6 and (for comparison), the MR guidelines derived using short-term lethal data with an AF of 10 applied. Two sets of MR guidelines are presented: one derived using all lethality data from 48 hr and 96 hr exposure trials and another set of guidelines with just 96 hr data. The latter are included because it appears that the lethal effects of LAS are considerably less at 48 hr so including these data may result in an underestimation of LAS toxicity over longer exposure periods.

The PCx values derived using lethality data subjected to an AF of 10 were more conservative than PCx values derived from sub-lethal data (except at the PC99). Thus, in the absence of a HR guideline, this approach (to derive a MR guideline) is suitably precautionary and likely to protect the ecosystem, at least at the PCx values likely to be utilised in the WQGs (e.g. PC95-PC70) (Table 4.6). The exclusion of the 48 hr toxicity data from the SSD derivation resulted in only slightly lower PCx values at percentages of species protection ranging from 80-95%, while at lower species protection percentages the differences in values increased. It thus appears that utilising 48 and 96 hr short-term lethal data together in the determination of a MR guideline is acceptable, especially if the amount of available short-term lethal data is low.

Table 4.6 High (HR) and medium reliability (MR) guidelines derived for LAS at a variety of PCx (PCx = protective concentration for x percentage of species from a specific effect).

Protective concentration for x% of species	SSD of sub-lethal data. HR guideline ($\mu\text{g.L}^{-1}$) <i>n</i> = 11	SSD of all lethal data (AF of 10 applied). MR guideline ($\mu\text{g.L}^{-1}$) <i>n</i> = 31	SSD of 96 hr lethal data (AF of 10 applied). MR guideline ($\mu\text{g.L}^{-1}$) <i>n</i> = 16
PC99	90	129	160
PC95	304	193	210
PC90	519	247	245
PC85	714	296	273
PC80	901	346	300
PC75	1087	398	325
PC70	1276	454	351
PC60	1674	587	407
PC50	2126	763	472

The resultant PC95 of 304 $\mu\text{g.L}^{-1}$ (Table 4.6) derived using the sub-lethal data falls within the range of PC95s of 250-351 $\mu\text{g.L}^{-1}$ which were derived by Van de Plaasche et al. (1999), ANZECC and ARMCANZ (2000) and Dyer et al. (2003) (Table 4.1). These derived PC95s are remarkably similar, despite the use of different statistical extrapolations, and in some cases different species data, between studies. The ANZECC and ARMCANZ (2000) derivation method is the same as that used in this thesis (Burr type III). The derivation methods of the other two studies (Van de Plaasche et al. 1999; Dyer et al. 2003) were, however, logistic and log-logistic statistical extrapolations, respectively. Although some of the sub-lethal species data were commonly used in all studies, data from different species were also included in the SSDs of these studies. For example, the species list of sub-lethal data utilised in this thesis contained only one macrophyte and no algae, while Van de Plaasche et

al. (1999), ANZECC and ARMCANZ (2000) and Dyer et al. (2003) included six algal species (Appendix 6). The algal sub-lethal toxicity data were not utilised in this thesis as the original reports by BKH Consulting Engineers (1993), which provided details of these results, are no longer available and could not be assessed for data quality. Another potential source of variation in the derived PC95s was the inclusion of marine species toxicity response data by Van de Plaasche et al. (1999). The remaining PC95s were derived exclusively from toxicological responses of freshwater taxa.

The PC95 of $304 \mu\text{g.L}^{-1}$ derived in this thesis (Table 4.6) is more conservative than the multiple species NOECs determined by Fairchild et al. (1993) and Belanger et al. (2002) of 472 and $384 \mu\text{g.L}^{-1}$ respectively (Table 3.5). Although Lewis et al. (1993) determined a multiple species NOEC of $1441 \mu\text{g.L}^{-1}$, exposed taxa were only benthic algae, whereas the Fairchild et al. (1993) and Belanger et al. (2002) studies included benthic microbial and invertebrate communities and are thus more likely to be environmentally realistic. In addition, only two cellular and sub-cellular NOECs below $304 \mu\text{g.L}^{-1}$ LAS are recorded in the LAS hazard characterisation chapter (Table 3.10), being 200 for phagocytotic activity and $262 \mu\text{g.L}^{-1}$ for catalase activity, respectively. Overall, a LAS concentration of $304 \mu\text{g.L}^{-1}$ appears a robust estimate of the PC95 for LAS, and a suitable protection level for the threshold between water resource management Classes of A and B.

It is more difficult to determine the suitability of other PCx estimates as appropriate protection levels for specific water resource classes (Table 4.5). As discussed earlier, Jooste and Rossouw (2002) suggested that a potentially suitable indicator of the threshold between Class D and E could be the 5th percentile of long-term lethality LC50 data. It was, however, not possible to use this approach in the case of LAS. The availability of long-term lethal NOEC data for LAS, however, allowed an assessment of the ecological relevance of the PC80. Available NOEC values were as follows: $646 \mu\text{g.L}^{-1}$ for *Pimephales promelas*, $5987 \mu\text{g.L}^{-1}$ for *Daphnia magna* and $3200 \mu\text{g.L}^{-1}$ for *C. nilotica*. An EC20 value of 1362 for *Hyaella azteca* was also available (Table 3.3). The EC20 estimation has been used as an alternative long-term endpoint to the NOEC (Versteeg and Rawlings 2003) and, if used with the above NOEC data, would provide enough data for an SSD assessment. The resultant 5th percentile of the Burr Type III SSD of the long-term lethal NOEC data is $578 \mu\text{g.L}^{-1}$, which is more conservative than the PC80 of $901 \mu\text{g.L}^{-1}$ derived for the threshold of Classes D and E using sub-lethality data. However, the 5th percentile of

the long-term lethality NOEC data is probably not an appropriate endpoint to define the D/E class boundary. Indeed, Jooste and Rossouw (2002) utilised LC50 data to determine the D/E class boundary, which would have resulted in a less conservative value. The narrative description of Class D states that a large loss of biota and basic ecosystem function has occurred, and for Class E that loss of biota and basic ecosystem function is extensive (Kleynhans and Louw 2008) (Table 4.4). These descriptions seem to suggest a loss of greater than 5% of species. Coincidentally, the PC80 of the sub-lethal data, 901 $\mu\text{g.L}^{-1}$ corresponds with the 19th percentile (i.e. PC81) of the long-term lethal NOEC data (Table 4.7). Of course, as there are only four long-term lethal NOEC data available the results of the SSD should be interpreted with caution. In the case of LAS, however, the choice of PC80 of sub-lethal data for the threshold between Class D and E is probably not inappropriate and in fact may be to some extent conservative.

Table 4.7 Protective concentration percentiles (PCx) produced from sub-lethal and long-term lethal LAS toxicity data.

Protective concentration for x% of species	SSD of sub-lethal data. HR guideline ($\mu\text{g.L}^{-1}$) <i>n</i> = 11	SSD of long-term lethal NOEC data ($\mu\text{g.L}^{-1}$) <i>n</i> = 4
PC99	90	417
PC95	304	578
PC90	519	705
PC85	714	817
PC80	901	925
PC75	1087	1036
PC70	1276	1153
PC60	1674	1418
PC50	2126	1752

The ecological or biological attributes for water resource class thresholds B/C, C/D and E/F are less explicit than for the class thresholds A/B and D/E, discussed in the above paragraphs. It was thus not possible to use the available LAS toxicological and biological monitoring data to assess the alignment of the proposed protection levels (PCxs) with resource classes. A decision was, however, taken to retain the arbitrarily-assigned protection levels for class thresholds B/C, C/D and E/F proposed in Table 4.5. The resultant LAS WQGs for South African freshwaters proposed by this thesis are presented in Table 4.8.

Table 4.8 Derived LAS water quality guidelines (WQG) for South African water resource classes.

Water resource class	Level of protection (PCx) threshold	LAS WQG ($\mu\text{g}\cdot\text{L}^{-1}$)
A	PC95	304
B		519
C	PC85	714
D	PC80	901
E	PC70	1276
F		

4.8 Conclusion

In the derivation of WQGs, the aim among all jurisdictions is to reduce the uncertainties involved in using a limited number of toxicological data to predict an ecosystem effect. Particular emphasis is placed on the quality, quantity and type of toxicity data employed, and on the statistical method used to combine the data and make the extrapolation to a specific ecological effect. The often-limited number of toxicological data available for most chemicals means a compromise has to be reached regarding the above issues. This is where the derivation approaches of jurisdictions differ.

After a review of derivation approaches from different jurisdictions it was concluded that the Australian and New Zealand approach (ANZECC and ARMICANZ 2000) was generally the best at reducing the uncertainty of ecological relevance inherent in WQGs. This approach adopts reasonable compromises between the quantity and type of toxicity data ultimately desired for use in WQ derivation and the reality of available toxicity data for most chemicals. A further advantage of this approach was the ability to derive guidelines at lower levels of confidence if insufficient data were available for derivations of higher reliability. This approach enables WQGs to be derived for many more chemicals, but with confidence levels explicitly stated.

In most circumstances, water resources in Australia and New Zealand are managed as slightly- to moderately-disturbed systems with a protection level of PC95, i.e. the 5th percentile of an SSD of relevant toxicity data. Some river systems are considered to be of high conservation value. The protection of such rivers would receive a high priority, such as a prohibition of inputs of synthetic compounds and requirements that

natural chemicals should not exceed background levels. South Africa has six water resource classes: water resources are managed according to Classes A-D, with Classes E and F being considered unacceptable and the resource managed as a Class D. The implementation of these regulations therefore requires a derivation approach that will provide guidelines for each of the classes. As the PC95 is regularly used internationally as the protection level indicating an acceptably small effect but with no overall significant ecosystem, this approach was adopted as the threshold level between resource Classes A and B. An analysis of toxicological data for LAS showed that this level of protection was indeed applicable as a WQG for these two classes.

Very little international research can be used to help determine appropriate levels of protection for threshold values of lower classes. Jooste and Rossouw (2002) utilised the 5th percentile of LC50 lethality data extrapolated to 336 hr to determine the protection level separating management Class D from the unacceptable Classes of E and F, while Warne et al. (2005) suggested the use of the PC80 of sub-lethal data. While the Jooste and Rossouw (2002) approach is attractive as it provides a definite biological endpoint, the LAS lethality data did not fit the exponential model it employs. The PC80 of sub-lethal LAS data was found to correspond with the PC81 of available long-term lethal NOEC data for LAS. As the narrative description of ecological effects that constitute a Class D water resource suggests a large loss of biota has occurred, this level of protection can probably be considered as suitably protective. With only the broad narrative descriptions of biota loss and ecosystem effects available for defining resource classes, it was difficult to rationalize levels of protection for the remaining resource classes and, consequently, the protection levels were arbitrarily assigned around the PC95 and PC80 levels (Table 4.8).

It is generally accepted that WQGs should not be seen in the light of a pass-or-fail scenario, since moderate short-term exceedance of the guideline does not necessarily imply environmental degradation (Matthiessen et al. 2010). Consequently, the exceedance of the proposed LAS WQGs should initiate, or trigger, further investigation using tools such as on-site biological monitoring and, where appropriate, direct toxicity assessment and toxicity identification and evaluation (ANZECC and ARMCANZ 2000; Matthiessen et al. 2010).

Chapter 5 In-stream LAS concentrations in the Balfour River

Preface: This chapter, in more abbreviated form, was been published in the scientific journal *Science of the Total Environment* (Gordon et al. 2009). The data presented here have also been presented in a poster paper at the International Conference on Implementing Environmental Water Allocations, Port Elizabeth, South Africa (February 2009), and an oral paper at the 14th International Symposium on Toxicity Assessment, Metz, France (September 2009).

5.1 Introduction

As discussed in Chapter 1, large quantities of detergent ingredients enter the environment continuously via treated wastewater streams. However, due to the high removal rates of the predominant detergent surfactant ingredient LAS through precipitation, adsorption and biodegradation — both within the sewer system and within the waste water treatment works — environmental risks have been shown to be acceptable (Van de Plassche et al. 1999; Sanderson et al. 2006; HERA 2007).

In urban areas of the developing world, poor provision of wastewater treatment means that municipal and household wastewater is often discharged directly to receiving waters. Under these circumstances, measured surfactant concentrations are highly variable (Table 5.1). McAvoy et al. (2003) and Whelan et al. (2007) have shown that high surfactant concentrations often occur in urban water courses close to emission points and that concentrations decrease rapidly beyond the urban fringe.

The fate and potential effects of laundry detergents in the rural areas of the developing world are not well understood. In rural areas of South Africa, some homesteads and villages do not yet have piped water, necessitating the utilisation of nearby surface water resources. Consequently, laundry washing is sometimes undertaken alongside rural rivers, resulting in the potential for detergent ingredients to be introduced directly into rivers. The levels of LAS in rural South African rivers used for laundry washing are yet to be determined. A study of the washing habits and detergent usage of a rural village situated alongside a small river in the Eastern Cape Province of South Africa (Soviti 2002) suggested intense loading of the river with laundry detergent on a specific temporal basis (it was estimated that approximately 55.2 kg of detergent entered the river each Saturday, with little direct input during the

rest of the week). The effect of season on detergent input was not considered in Soviti's study (2002).

Table 5.1 Receiving water LAS concentrations from direct discharges of untreated domestic and industrial effluents in developing countries.

Country	River	Low - high total LAS conc ($\mu\text{g.L}^{-1}$)	Detection method	Reference
Lao PDR	Houay Mak Hiao River	14 \pm 3 - 513 \pm 25	LC-MS	Whelan et al. 2007
Philippines	Balatuin	3 \pm 1 - 1227 \pm 465	HPLC	McAvoy et al. 2003
	Bucal	14-25	LC-MS	Eichhorn et al. 2001
	San Pedro	73-102		
	San Cristobal	8-9		
	Napindan channel	1-2		
	Pasig (Guadalupe)	6-8		
	Pasig (Manila Bay)	27-73		
Thailand	Maeklong	<2	LC-MS	Kruawal et al. 2005
	Chao Praya	<2		
Brazil	Macacu	14-155	LC-MS	Eichhorn et al. 2002
Taiwan	Lao-Jie	12-135	GC/MS	Ding et al. 1999
Nigeria	Ubu	130 \pm 40 - 270 \pm 140	MBAS	Uzoukwu et al. 2004
	New Calabar	0.1-30	MBAS	Odokuma & Okpokwasili 1997

Notes: * If multiple samples at a site, an estimate of variability is provided, all authors used \pm one standard deviation.

HPLC = high-performance liquid chromatography

LC-MS = liquid chromatography – mass spectrometry

GC-MS = gas chromatography – mass spectrometry

MBAS = methyl blue active substances (measures all anionic surfactants)

To address the research question of this thesis, an understanding of the range of LAS concentrations to which in-stream biota are exposed is required. From Soviti's (2002) work it was apparent that the Balfour River, which runs through the Balfour Village in the Eastern Cape, was an ideal location for this type of study. There were no other land uses or activities that would contribute to LAS input to the river, there were clearly-identified washing sites and a suitable reference site upstream of washing sites. Potential downstream monitoring sites were available, and there was a nearby hydrological gauging weir.

In addition to obtaining in-stream LAS information through an intensive monitoring programme of river water and sediment, an attempt was made to predict LAS concentrations in-stream, based on information on laundry washing practices and detergent use of residents living alongside the river. The ability to predict in-stream

LAS concentrations from washing practises and detergent usage of riverside residents could prove a useful tool in attempting to determine potential ecological harm of near-stream laundry washing to aquatic biota in other catchments, thus reducing the need for costly monitoring programmes.

Consequently, the aims of this chapter were to (1) determine temporal patterns of in-stream LAS concentrations within the Balfour River, so as to contribute to an assessment of the risk that this activity poses to aquatic biota and (2) to ascertain whether in-stream LAS concentrations could be predicted from detergent consumption data obtained from households alongside the river.

5.2 Materials and methods

5.2.1 Study area

The Balfour River is a small tributary of the Kat River, situated within the Eastern Cape Province of South Africa (Figure 2.1). A description of the river and catchment is presented in Section 2.2. Briefly, the river is small, approximately 9 km long. The river morphology is typical of low order streams: the depth is generally shallow (ranging between about 0.2 m and 1 m in riffles and pools, respectively) and the bed material is mostly coarse (boulders, gravel and sand). Land use within the catchment appears to comprise limited subsistence livestock and subsistence agriculture, with indigenous and limited commercial forestry in the headwaters of the catchment. The Balfour village is the only settlement situated alongside the Balfour River and is composed predominantly of residential buildings. A limited number of homesteads are scattered throughout the catchment.

5.2.2 Water and sediment chemistry sampling

River water and sediment were sampled on an arbitrarily-chosen Wednesday and Saturday in the winter and summer of 2004, and in the autumn of 2005 at six sites, one upstream and five downstream of identified washing sites along the Balfour River (Figure 2.1). The objective was to take samples when LAS input to the river was highest and lowest, to determine the full range of potential concentrations in-stream. Wednesday and Saturday were chosen as suitable days for sampling after a preliminary investigation by Soviti (2002) suggested more laundry washing occurred over the weekend and less in the middle of the week. On each sampling day, 1 L

grab samples of river water were collected, in pre-washed glass Schott bottles, by opening the Schott bottles at a depth of 10 cm below the river water surface. The samples were collected on the hour between 8 am and 5 pm at all six sites by employing independent samplers for each site. In addition, three one-off sediment samples (approximately 120 g per sample) were collected in glass jars from each site.

Prior to field sampling, sample bottles and jars were pre-washed in the laboratory with 10% HCl, rinsed in deionised water, and left to dry. They were then rinsed with 50 – 100 mL methanol, rinsed again in deionised water, and then dried. Also in the laboratory, 30 mL of 37-40% formaldehyde (i.e. 3% v/v formalin) was placed in the Schott bottles to preserve the water sample and prevent further biological degradation of the LAS by micro-organisms (e.g. Eichhorn et al. 2002). The Schott bottles were then sealed and packed for transport to the field. Sediment samples were obtained by taking three grab samples per site of in-stream river sediment to a depth of 5 cm. Excess water was allowed to drain out for 1 minute. Sediment was transferred to the glass jars and preserved with 10% formalin (enough volume was added to immerse the sediment). Methanol-washed aluminium foil was placed over the mouth of the jar and then fastened with the lid to prevent sample contamination. Samples were kept on ice. In the laboratory, water and sediment samples were maintained at approximately 4 °C until transport in polystyrene boxes with dry ice to the Safety and Environmental Assurance Centre (SEAC), Unilever Colworth, United Kingdom, for analysis of LAS. The analysis of LAS in the Balfour River water was undertaken by SEAC staff.

5.2.3 Extraction of water samples

Typically, 500 mL of river water was mixed with 25 mL of methanol before loading onto a methanol-conditioned C₁₈ 1 g / 6 mL Isolute solid phase cartridge (Kinesis, Bolnhurst, UK). After drying for 1 hour under vacuum, the solid phase cartridges were eluted with methanol (approximately 20 mL). The eluent was taken to dryness under nitrogen and resuspended in 1 mL of methanol to form the final extract.

5.2.4 Extraction of sediment samples

Overlying water was removed prior to oven drying at 80 °C for 16 hr. The dry sediment (10 g, large stones and grit not included) was extracted by sonication with

methanol at 50 °C in a Decon FS 200b Ultrasonic bath (240 V, 3 A, 50 Hz) (Decon Ultrasonics Ltd, Hove, Sussex, UK). Three 10 min extractions (50 mL and 2 x 40 mL) were carried out, with the sediment separated from the extract by means of a centrifugation step. The combined extract was normally concentrated to 2 mL to form the final extract.

5.2.5 Liquid chromatography/mass spectrometry

Final extracts from the water and sediment samples were analysed using liquid chromatography/mass spectrometry as described in Whelan et al. (2007). The detection limit of the method was typically 1 µg.L⁻¹ and 100 µg.kg⁻¹ (dry weight) for LAS in water and sediment respectively.

5.2.6 Statistical analysis

Measured LAS concentrations were analysed using the STATISTICA™ Version 7 statistical package. The data were checked for normality and homogeneity of variance and, as a consequence, then subjected to the nonparametric Kruskal-Wallis ANOVA (analysis of variance) and median test.

5.2.7 Obtaining data on laundry washing practices and detergent usage

Two types of interview methods were conducted to obtain data regarding the frequency, periodicity and location of laundry washing and the type and quantity of laundry detergent used by rural villagers. The first method was an unstructured community workshop organised within the Balfour Village. The second method was a semi-structured interview of 40 targeted individual households within the village. An interviewer of the same ethnicity as the local residents was employed to conduct interviews and to run the workshop.

5.2.8 Calculating potential in-stream LAS concentrations

The potential in-stream LAS concentration was predicted from the detergent usage estimates. Although there were a number of laundry washing sites along the Balfour River, the predicted in-stream LAS concentrations were determined for the lowermost washing site, situated immediately above Site 2 (Figure 2.1), assuming all washing

occurred here. The predicted in-stream LAS concentrations (C_0 , $\text{mg}\cdot\text{L}^{-1}$) were calculated using the following equation:

$$C_0 = \frac{L_P}{Q} \quad (1)$$

where Q is discharge ($\text{L}\cdot\text{s}^{-1}$) and L_P is average input of LAS to the river ($\text{mg}\cdot\text{s}^{-1}$), which in turn is determined by L_D (total LAS input, mg) over the period (P) in which laundry is typically performed (assumed to be 4 h):

$$L_P = \frac{L_D}{P \cdot 3600} \cdot F \quad (2)$$

where F is the proportion of LAS used which actually enters the river. The input of LAS to the river during near-stream laundry washing (L_D) was estimated using detergent consumption data obtained from individual household interviews for the Balfour Village.

The LAS component of the daily detergent input was calculated by determining the proportion of LAS in the various detergent products and the activity of the LAS used to manufacture these products. Washing powders Omo[®], Sunlight[®], and Surf[®] consist of 25%, 19% and 23.5% LAS respectively (Pers. comm. Warren 2004). The activity of LAS powder used in the manufacture of these washing powders is approximately 84% (Pers. comm. Ramsamy 2004). An initial assumption was made that 100% of the LAS component entered the river (i.e. $F = 1$). However, a significant fraction of laundry is washed on the river bank rather than in the river itself, so this assumption is conservative. A lower estimate of $F = 0.3$ was considered to be more realistic, whilst still being conservative. An attempt was also made to estimate F by back calculating from the observed LAS concentration (C_{OBS} , $\mu\text{g}\cdot\text{L}^{-1}$) in the Balfour stream at Site 2. This was achieved by combining Equations (1) and (2):

$$F = \frac{C_{OBS} \cdot Q \cdot P \cdot 3600}{L_D} \quad (3)$$

in which L_D is expressed in $\mu\text{g}\cdot\text{d}^{-1}$. River discharge data (from 1972–2005) were obtained from the Department of Water Affairs and Forestry, measured by the hydrological weir Q9H019 situated within the Balfour River at 32°33'05" S 26°40'17" E (Figure 2.1). In order to reflect the seasonality in river discharge, mean monthly flow data were utilised to calculate predicted seasonal in-stream LAS concentrations.

These concentrations at downstream Sites 3-6 were not calculated because of the difficulties of defining the upstream boundary conditions (characterized by highly variable temporal loading) and subsequent advection, dispersion and degradation.

5.3 Results

5.3.1 LAS concentrations measured in the Balfour River

Laboratory quality control analyses showed good average recoveries of LAS spiked at $10 \mu\text{g.L}^{-1}$ in MilliQ Ultrapure water in the laboratory, averaging 87% ($n = 28$). Average recovery for $10 \mu\text{g.L}^{-1}$ LAS laboratory-spiked river water was 101% ($n = 10$). Average recoveries for laboratory-spiked river sediment at $1000 \mu\text{g.kg}^{-1}$ and $200 \mu\text{g.kg}^{-1}$ LAS averaged 92% and 88%, respectively ($n = 4$ for both analyses). River water was also field-spiked as part of the quality control procedure to test whether adequate preservation had taken place. The average LAS recovery of spiked river water was 107% for a $1000 \mu\text{g.L}^{-1}$ spike and 91% for a $10 \mu\text{g.L}^{-1}$ LAS spike ($n = 9$ and $n = 10$ respectively).

Measured LAS concentrations, organised in terms of site, day of the week, time of sampling and season, are summarised in Figure 5.1. Changes in average LAS concentration (all data) at various distances downstream of the lowermost washing site, are shown in Figure 5.2. Although LAS was detected at the reference site (Site 1), concentrations were consistently below $4 \mu\text{g.L}^{-1}$ (Figure 5.1a and b; Table 5.2). At Site 2, the first sampling site below the laundry washing sites, considerably higher concentrations of LAS were observed during summer, with a peak concentration of $342 \mu\text{g.L}^{-1}$ measured at 10h00 on Saturday (Figure 5.1c and d). LAS concentrations at sampling Sites 3-6, further downstream, were much lower (Figure 5.1e-l; Figure 5.2), with the highest concentration being $11 \mu\text{g.L}^{-1}$ at Site 3 on Saturday during summer at 16h00 (Figure 5.1e). Mean LAS concentration measured at Site 2 was considerably higher, although not significantly different (Kruskal-Wallis; $p \geq 0.05$) from concentrations at sampling sites further downstream (Table 5.2). The lack of statistical significance in the differences between concentrations at the different downstream sites was largely a consequence of the range in concentrations measured. Measured in-stream LAS concentrations at all six sites were significantly higher on Saturday (particularly in summer) compared to Wednesday (Kruskal-Wallis; $p \leq 0.05$) (Figure 5.1; Table 5.2). It was also evident that measured LAS concentrations were significantly higher during summer compared to autumn and

winter (Kruskal-Wallis; $p \leq 0.05$) (Table 5.2). The highest concentrations of LAS were measured at Site 2 in the morning (Figure 5.1c and d). At sampling sites further downstream there was no distinct pattern in terms of the timing of peak LAS concentrations (Figure 5.1e-l).

The highest concentration of LAS measured in river sediment was $186 \mu\text{g.kg}^{-1}$ at Site 3 on Saturday during summer. Samples from other sites were below the detection limit ($100 \mu\text{g.kg}^{-1}$). Samples obtained four days later, on Wednesday, were all below the detection limit. During the winter sampling, elevated concentrations of LAS (between 101 and $176 \mu\text{g.kg}^{-1}$) were measured in sediment from Sites 2 to 4 on Saturday and Sites 2 to 5 on Wednesday. Samples from all sites, taken on Saturday and Wednesday in August, were below the detection limit.

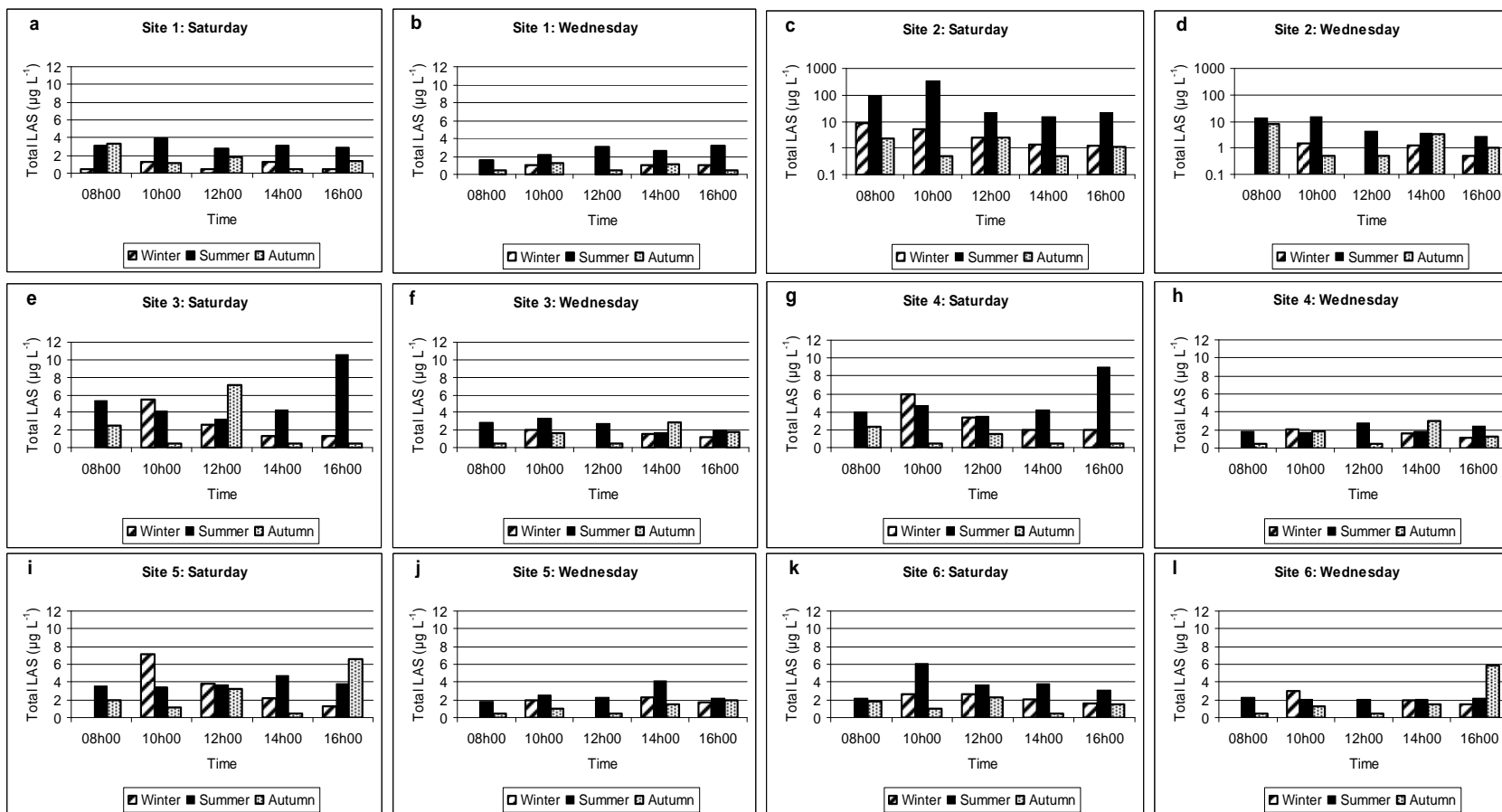


Figure 5.1a-I LAS measured at sampling Sites 1-6 in the Balfour River on a Saturday and Wednesday in winter, summer and autumn. Note: Log y-axis in panels C & D.

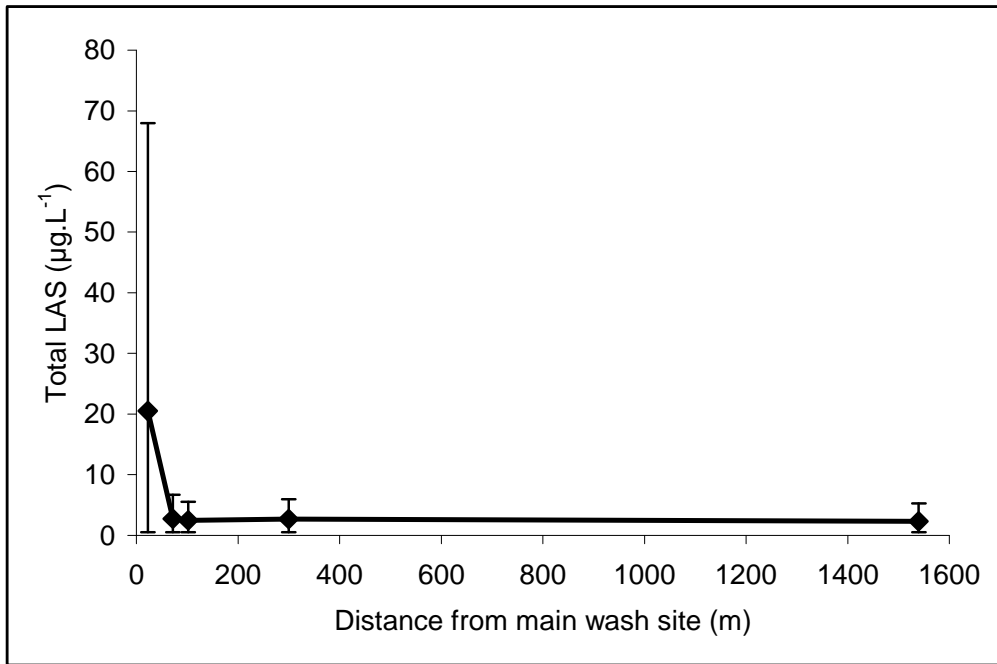


Figure 5.2 Average LAS concentrations in the Balfour River (all data) at monitoring sites downstream of main laundry washing site. Error bars indicate 95th and 5th percentile concentrations.

Table 5.2 Mean LAS concentrations measured per site, day and season sampled.

Variable	Mean LAS concentration (µg.L ⁻¹)	Standard deviation	Maximum LAS concentration (µg.L ⁻¹)	Minimum LAS concentration (µg.L ⁻¹)	n
Site 1	2	1	4	0.5	28
Site 2	21	65	342	0.5	28
Site 3	3	2	11	0.5	27
Site 4	3	2	9	0.5	27
Site 5	3	2	7	0.5	27
Site 6	2	1	6	0.5	27
Saturday	8	38	342	0.5	86
Wednesday	2	3	14	0.5	78
Autumn	2	2	8	0.5	60
Winter	2	2	9	0.5	44
Summer	12	45	342	2	60

Note: Detection limit = 0.5 µg.L⁻¹

5.3.2 Balfour community profile

Twelve community representatives from various community structures (sub-village committees and street committees) attended the community workshop interview, and provided information for the profile of Balfour Village. At the time of this workshop, the Balfour Village consisted of four sub-villages: Phase 4 (30 households); Mandela Park (164 households); Chris Hani (151 households) and Phola Park (113 households) (Figure 2.1). Phase 4 was the only sub-village on the west bank of the Balfour River and had not been provided with communal taps. Mandela Park, lying opposite Phase 4, was supplied with a few communal taps. Chris Hani sub-village, which lies alongside the river to the south of Mandela Park, had been supplied with only a few communal taps. Residents of these three sub-villages were generally poorer than those from Phola Park and thus possessed few rainwater tanks. Consequently they relied extensively on river water for consumption, cooking and laundry washing. In contrast, Phola Park, situated approximately 1 km from the river, had been provided with more communal taps than the other sub-villages. The taps also worked more frequently than those in the other sub-villages, although residents collected water from the river if necessary. It was thus decided to exclude the Phola Park from the individual household interviews when determining washing practices and in-stream LAS predictions. The focus was therefore on the sub-villages located alongside the river.

5.3.3 Washing practices

Laundry washing was generally undertaken by respondents once (35%) or twice a week (43%), with 18% of respondents undertaking washing three times a week (see Appendix 7 for a summary of all data obtained from interviews and the workshop). The most popular days for washing were Saturday (27%), Monday (20%), Friday (18%) and Wednesday (16%). Washing was not purportedly undertaken on Sundays. The duration of washing activity generally varied between 1 and 4 hours and was usually undertaken in the morning. Monthly laundry detergent usage per household was determined to be 1.5 kg. Taking into consideration the quantity of LAS in each detergent brand used, the activity of the LAS, and the number of households in the three villages alongside the Balfour River (345), the total monthly LAS consumption by the communities living

alongside the Balfour was determined to be 58.6 kg. Seasonal difference in consumption of laundry detergent was not determined.

The location of laundry washing varied seasonally. During summer, 25% of respondents undertook washing at the river, and 75% at home. During winter, however, only 8% of respondents washed at the river, and 92% washed at home. There was thus potentially more LAS entering the river during summer than in winter.

The fate of the discarded washing water was found to be dependent on the position of the washing site in relation to the river. If directly beside the river, the discarded wash liquor could potentially enter the river when poured on the ground. If the site was positioned some way from the river (usually because of a shortage of available comfortable places beside the river) the discarded liquor often drained into the ground before reaching the river. If washing was undertaken at the homestead, the wash liquor was discarded on (and usually infiltrated into) the ground. All the above information and data were used in predicting potential in-stream LAS concentrations in the Balfour River.

5.3.4 Predicted in-stream LAS concentrations

Potential in-stream LAS concentrations at Site 2, based on the washing practice survey, assuming $F = 1$ and $F = 0.3$, for different days of the week and different months of the year, were assessed (Figure 5.3). The predictions reflect the periodic nature of near-stream laundry practices and the seasonality of river flow (Figure 5.4). Predicted peak-concentrations varied from day to day, depending on the number of washers using the river bank. The highest concentration each week was predicted to be on a Saturday, reflecting the results of the laundry washing survey. In-stream LAS peak-concentrations on other days of the week were predicted to be considerably lower. Potential in-stream LAS concentrations were predicted to be highest in July, August, and October. To give an indication of potential occurrence of hazardous LAS concentrations in the Balfour River, predicted in-stream LAS concentrations are compared to the LAS WQG of $304 \mu\text{g.L}^{-1}$ determined in Chapter 4 of this thesis. Even under the unrealistic assumption of $F = 1$ (i.e. all discarded laundry liquor is poured directly into the river), predicted peak LAS concentrations only exceed the LAS WQG of $304 \mu\text{g.L}^{-1}$ on certain days in July, August

and October (Figure 5.3a). Based on the assumption of $F = 0.3$, the predicted peak LAS concentrations never exceed the LAS WQG (Figure 5.3b).

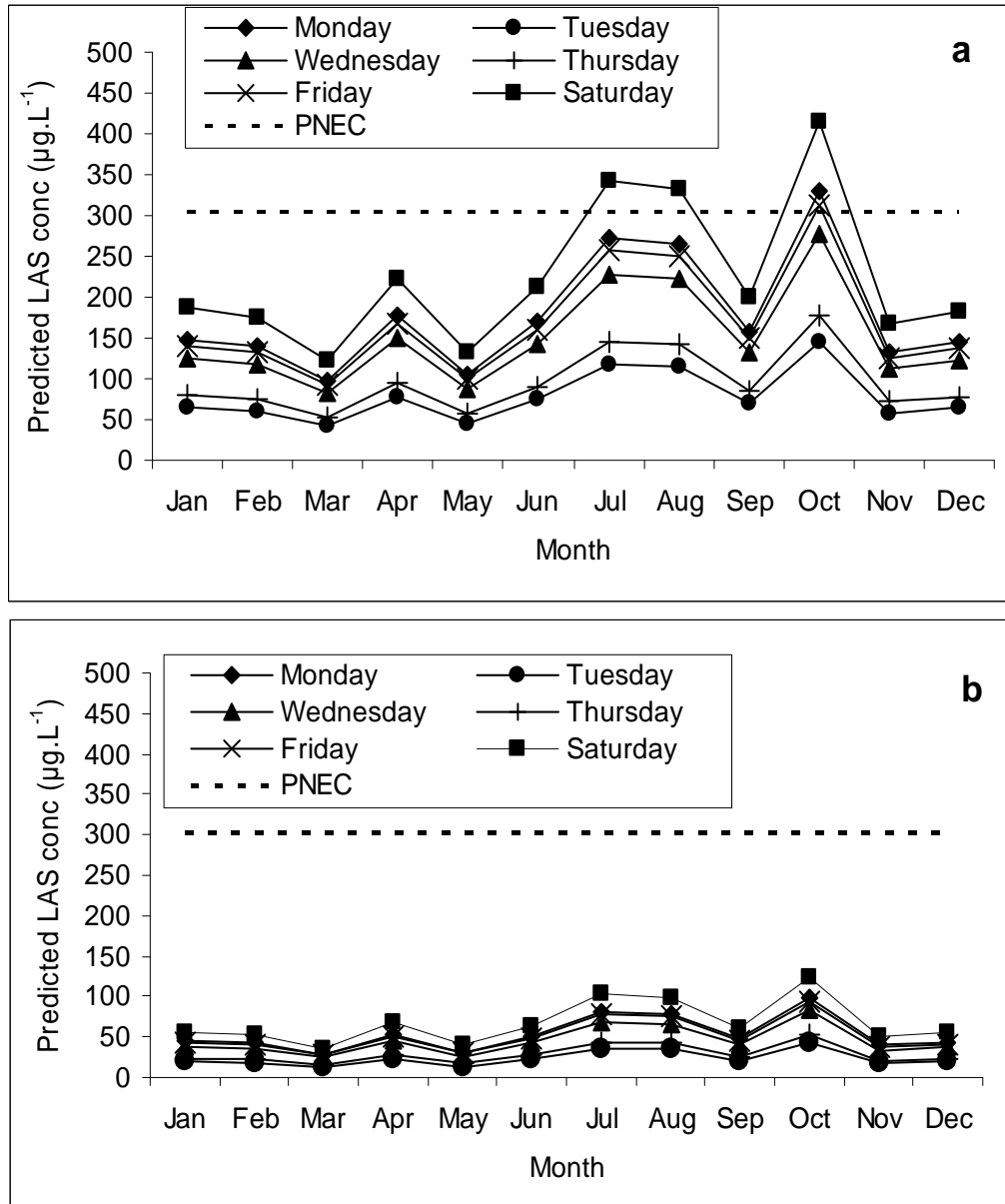


Figure 5.3a-b Potential peak-concentration of LAS expected within the Balfour River at Site 2 each day of each month, assuming (a) 100% LAS input to the river ($F = 1$) and (b) 30% LAS input to the river ($F = 0.3$).

Note: the LAS WQG of 304 µg.L⁻¹ was determined in Chapter 4 of this thesis.

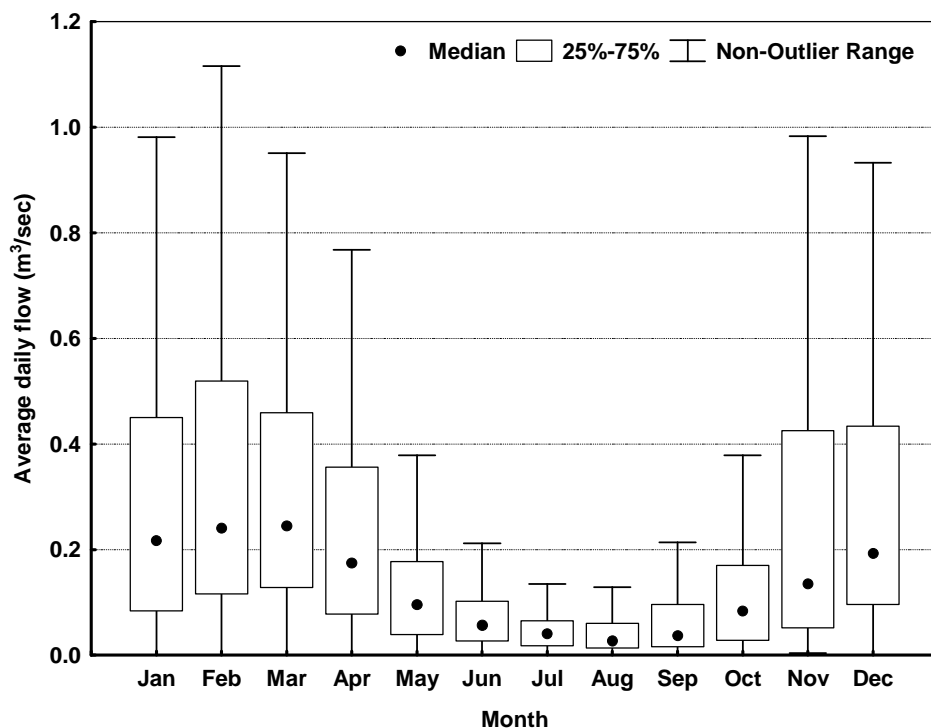


Figure 5.4 Average median monthly flow rates ($\text{m}^3 \cdot \text{s}^{-1}$) measured at the Balfour River Weir (April 1972–August 2005)

Values for F determined from the observed concentration data at Site 2 and the corresponding load estimates for day of week and season (Equation 3) had a mean value of 0.11, a median of 0.018, and a 95th percentile value of 0.37. Overall, adopting a value for F of 0.3 is probably a reasonable worst-case assumption for this site, although on one occasion at Site 2 the peak measured concentration (at $342 \mu\text{g} \cdot \text{L}^{-1}$) exceeded the predicted concentration, suggesting that locally-high concentrations are experienced intermittently.

5.4 Discussion

The good LAS recoveries measured in field-spiked river water suggest that the methods used for sample preservation, storage and transportation to the United Kingdom were effective. Furthermore, the good LAS recovery rates observed for laboratory-spiked water and sediment samples indicate that the sample preparation and LAS analysis methods were of a high standard.

Site 1 was chosen as a reference site, with no apparent evidence of laundry washing occurring along the banks upstream. No LAS was measured within sediments at this site over the three seasons sampled. However, low in-stream LAS concentrations were measured at this site, suggesting some washing activity upstream. Most of these concentrations were just above the detection limit. The ecological impact of LAS at this site is thus likely to be inconsequential as concentrations are far below the derived WQG for LAS determined in Chapter 4.

The apparent variability observed suggests that LAS in sediment are transient, although high spatial variability could also be an important explanatory factor. The higher concentrations observed during winter could be related to the lower river flows experienced at the time, possibly resulting in increased particulate deposition (and a deposition of finer sediment size fractions) with associated sorbed-phase LAS. In contrast, the lack of LAS within sediment samples during summer (except for the one measurement of $186 \mu\text{g.kg}^{-1}$ at Site 3 on Saturday when there was unseasonably low flow) could be a result of higher flows within the river at that time of year, resulting in higher sediment entrainment rates and lower deposition rates. Lower water temperature in winter (average $13 \text{ }^{\circ}\text{C}$) compared to summer (average $22 \text{ }^{\circ}\text{C}$) should result in reduced LAS degradation rates in sediment. A sediment quality guideline for LAS has yet to be derived. However, HERA (2007) proposed a sediment PNEC of $8100 \mu\text{g.kg}^{-1}$ from available toxicity test data. This value is considerably higher than the LAS concentrations measured in Balfour River sediments, suggesting little anticipated effect on sediment-dwelling organisms within this river.

The highest pelagic LAS concentrations were measured at Site 2, the closest sampling site to areas where laundry washing took place. Considerably lower concentrations were measured at Site 3, just 49 m downstream from Site 2. This was most clearly seen on Saturday during summer, when $342 \mu\text{g.L}^{-1}$ LAS was measured at Site 2, and a maximum of $11 \mu\text{g.L}^{-1}$ was measured at Site 3. The pattern of LAS concentrations with distance downstream (Figure 5.2) reflects an intermittent emission of wash water to the river (characterised by high concentration variability at Site 2) followed by relatively rapid mixing and a high degree of hydrodynamic dispersion. This phenomenon is particularly important in 'spill' scenarios (e.g. Gandolfi et al. 2001) when solute plumes spread out relatively quickly, reducing peak solute concentrations significantly, even in the absence

of loss mechanisms (such as sorption or degradation). In the case of LAS, degradation is likely to enhance the effects of dispersion in terms of reducing concentrations. Some LAS may also have been advected or diffused into the sediment. Fox et al. (2000) reported a first order rate constant of 0.3 h^{-1} for a stream in Yorkshire, UK with similar channel morphology to that of the Balfour River. This corresponds with a half life of about 2.3 h. The removal was attributed to primary biodegradation and the deposition of suspended matter onto which the LAS had adsorbed. Similar reductions in LAS, with increasing distance downstream from a LAS input, have been observed by McAvoy et al. (2003). Such rapid degradation has a significant effect on concentration changes in the monitored reach. Other workers — including Whelan et al. (1999), Eichhorn et al. (2002) and Whelan et al. (2007) — have observed lower rate constants, probably because of deeper water and a lower degree of contact with bed and bank sediments.

Significantly higher in-stream LAS concentrations were measured on Saturday compared to Wednesday. This was particularly evident during summer, reflecting more laundry washing on Friday and Saturday compared to midweek. Higher in-stream LAS concentrations on Saturday corroborate information provided by residents of Balfour Village, which indicated that Saturday was the most preferred day for laundry washing. Predicted in-stream LAS concentrations were based on the information from the washing practices survey and, hence, were also estimated to be highest on Saturdays.

The measured in-stream LAS concentrations were significantly higher during the summer sampling campaign compared to concentrations measured in autumn and winter. Higher concentrations on summer Saturdays can be attributed to a combination of high laundry activity and lower-than-expected flows for this time of the year. Although hydrological data (Figure 5.4) from the gauging station on the Balfour River suggest that lowest flows normally occur during the winter months (June–September) and highest flows during the summer months (December–March), the daily flow rate measured on the Saturday sampled in summer 2004 was much lower ($0.05 \text{ m}^3 \text{ s}^{-1}$) than would normally have been expected for that time of year (Table 5.3). A rain storm on the following Tuesday caused the higher flow of $0.72 \text{ m}^3 \text{ s}^{-1}$ measured on Wednesday. This flow rate was more reflective of the expected summer flow rates, and partially explains the much lower LAS concentrations measured on Wednesday. Flow rates measured during winter 2004 and autumn 2005 sampling campaigns corresponded with the

average median flows expected during this season (Table 5.3). It thus appears that low flow conditions during winter are associated with lower in-stream LAS concentrations compared with similar flow conditions during the summer months (when they occur) because the potential for LAS input is much higher in summer.

The highest concentrations of LAS at Site 2 were measured in the morning. The survey of Balfour residents revealed that laundry washing most often took place when climatic conditions beside the river were most comfortable. During summer, temperatures can be uncomfortably high during the middle part of the day, so the washing generally takes place during the early morning. Further downstream, there is no distinct temporal pattern for the highest measured LAS concentrations.

Table 5.3 Average daily flow rates ($\text{m}^3 \cdot \text{s}^{-1}$) measured on sampling occasions.

Sampling season	Date	Flow rate ($\text{m}^3 \cdot \text{s}^{-1}$)
Winter 2004	Saturday 31/7/2004	0.08
Winter 2004	Wednesday 4/8/2004	0.05
Summer 2004	Saturday 4/12/2004	0.05
Summer 2004	Wednesday 8/12/2004	0.72
Autumn 2005	Saturday 30/4/2005	0.23
Autumn 2005	Wednesday 4/5/2005	0.27

In-stream LAS concentrations predicted from the washing surveys suggested that the winter and spring months were likely to result in the highest in-stream LAS concentrations, due to the low flow at this time. This was not however reflected in the measured in-stream LAS concentrations. Possible explanations for this discrepancy are that a lower percentage of potential washers than determined from the survey (i.e. 8%), may have actually washed clothing beside the river, or perhaps laundry washing was undertaken further from the river, resulting in less discarded laundry liquor reaching the river in winter than initially assumed. Calculated values of the fraction of estimated LAS entering the Balfour River (F), based on the observed concentration data (Equation 3), suggest that only a small fraction of laundry liquor (<10%) is discarded close enough to the river to allow it to make a significant contribution to in-stream LAS concentrations.

5.5 Conclusion

Linear alkylbenzene sulfonate was rarely present in Balfour River sediments and —when present — was at low concentrations. The potential effect of sediment- associated LAS on benthic organisms is, thus, likely to be limited.

Concentrations of LAS in Balfour River water were generally low, with the mean LAS concentration measured at Site 2 being $21 \pm 65 \mu\text{g.L}^{-1}$ (\pm standard deviation). Mean LAS concentrations at sites further downstream ranged between 2 and $3 \mu\text{g.L}^{-1}$, suggesting limited risk to aquatic organisms. Peak LAS concentrations as high as $342 \mu\text{g.L}^{-1}$ were measured at Site 2 however. This would have exceeded the LAS WQG, suggesting possible sub-lethal effects on resident aquatic organisms. It appears, however, that these higher LAS concentrations, with the potential to cause sub-lethal ecological effects, are likely to occur only occasionally — for short periods of time and for a limited distance downstream — in parts of the Balfour River that are located close to laundry washing sites. Laundry washing habits and river flow rates suggest that these higher LAS concentrations are likely to occur in the morning, during the warmer months and when the river flow is unseasonably low.

An assumption of 100% detergent discharge directly to the river by near-stream laundry washers significantly over-predicted in-stream LAS concentrations based on detergent usage and washing practice information. The most likely explanation is that a significant fraction of the laundry liquor discarded on the river bank infiltrates the soil rather than running into the river. At the Balfour River site, the transfer of LAS to the river was estimated to be <10 % of the LAS used by near-stream washers, depending on season.

Chapter 6 Biological monitoring of the Balfour River to assess effects of near-stream laundry washing

6.1 Introduction

Chemical monitoring for the primary detergent ingredient linear alkylbenzene sulfonate (LAS) in the Balfour River water (Chapter 5) showed that concentrations varied temporally during a typical day, week, and over different seasons. Measured LAS concentrations in the river water were generally higher during the morning, at the end of the week (Friday and Saturday), and during the warmer summer months. Concentrations also varied spatially, with the higher concentrations occurring immediately below washing sites and decreasing rapidly downstream. Although the average LAS concentrations measured were low ($21 \pm 65 \mu\text{g.L}^{-1}$ immediately below the washing site to $2 \pm 1 \mu\text{g.L}^{-1}$ further downstream), the peak concentrations immediately below the washing site ($342 \mu\text{g.L}^{-1}$) were above the established predicted no-effect concentrations (PNEC) determined for LAS of $245 \mu\text{g.L}^{-1}$ (Dyer et al. 2003) and $250 \mu\text{g.L}^{-1}$ (Van de Plassche et al. 1999), the Australia and New Zealand water quality guideline (WQG) for freshwaters of $280 \mu\text{g.L}^{-1}$ (ANZECC and ARMCANZ 2000) and the WQG derived in this thesis of $304 \mu\text{g.L}^{-1}$ (Chapter 4).

However, comparing measured in-stream LAS concentrations to PNECs and WQGs, especially when concentrations were fluctuating over time, is not considered a reliable way of assessing biological effects of contaminant exposure (Adams and Tremblay 2003; Van der Oost et al. 2003; Galloway et al. 2006). In addition, PNECs and WQGs were derived using only a limited number of organisms, which are assumed to be representative of all organisms in the environment (Selck et al. 2002; ANZECC 2000). There is also concern that results determined in the laboratory cannot be accurately extrapolated to the field as conditions in laboratories and the organisms used there can be different to those found in natural environments (Connel et al. 1999; Jorgensen and Christoffersen 2000). Biological monitoring is considered to have the advantage of integrating temporally-variable contaminant concentrations (Suter 2001; Karr and Chu 2000).

Biomonitoring of water resources exposed to LAS has only been undertaken on a few occasions (Dyer et al. 2003; Sanderson et al. 2006), probably because LAS usually enters the aquatic environment via wastewater treatment works along with many other xenobiotics. In such a scenario, ascribing observed biological effects to LAS is not possible. In the Balfour River catchment, however, there appears to be no other chemical input other than

laundry detergent to the river, providing an opportunity to assess the specific effects of laundry detergent ingredient LAS on in-stream organisms.

Aquatic macroinvertebrates were the biota selected to investigate the potential effects of near-stream laundry washing and associated LAS input to the Balfour River. They are the most widely-used organisms in bioassessments in South Africa and internationally (Ollis et al. 2006). The advantages of using macroinvertebrates for this purpose have been extensively discussed (Reynoldson and Metcalfe-Smith 1992; Rosenberg and Resh 1993; Downes et al. 2002; Hodkinson and Jackson 2005; Bonada et al. 2006; Ollis et al. 2006) and a summary is provided in Chapter 1. Fish were not considered suitable as previous bioassessments of this river failed to record any fish species above the washing sites, probably due to the steep gradient of the river and frequent cascades in these upper reaches (Lerotholi 2005).

Stress responses, used to measure the effects of contaminant exposure on biota, can be undertaken at various levels of biological organisation. This concept is discussed in more detail in Chapter 1 (see Table 1.2). In summary, stress responses measured at lower levels of biological organisation represent responses to contaminant exposure that are manifested at a small spatial scale (in a cell or organ of an organism) and occur within a short period of time after exposure. It is difficult, however, to link a response at this low level of organisation with an effect that might be regarded as ecologically relevant (Adams et al. 2000; Triebskorn et al. 2001; Johnson and Collier 2002). Stress responses measured at higher levels of organisation (population or community responses) are, however, considered more ecologically relevant (Mayon et al. 2006), which is considered desirable when assessing impacts on aquatic ecosystems. Unfortunately, at these higher levels of organisation, it becomes difficult to establish exactly what the causal relationships between the contaminant exposure and the measured ecosystem effects are (i.e. specificity of the measured stress response decreases and it becomes difficult to attribute impairment to the presumed contaminant) (Hodkinson and Jackson 2005). There is an advantage, therefore, to utilising stress responses from a number of levels of organisation: each response provides a different type of information necessary to understand the dynamics of the exposure response situation and to make an accurate assessment of the impact of the contaminant (Clements 2000; Ross and Bidwell 2006; Yeom and Adams 2007).

Biological stress responses from low to high levels of biological organisation have been utilised to measure the effects of LAS exposure in the laboratory (described in greater detail in Chapter 3). At low levels of organization, various enzymes such as cholinesterase (ChE), glutathione-S-transferase, cytochrome P-450 and other gill, liver and kidney enzymes, as

well as protein synthesis and phosphate activity have been measured (Table 3.9). At higher levels of organization, growth, reproduction and various community responses have been used to determine the hazard of LAS (Tables 3.4 and 3.5). These studies, together with the various measures of macroinvertebrate taxa richness and abundance employed by Dyer et al. (2003) and Sanderson et al. (2006), informed the choice of which stress responses would be employed in the biological monitoring of the Balfour River, upstream and downstream of near-stream laundry washing. Stress responses from low levels of biological organisation included the commonly-applied assays of ChE inhibition and lipid peroxidation (LPx). At higher levels of biological organisation, measures of macroinvertebrate tolerance to water quality impairment, measures of community composition, measures of community richness, together with macroinvertebrate functional feeding groups (FFG) (a measure of ecosystem function) were utilised (Table 6.1). The objective for this chapter was thus to measure the potential effects of near-stream laundry washing on the macroinvertebrates of the Balfour River by investigating biological stress responses at different levels of organisation (Table 6.1). In particular, the use of two simple and frequently-applied cellular and sub-cellular stress responses, as early warning indicators of LAS exposure, was assessed.

Table 6.1 Macroinvertebrate stress responses measured in this study: adapted from Barbour et al. (1996), Baptista et al. (2007) and Brossett et al. (2010).

Biological stress response	Predicted response to stress (when compared to reference site)	Description
Lower levels of organisation		
Cholinesterase activity	Inhibition	Evidence of effects of contaminant stress on organisms at a cellular level
Lipid peroxidation	Increase	
Higher levels of organisation		
Measures of macroinvertebrate taxa tolerance		
SASS5	Decrease	Measures of the sensitivity of taxa to contaminant stress. Baetidae / Ephemeroptera and EPT / Chironomidae represent a ratio of abundance of organisms from these taxa to one another
Baetidae / Ephemeroptera	Increase	
EPT / Chironomidae	Decrease	
Measures of macroinvertebrate community composition		
% EPT abundance	Decrease	Indicates changes in percentage abundance of indicator species (sensitive or tolerant) to contaminant stress
% Ephemeroptera abundance	Decrease	
% Trichoptera abundance	Decrease	
% Diptera abundance	Increase	
NMDS of all macroinvertebrate taxa	Differentiation	
Measures of macroinvertebrate community richness		
Number of EPT families	Decrease	High richness is often used to indicate good habitat availability (water physicochemical, substrate, food resources)
Shannon-Weiner Diversity Index	Decrease	
Margalef's Taxon Richness Index	Decrease	
Measures of trophic level representation in macroinvertebrate community		
NMDS of functional feeding groups	Differentiation	Potential measure of changes in trophic dynamics of water resource by simplifying macroinvertebrate into trophic guilds according to feeding strategy

Notes: SASS5 = South African Scoring System Version 5.

EPT = Ephemeroptera, Plecoptera and Trichoptera.

NMDS = Non-metric multi-dimensional scaling.

6.2 Materials and methods

6.2.1 Study area

A description of the Balfour River and surrounding area is provided in Chapter 2, and a map of the sampling sites depicted in Figure 2.1. Collection of organisms for analysis of stress responses at low levels of biological organisation was undertaken at the reference site upstream of any laundry washing (Site 1), then immediately below the most popular and lowermost washing site (Site 2) and also at the furthestmost site downstream (Site 6) to determine the spatial extent of possible LAS effects on the sub-cellular responses of selected organisms in-stream.

Sampling of the macroinvertebrate community for determination of effects at higher levels of biological organisation was undertaken at five sites (Sites 1-2 and 4-6) (Figure 2.1). Site 3 was situated at the overflow from a hydrological weir and was used for the monitoring LAS concentrations in Balfour River water (Chapter 5) but was not suitable for biological monitoring due to a lack of sampling habitat.

6.2.2 Water quality parameters

Water quality parameters measured included temperature ($^{\circ}\text{C}$), dissolved oxygen (DO in mg.L^{-1}), pH, electrical conductivity (EC in mS.m^{-1}) and laundry detergent LAS concentration ($\mu\text{g.L}^{-1}$). Temperature, DO, pH and EC were measured in-stream using handheld field meters (described in Chapter 2). The concentration of LAS in the river water was estimated using the Spectroquant® Surfactants (anionic) Cell Test (also described in Chapter 2). For this test, river water was collected in pre-washed glass Schott bottles. Schott bottles were pre-washed in 10% HCl, rinsed in deionised water and left to dry. Once dry, they were rinsed with 50–100 mL methanol, and again rinsed in deionised water and dried. In the field, river water samples were preserved with 30 mL of 37-40% formaldehyde (i.e. 3% v/v formalin) and stored on ice at 4 $^{\circ}\text{C}$. On returning to the laboratory, the samples were analysed using a Merck Spectroquant® NOVA 60 at a detection limit of 50 $\mu\text{g.L}^{-1}$ methyl blue active substances (MBAS). A number of naturally-occurring inorganic cations, anions and organic compounds (phenols and humic acid) can sometimes interfere with the test. This should be borne in mind when interpreting results from natural waters (Pedraza et al. 2007).

6.2.3 Methods for determining stress responses at low levels of biological organisation

The organisms selected for analysis of stress responses at low levels of biological organisation were the flatworm (Class Turbellaria, Genus *Dugesia*), Tricorythidae mayfly *Tricorythus discolor* and Ancyliidae limpet *Burnupia stenochorias*. An attempt was made to collect the Leptophlebiidae mayfly *Adenophlebia auriculata*, which had been exposed in toxicological experiments to LAS in the laboratory, so as to allow for a comparison of sub-lethal stress responses observed in the Balfour River and those measured in the laboratory. However, insufficient numbers were available for collection during sampling occasions and the more abundant *Tricorythus discolor* was collected instead. An attempt was made to collect 20 individuals of each species from each of the three sites but on some occasions insufficient organisms were available at the site for sampling. In such instances, as many specimens as possible, were collected. Ten individuals per species, per site, per sampling occasion were allocated for analysis using the two biochemical assays, ChE and LPx. Sampling was undertaken on three occasions: during winter (17 June 2008); spring (9 October 2008); and summer (10 February 2009). Collected specimens were immediately frozen in 1.5mL Eppendorf microcentrifuge tubes with liquid nitrogen and stored in liquid nitrogen while in the field. On return to the laboratory, the Eppendorf tubes were placed in a freezer and stored, at -70 °C, until biochemical assays took place. The procedure for undertaking the biochemical assays for the determination of ChE activity and LPx are described in detail in Chapter 2.

6.2.4 Methods for determining stress responses at higher levels of biological organisation

Sampling of aquatic macroinvertebrates was undertaken using the SASS5 rapid bioassessment index methodology (Dickens and Graham 2002) on six occasions: winter (4 August 2004); summer (8 December 2004 and 7 February 2007); autumn (4 May 2005 and 11 April 2006); and spring (27 October 2006). Using a collection net (300mm x 300mm), three biotopes were sampled individually: stones in-and-out-of-current; vegetation (marginal and aquatic if available), and gravel/sand/mud (GSM). According to the sampling protocol: stones were kicked for two minutes while holding the net downstream to collect dislodged organisms; two meters of marginal vegetation was swept; and GSM was stirred with the sampler's feet and swept with the net for one minute. After each biotope was sampled, the contents of the net were emptied into a tray, cleaned of leaves and twigs, and macroinvertebrates identified and recorded on the SASS5 score sheet. The SASS5 rapid bioassessment index assigns scores to taxa, based on perceived sensitivity to water quality

impairment. The highest score (15) represents especially sensitive taxa and the lowest score (1) represents especially tolerant taxa. The SASS score is the sum of the sensitivity scores of taxa identified from the sample. A further metric is obtained by dividing the SASS score by the total number of taxa sampled in order to obtain the Average Score per Taxon (ASPT) (Dickens and Graham 2002). Once the SASS evaluation was complete, a further two samples from each of the biotopes were collected (replicate samples) and all samples were preserved in 80% ethanol. All samples were further enumerated in laboratory, providing accurate counts for each of the taxa for use in the assessment of the macroinvertebrate community using the various high-level stress responses listed in Table 6.1. These measures, at high levels of organisation, were chosen from literature which detailed bioassessments similar to those of this study. Measures of Baetidae/Ephemeroptera, and EPT/Chironomidae describe the ratio of abundances of these taxa to one another, while % EPT (% Ephemeroptera, % Trichoptera and % Diptera) describes the abundance of the particular taxa to all taxa sampled, expressed as a percentage.

The classification of macroinvertebrates into functional feeding groups (FFG) was developed by Cummins (1973, 1974) to describe macroinvertebrates that use similar resources in similar ways (i.e. feeding strategies). These guilds are ecologically meaningful units that facilitate an understanding of organic matter processing in streams (Vannote et al. 1980). The designation of macroinvertebrates into FFG can be based on feeding apparatus morphology, behaviour, and/or gut contents. A number of studies indicate that the use of FFG designations for North American taxa, as described in Merritt and Cummins' (1984), is inappropriate for other geographical regions (King et al. 1988; Tomanova et al. 2006). In addition, macroinvertebrate taxa are flexible in feeding strategy and their grouping into a single FFG can be idealistic. There also appears to be a common affinity among taxa for fine detritus (Palmer et al. 1993a; Tomanova et al. 2006). Consequently, the assignment of taxa to FFG needs to be undertaken on a site-specific basis, involving determination of gut contents, a study of mouthpart morphology, and observations of feeding behaviour. The response of macroinvertebrate FFGs to water quality indicators in the Buffalo River (in the Eastern Cape province, within the same ecoregion as the Balfour River) was determined by Palmer et al. (1996). The designations of taxa to FFG were determined through gut contents analysis (Palmer et al. 1993a) and studies on functional feeding morphology and behaviour (Palmer et al. 1993b). These designations were adopted for use in this thesis and applied to the macroinvertebrates sampled in the Balfour River.

6.2.5 Habitat assessment

A habitat assessment was undertaken at each site, using the Integrated Habitat Assessment System (IHAS; McMillan 1998). Although IHAS was initially developed for use with SASS4 (i.e. to adjust the SASS4 score), it nevertheless provides a useful assessment of the habitat available at a site as the diversity of macroinvertebrates can be influenced by availability of biotopes and physical characteristics of the river, and surrounding land-use impacts.

6.2.6 Statistical analysis

Cellular and sub-cellular data were tested for normality using the Kolmogorov-Smirnov test, log transformed and statistically analysed using the ANOVA test. Statistical analyses of biological indices were undertaken using the STATISTICA software package, the ANOVA test for normally distributed data, and Kruskal-Wallis ANOVA and Median Test for non-normal data. Significance was measured at $p < 0.05$. To analyse for macroinvertebrate community structure and FFG representation at different sites, multivariate statistical analysis was undertaken using non-metric multi-dimensional scaling (NMDS) provided for in the PRIMER V5 programme (Clarke and Warwick 2001). The NMDS ordination plot represents the similarity of abundances of family-level taxa, or FFG guilds, between samples. To statistically analyse for these similarities, an analysis-of-similarity (ANOSIM) test was undertaken on the seasonality data that had been replicated over subsequent years. As only one sample per site was taken on each occasion due to the limited availability of habitat at sites a two-way crossed analysis-of-similarity (ANOSIM2) test with no replication was used for assessing differences between sites (also provided for by the PRIMER v5 programme). In addition to the significance value, the Global R value indicates the degree to which the samples are similar or dissimilar. An R value of 1 indicates complete separation of groups, whereas an R value near 0 implies little or no segregation.

Multiple regression analyses were undertaken using STATISTICA software package in an attempt to discern which of the environment variables, measured during sampling, could best describe the observed biological responses (measured at different levels of organisation), and to quantify this relationship. Multiple regression analysis is more complex than simple linear regression as interactions between the various environmental variables are taken into consideration when investigating the relationship between environmental variable and biological response (Smith and Anderson-Cook 2000). Corresponding environmental variables and biological response data from all sites were utilised. To ensure that the test assumptions were met, residual values were checked for normality and the overall goodness-of-fit of data to the model. The direction of the relationship between a

particular environmental factor and the respective observed biological response is reported in brackets in the text as either a proportional (+, i.e. positive relationship) or inversely proportional (-, i.e. negative relationship). The statistical significance of this relationship, determined at $p \leq 0.05$, reflects the consistency of the relationship over numerous sampling occasions at different sites and is not related to the strength of the positive or negative relationship. An R^2 value is also provided, indicating how well the model fitted the data (for example, an R^2 of 1.0 indicates that all of the variability has been accounted for: Smith and Anderson-Cook 2000).

6.3 Results

6.3.1 Stress responses at low levels of biological organisation

Water quality parameters

Biological monitoring at different levels of organisation was undertaken on separate occasions. Water quality parameters measured on each occasion are thus presented separately. Water temperatures taken during the sampling of *Dugesia* sp., *T. discolor*, and *B. stenochorias* (for cellular and sub-cellular response analysis) were considerably lower in winter compared to those measured in spring and summer and tended to increase with distance downstream from Site 1 (Figure 6.1a). The DO was higher during cooler winter-sampling occasions and lower during spring and summer (Figure 6.1b); pH was less variable, being only slightly lower during summer sampling (Figure 6.1c). The highest EC was measured during spring and increased with distance downstream of Site 1 (Figure 6.1d). Concentrations of LAS were below the detection limit ($50 \mu\text{g.L}^{-1}$) during winter and spring, but were measurable at all sites during summer: the highest concentration was at Site 2 ($100 \mu\text{g.L}^{-1}$); the lowest at Site 6 ($52 \mu\text{g.L}^{-1}$); at Site 1 the concentration was $80 \mu\text{g.L}^{-1}$ (Figure 6.1e).

Cholinesterase activity

The ChE data for all three organisms were log transformed and subjected to ANOVA statistical analysis; consequently, the results are presented as means. There was significant seasonal variation in ChE activity for *Dugesia* sp. collected from Site 2, with ChE activity in winter and spring being significantly more inhibited than in summer (Figure 6.2a). There was no significant seasonal variation at the remaining two sites. Seasonal ChE activity between sites indicated that the only significant difference was observed during summer when there was significant inhibition of ChE at Site 1 compared to Site 2 (Figure 6.2b). Multiple regression analysis indicated that EC, temperature and LAS were significantly correlated with ChE activity in flatworms; in all cases, this correlation was positive (+0.01, +0.32 and

+0.49 respectively). ChE activity thus increased when LAS concentrations were higher and was not inhibited, as had been expected. The regression model did not, however, fit the data well, with less than half of the variability in the data accounted for ($R^2 = 0.43$).

There was no seasonal variation in activity of ChE in *T. discolor* sampled from Site 2, while *T. discolor*, sampled from Site 1 and Site 6, exhibited significant seasonal variability, with ChE being significantly inhibited in spring and summer (Figure 6.2c), in contrast to the trends observed in *Dugesia* sp. (Figure 6.2a). Only during spring were there a significant difference in *T. discolor* ChE activity at different sites, with Site 1 being significantly inhibited (Figure 6.2d). Temperature (-0.58), DO (+0.31) and EC (-0.12) were significantly correlated with ChE activity in *T. discolor*, suggesting that, in the case of this organism, higher water temperature and EC, and lower DO occur in conjunction with ChE inhibition. Less than half of the variability in the data was accounted for ($R^2 = 0.46$).

In the case of ChE activity associated with *B. stenochorias*, significant inhibition occurred in winter and summer at Site 1, with no difference between seasons at remaining sites (Figure 6.2e). As was the case for *T. discolor*, significant differences in ChE activity between sites was only observed during spring (Figure 6.2f), with significant inhibition being observed at Site 6. This contrasted with the trends relating to ChE inhibition observed in the other two organisms. Multiple regression analysis revealed that DO (+0.31), temperature (-0.01) and LAS (-0.13) were significantly correlated with ChE activity, although the data did not fit the model well ($R^2 = 0.32$).

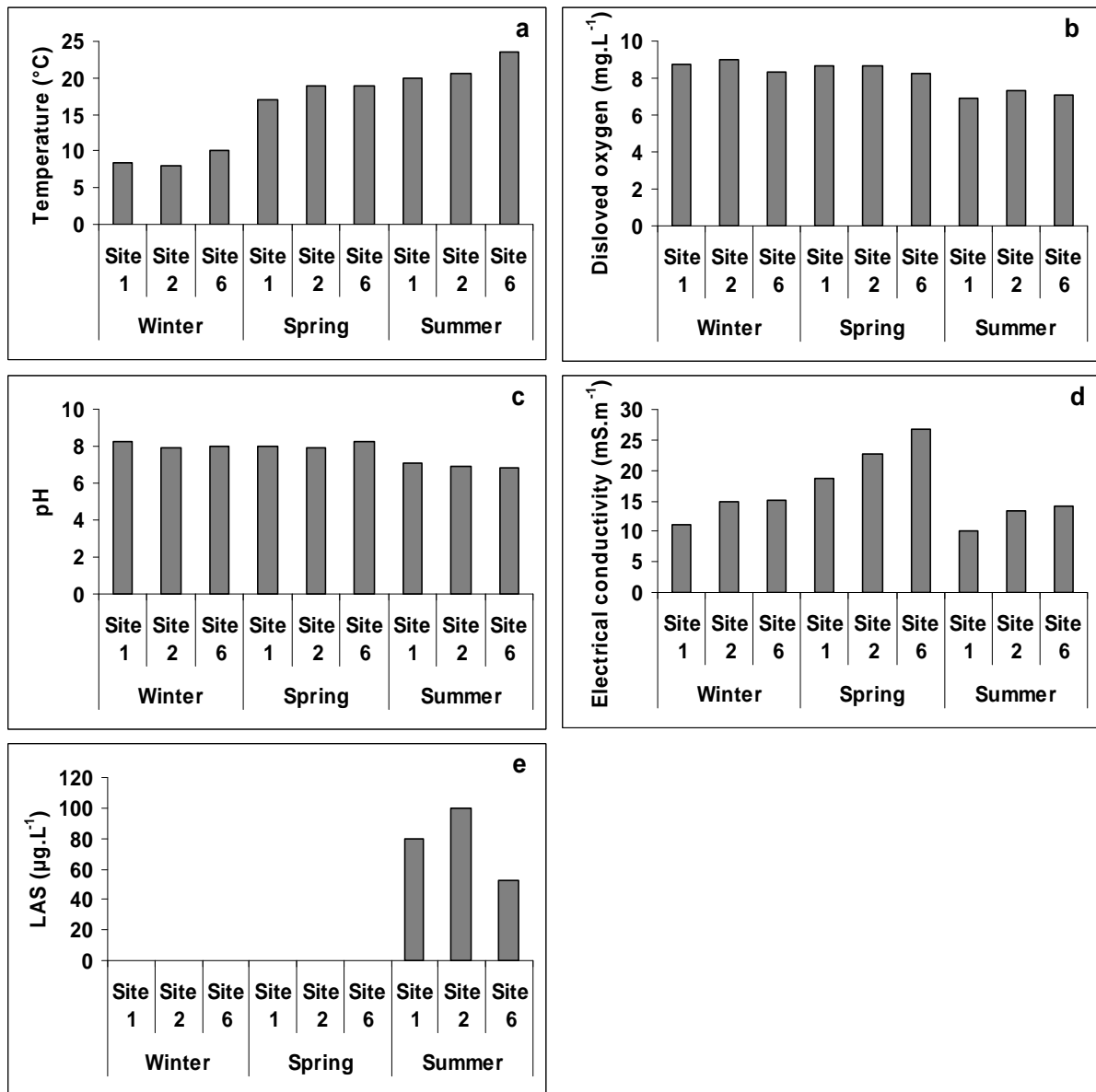
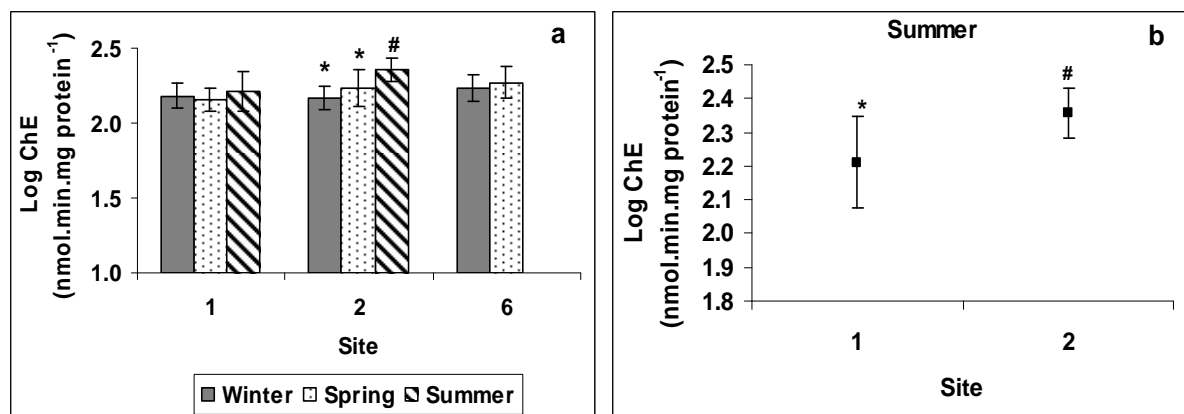
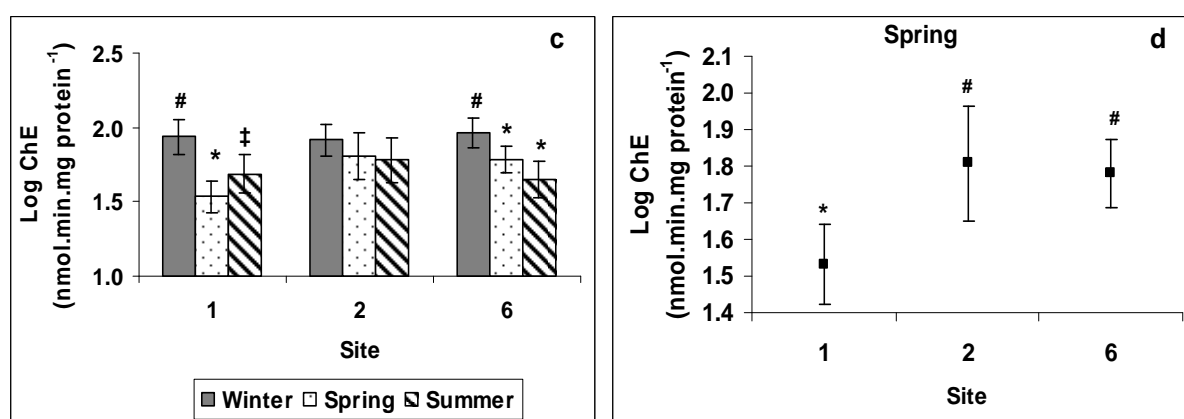


Figure 6.1 Water quality parameters, measured during the collection of flatworm *Dugesia* sp., mayfly *Tricorythus discolor* and limpet *Burnupia stenochorias*, for analysis of cellular and sub-cellular stress responses.

Flatworm *Dugesia sp.*



Mayfly *Tricorythus discolor*



Limpet *Burnupia stenochorias*

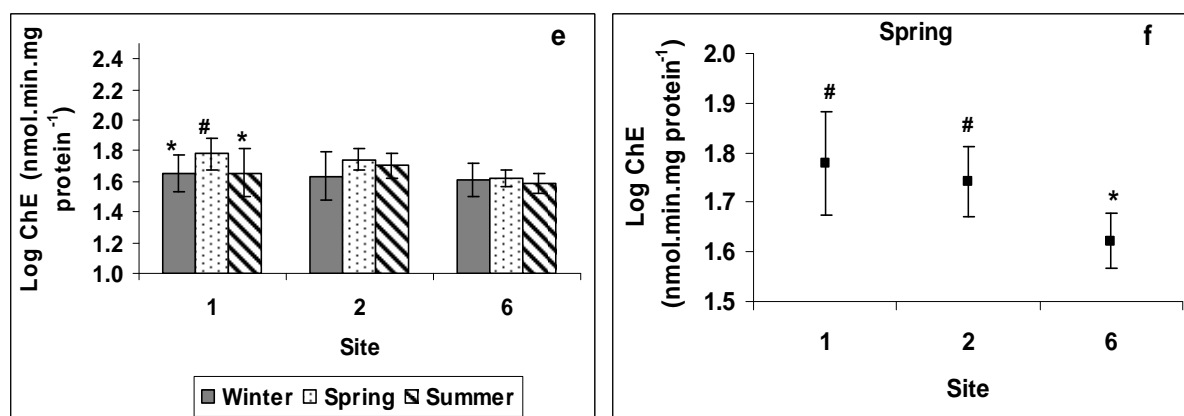


Figure 6.2a–f Log ChE activity (means with standard deviations) in flatworm *Dugesia sp.*, mayfly *Tricorythus discolor*, and limpet *Burnupia stenochorias* by season (Graphs a, c, e, respectively). Within each season, the only significant difference between sites was observed in summer for *Dugesia sp.* (Graph b) and spring for *T. discolor* and *B. stenochorias* (Graphs d and f).

Note: different symbols indicate significant difference at $p \leq 0.05$.

Lipid peroxidation

Much of the LPx data for the three organisms analysed had to be log transformed in order to achieve normal distribution for statistical analysis. Although some data did not have to be transformed they were, nevertheless, presented graphically as log transformed, to facilitate a uniform presentation of results.

Lipid peroxidation in *Dugesia* sp. from each of the three sites varied significantly between season, with elevated LPx being measured during spring and the highest value during summer (Figure 6.3a). When *Dugesia* sp. LPx data were analysed separately for each season, the only significant difference between sites was observed in spring when there was increased LPx in *Dugesia* sp. from Site 6 compared to Site 1 (Figure 6.3b). There was a significant positive correlation between recorded LPx values and both temperature (+0.76) and LAS (+0.48), and a significant negative correlation between LPx and both DO (-0.63) and pH (-0.53) ($R^2 = 0.66$).

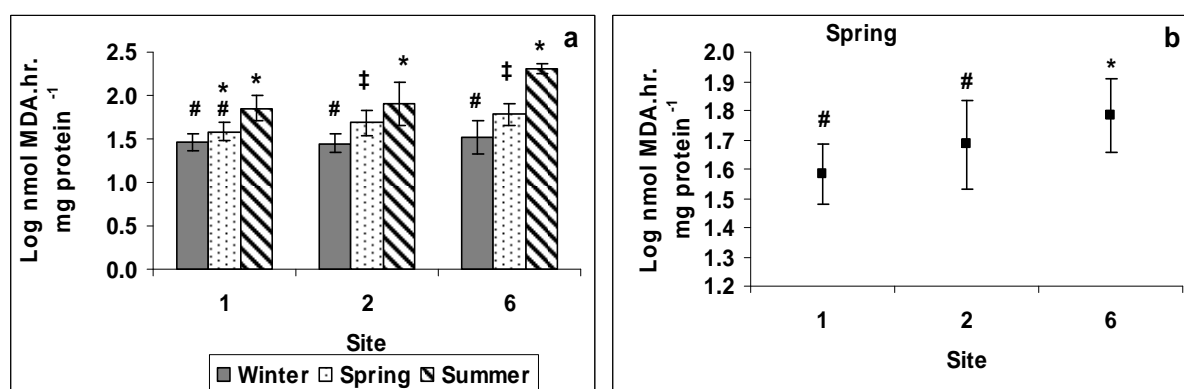


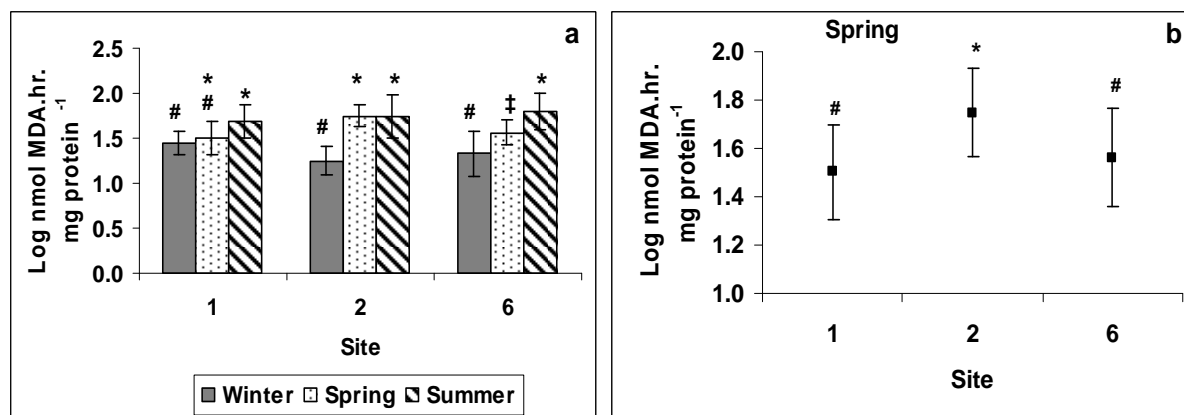
Figure 6.3a–b Lipid peroxidation (log means with standard deviations) measured in flatworms *Dugesia* sp. by season (Graph a). Within each season, the only significant difference between sites was observed in spring (Graph b).

Note: different symbols indicate significant difference at $p \leq 0.05$.

Levels of LPx measured in *T. discolor* and *B. stenochorias* exhibited a similar pattern to that observed in *Dugesia* sp., with significantly higher values during spring and summer (Figure 6.4a,c). In spring there was a significant difference in the LPx of *T. discolor* measured from different sites, with those from Site 2 exhibiting increased LPx (Figure 6.4b). In *B. stenochorias*, however, significant differences between sites were observed in spring and summer, with increased LPx recorded at Site 6 in spring (Figure 6.4d) and at Site 2 and Site 6 in summer (Figure 6.4e), a similar trend to that observed in *Dugesia* sp. (Figure 6.4b). The LPx values recorded in *T. discolor* were significantly correlated with temperature (+0.73), DO (-0.50), pH (-0.47) and EC (+0.05) while in *B. stenochorias* these values were significantly

correlated with temperature (+0.75) and LAS (+0.41). For both organisms the model fit to the data was fairly good, with R^2 values of 0.61 and 0.63, respectively.

Mayfly *Tricorythus discolor*



Limpet *Burnupia stenochorias*

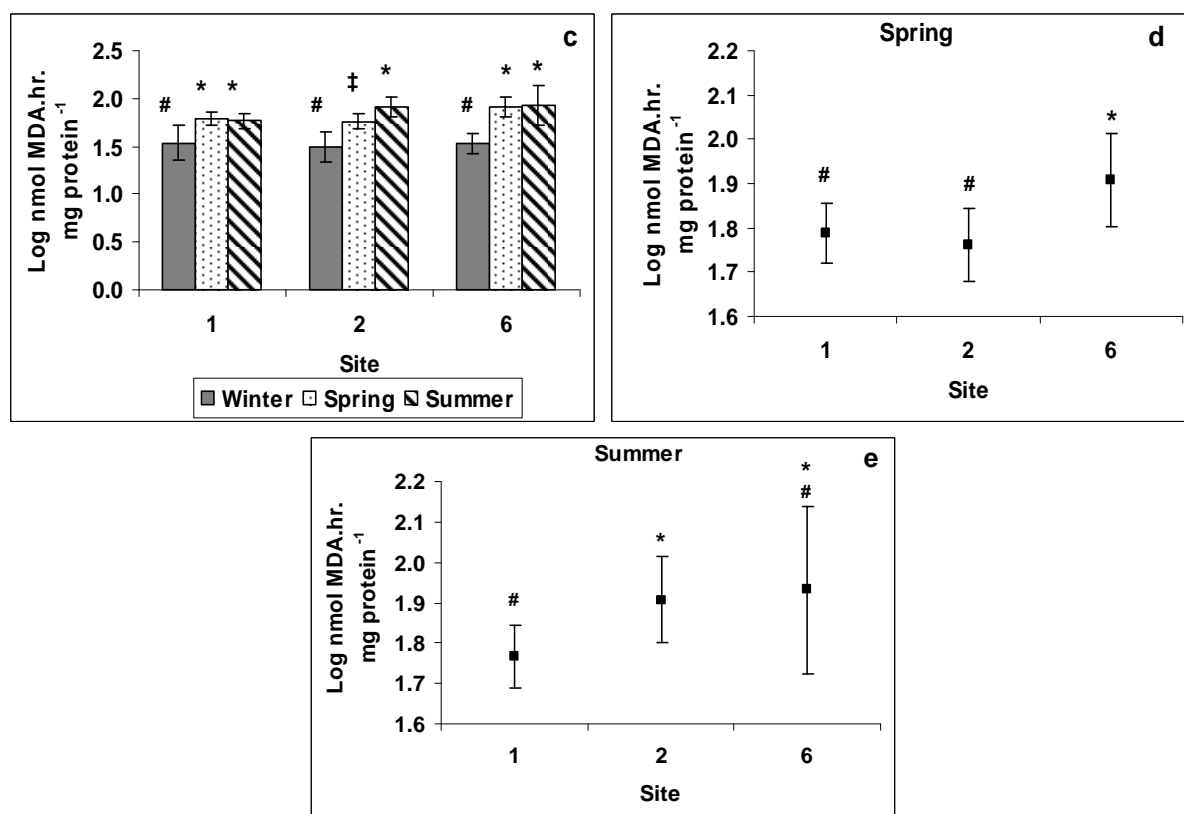


Figure 6.4a–e Log LPx (means with standard deviations) in mayfly *Tricorythus discolor* and limpet *Burnupia stenochorias* by season (Graphs a and c). Within each season, the only significant difference between sites was observed in spring for *T. discolor* (Graph b) and for *B. stenochorias* in spring and summer (Graphs d and e).

Note: different symbols indicate significant difference at $p \leq 0.05$.

6.3.2 Stress responses at higher levels of biological organisation

A diverse array of community-level bioassessment tools was used to determine if macroinvertebrate community structure was impacted by near-stream laundry washing. For all bioassessment tools, if data from a particular season were found to be statistically different from those of other seasons they were removed and the data from the remaining seasons re-analysed to determine differences between sites.

Water quality parameters

Monitoring of biological stress responses from higher levels of biological organisation was undertaken on different occasions to the collection of cellular and sub-cellular samples; consequently, separate water quality monitoring was undertaken. In cases when monitoring took place in the same season during consecutive years, standard deviations are presented. Water temperatures were highest in summer and lowest in winter, with a distinct trend of increasing temperatures from Site 1 to Site 4 and decreasing to Site 6 during all seasons (Figure 6.5a). The DO concentration was highest during the cooler winter months and decreased as the water temperature increased in spring and summer (Figure 6.5b); pH did not appear to exhibit any seasonal trend (Figure 6.5c), while EC appeared to increase during summer and was lowest in winter (Figure 6.5d). There was generally an increase in EC between Site 1 and Site 2, with concentrations at the lower sites remaining similar (Figure 6.5d).

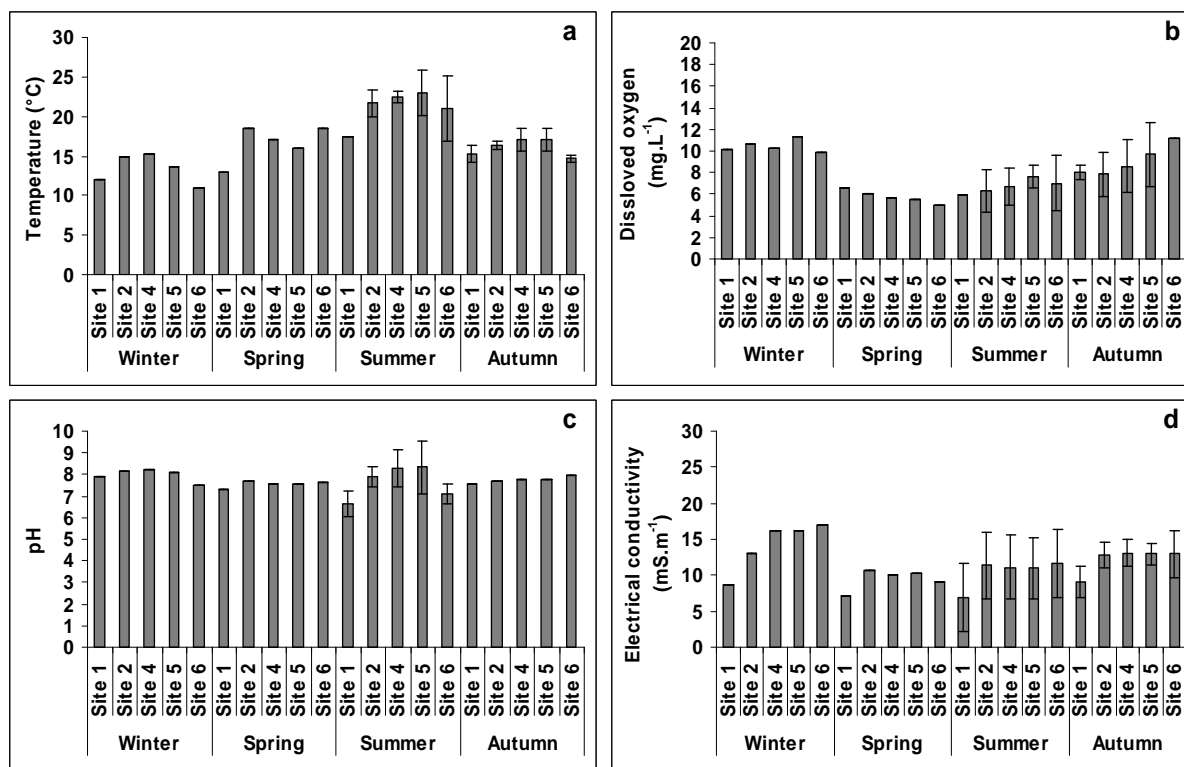


Figure 6.5a–d Water quality parameters measured during the biological monitoring of macroinvertebrates for assessment of stress responses at high levels of biological organisation. Data for summer and autumn represent means and standard deviations of two sampling occasions.

Note: the pH meter was faulty during one of the autumn samples.

Measures of macroinvertebrate taxon tolerance

Scores for the rapid bioassessment index SASS5 revealed no significant differences between Site 1, situated upstream of laundry washing, and Sites 2 and 4-6, situated downstream (Figure 6.6a). The number of taxa at each site did not differ significantly (Figure 6.6b). There was a slight decrease in the ASPT at Site 2, situated immediately below the laundry washing site, and a subsequent increase at sites further downstream. The ASPT values at these sites were not, however, significantly different from one another (Figure 6.6c). The ASPT values indicated a similar pattern to those of IHAS scores recorded, suggesting that habitat – rather than laundry washing detergent – may be the dominant influence on ASPT values (Figure 6.6d). For the above analyses, data from all seasons were combined, as no significant differences between seasons were observed for SASS scores and for the number of taxa, despite large variations around the mean values measured at some sites. The ASPT values were found to be significantly lower in winter compared to the other three seasons (ANOVA, $p \leq 0.05$) (Figure 6.7). Despite spring, summer and autumn data being re-analysed separately from winter data, no significant difference between sites emerged; consequently, the seasonal data were combined for the purpose of regression

analysis with water quality parameters. There were no significant correlations between water quality parameters measured and the SASS score and ASPT value. The EC was, however, significantly correlated with the number of taxa recorded at each site (+0.59), although the model fit of the data was not good ($R^2 = 0.39$).

The ratio of Baetidae abundance to total Ephemeroptera abundance did not differ significantly over the seasons sampled (Figure 6.8a) and although there was a decrease in the ratio at sites downstream from Site 2, these were not significant (Figure 6.8b). The ratio of EPT abundance to Chironomidae abundance was found to be significantly lower in winter (Figure 6.8c) but even when this was taken into account, there were no significant differences between the sites sampled (Figure 6.8d), although the mean log EPT/Chironomidae ratio increased slightly with distance downstream. These two indicators of macroinvertebrate taxon tolerance suggest a slight, although not statistically significant, improvement in water quality with distance downstream (Figure 6.8b and d). No significant correlation between the selected water quality parameters and either of these measures could be determined.

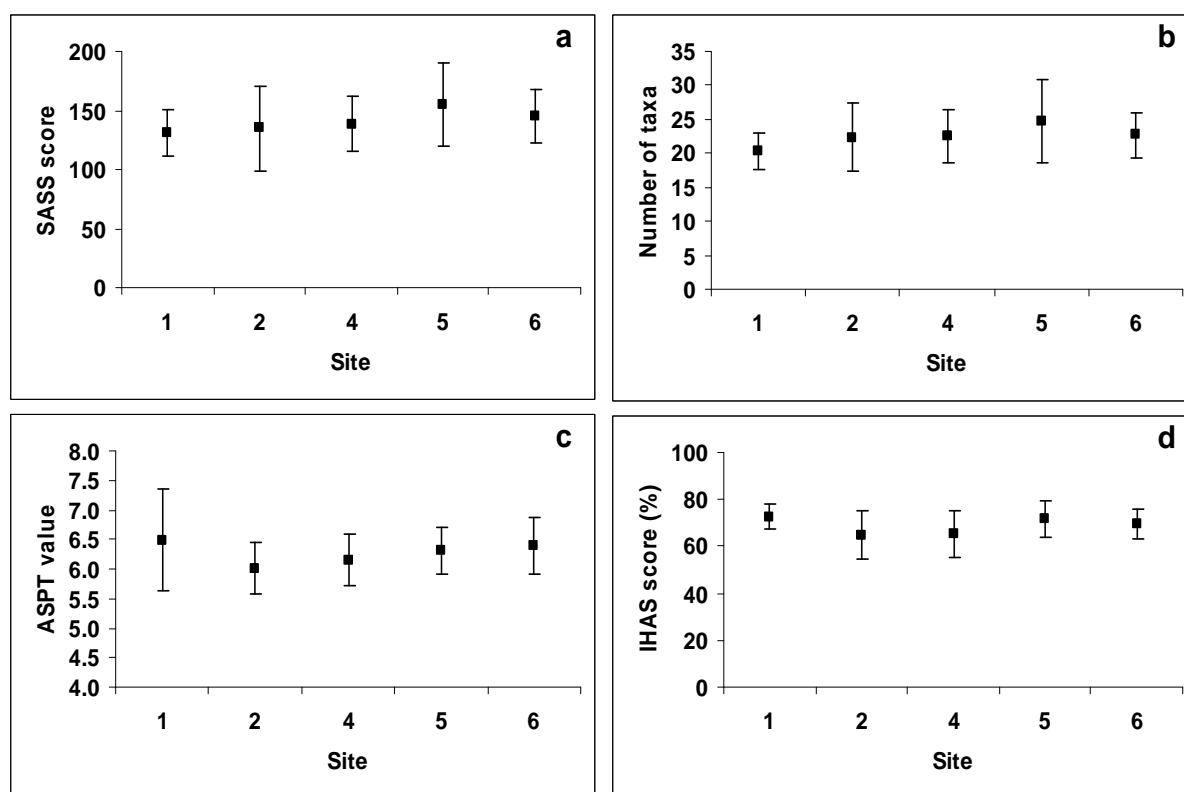


Figure 6.6a–d The means and standard deviations for total SASS5 score (Graph a), number of taxa sampled Graph b) and resultant average score per taxon (ASPT) (Graph c) are presented for sites where macroinvertebrates were sampled. Results from the integrated habitat assessment system (IHAS) are provided (Graph d) to enable interpretation of Graphs a–c.

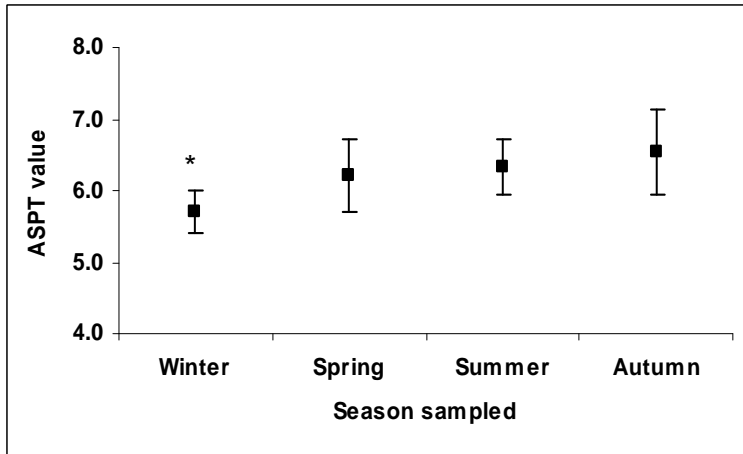


Figure 6.7 Mean seasonal variation in ASPT values recorded at all sites. Mean ASPT values in winter were determined to be significantly lower (ANOVA, $p \leq 0.05$).

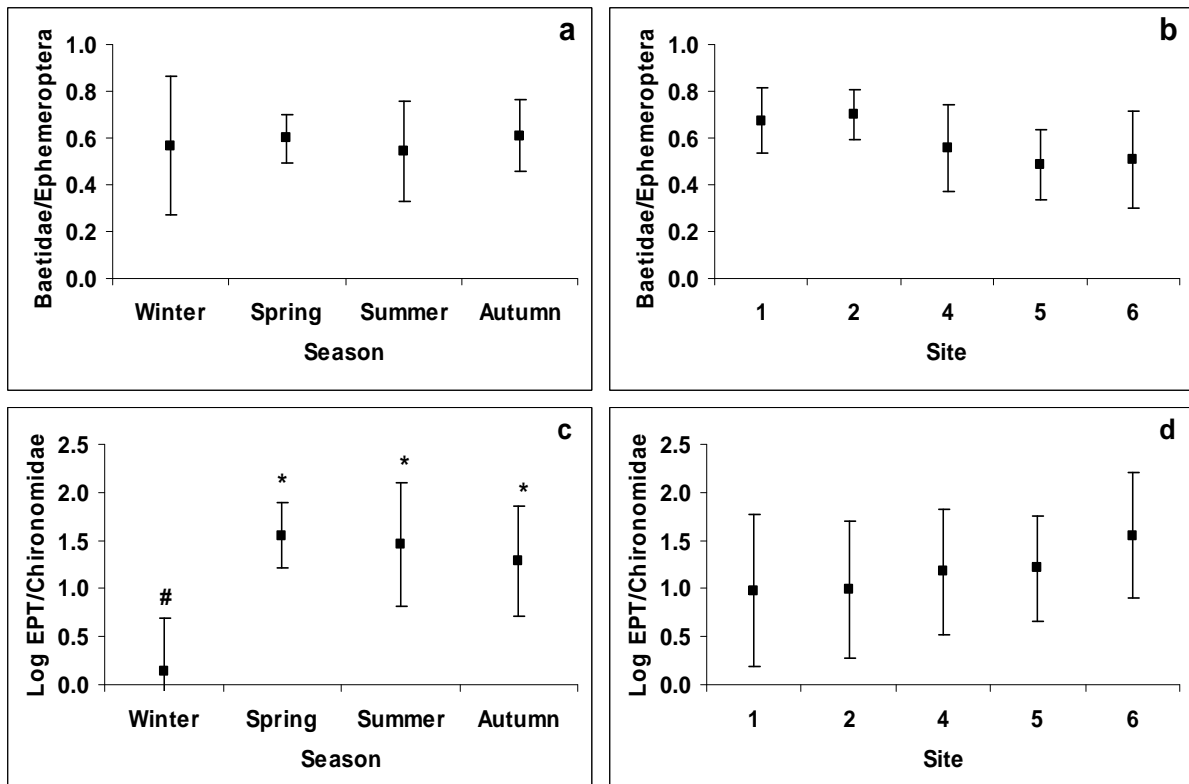


Figure 6.8a–d Mean ratios (with standard deviations) of Baetidae abundance to Ephemeroptera abundance (Graphs a and b) and EPT abundance to Chironomidae abundance (Graphs c and d) are presented as measures of tolerance to water quality impairment. An increase in the Baetidae/Ephemeroptera ratio and a decrease in the EPT/Chironomidae ratio are expected at sites with impaired water quality.

Note: different symbols indicate significant difference at $p \leq 0.05$.

Measures of macroinvertebrate community composition

The abundance of EPT taxa as a percentage of all taxa sampled at the sites was significantly higher in summer (Figure 6.9a). When summer data were removed, the % EPT abundance decreased at sites downstream of the laundry washing site (Site 2) as compared to the reference site (Site 1), although this decrease was not statistically significant (Figure 6.9b). Below Site 2, the % EPT abundance increased with distance downstream, with levels at Sites 4-6 being similar to that of the reference site (Figure 6.9b). The % EPT abundance data follow a similar trend to values recorded for ASPT (Figure 6.6c). Significant correlations were recorded for % EPT abundance and both EC (+0.66) and DO (+0.06) (R^2 value: 0.63).

To ensure that the combination of Ephemeroptera, Plecoptera and Trichoptera (EPT) data in the % EPT abundance measure was not masking a possible response by only one of the family groups, the % abundance of the Ephemeroptera and Trichoptera families were presented separately (Figure 6.9c-h). Very few Plecoptera were sampled and, consequently, these data were not considered. There was significant seasonal variation, with % Ephemeroptera abundance being statistically higher in summer and % Trichoptera abundance being statistically higher in autumn (Figure 6.9c and e). Although seasonal variation was taken into account, there were, nevertheless, no statistically-significant differences between sample sites (Figure 6.9d and f). The % abundance of both Ephemeroptera and Trichoptera were, however, noticeably lower at Site 2, a trend similar to that observed for % EPT abundance and ASPT values. While there was no significant correlation between the water quality parameters measured and % Trichoptera abundance, both EC (+0.66) and DO (0.04) were significantly correlated with % Ephemeroptera abundance ($R^2 = 0.63$). It is possible that poor habitat availability at Site 2 could have also had an impact on the relative abundance of Ephemeroptera and Trichoptera.

The % abundance for the Diptera family was also analysed separately. There was a significantly higher % abundance of Diptera in winter (Figure 6.8g), but no difference between sites (Figure 6.8h). In terms of this measure, significant correlation with EC was negative (-0.52) while DO remained positive (+0.35) ($R^2 = 0.75$).

Using NMDS, the abundance data of all macroinvertebrate taxa sampled were utilised to determine community composition changes at sampling sites (Figure 6.10). Although autumn and spring were not significantly different from one another, the remaining seasons were found to be statistically separate (Global $R = 0.50$) (Figure 6.10a), whereas no segregation could be determined among sites (Figure 6.10b), suggesting no statistical difference in community composition at the sites sampled.

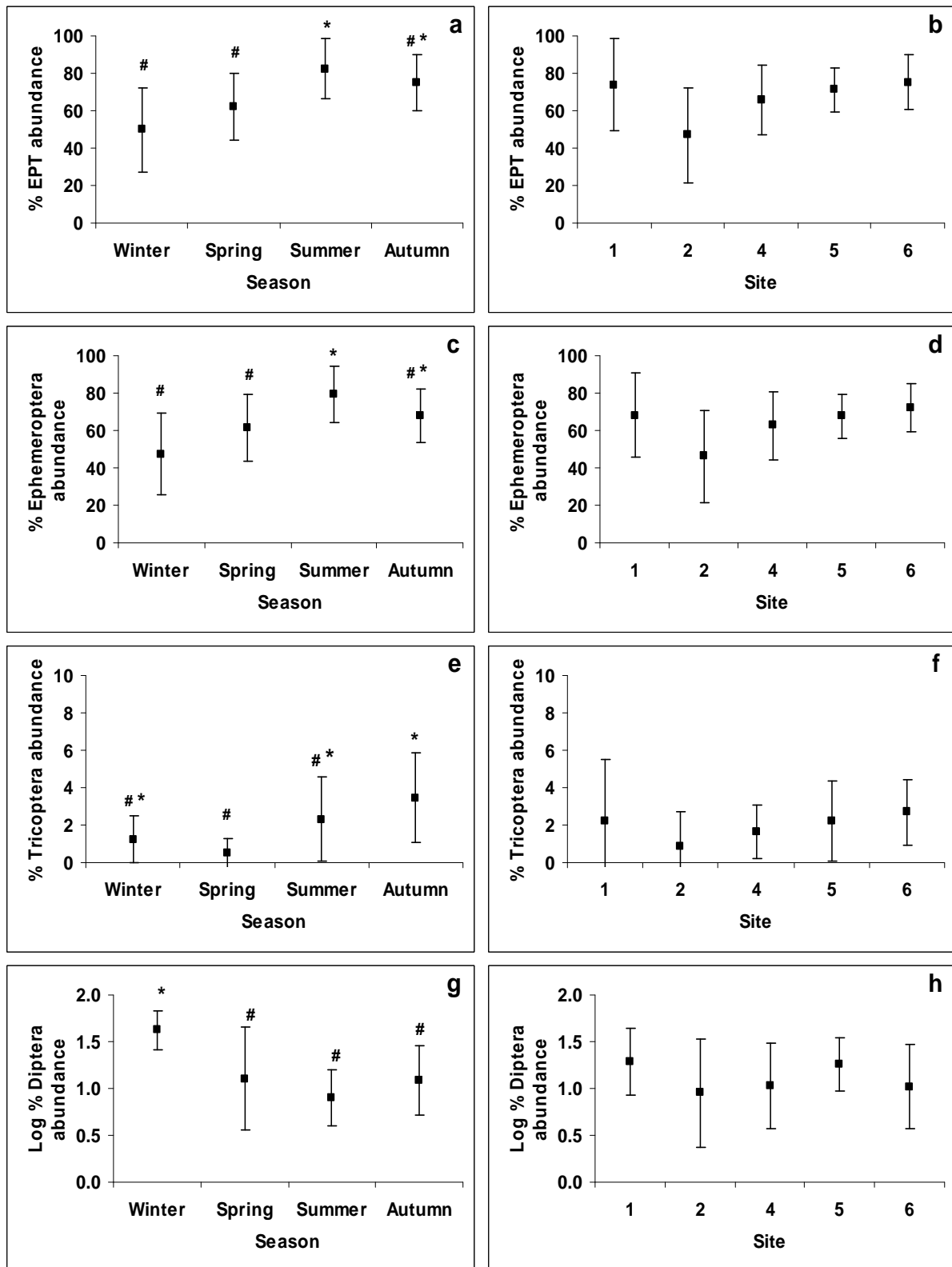


Figure 6.9a–h The above graphs represent measures of macroinvertebrate community composition. The mean (with standard deviation) abundance of EPT individuals (Graphs a and b) and individuals from the specific families Ephemeroptera (Graphs c and d), Trichoptera (Graphs e and f) and Diptera (Graphs g and h) as a percentage of total abundance of all macroinvertebrates sampled per season and per site, respectively.

Note: different symbols indicate significant difference at $p \leq 0.05$.

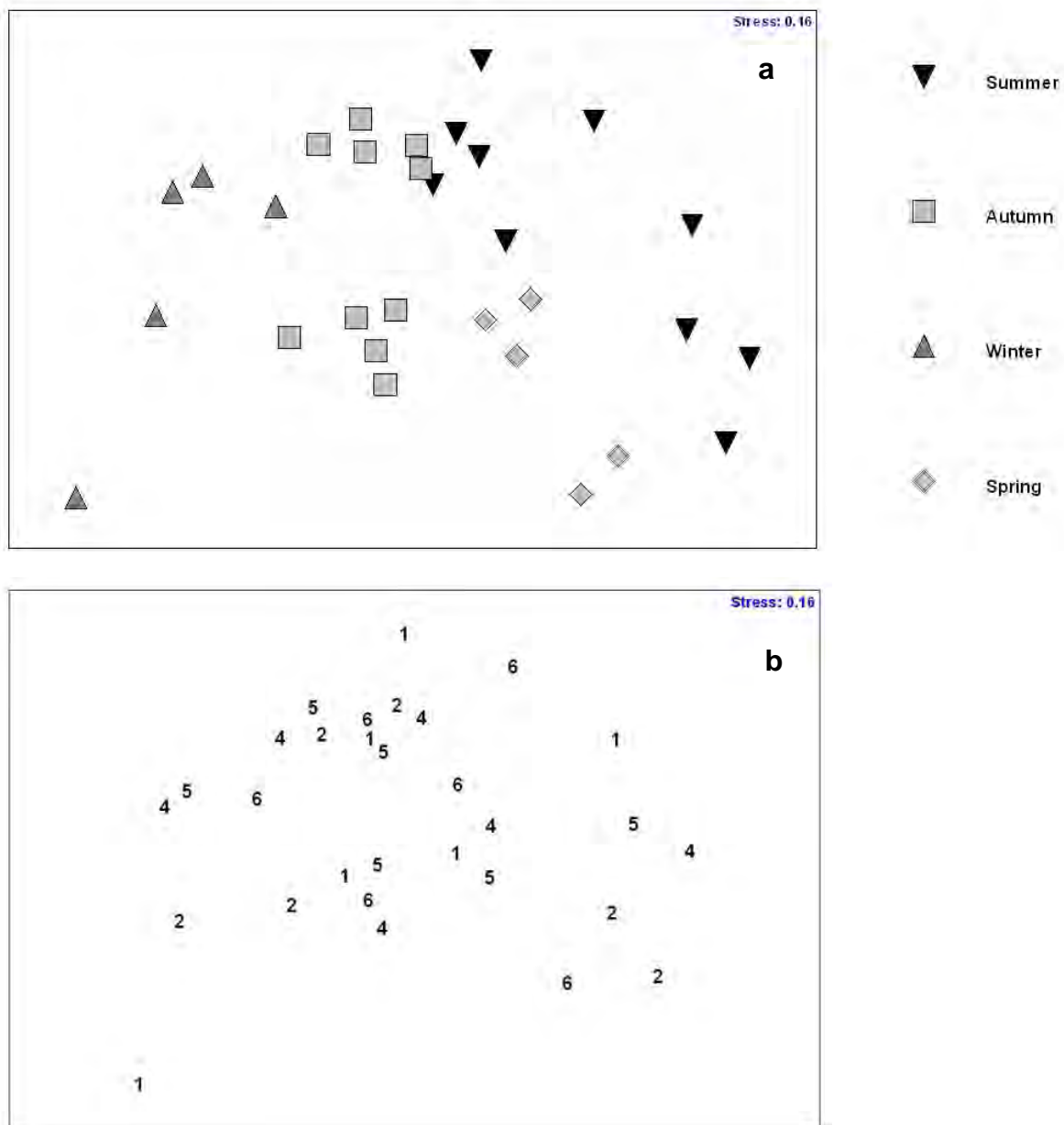


Figure 6.10 NMDS ordinations of enumerated macroinvertebrate data as measures of community composition by season (Graph a) and site (Graph b). The data are statistically different by season (although autumn and spring were not significantly different from one another) (ANOSIM: Global R = 0.50; $p \leq 0.05$), but not by site; (ANOSIM: Global R = 0; $p > 0.05$).

Measures of macroinvertebrate richness

In an effort to measure changes in macroinvertebrate taxa richness, the total number of families belonging to the orders Ephemeroptera, Plecoptera and Trichoptera were compared among sampling sites. There were no significant differences in the number of EPT families sampled during the four seasons or at the various sample sites (Figure 6.11a and b). The

Shannon-Weiner Diversity Index (Figure 6.11c and d) and Margalef's Taxon Richness Index (Figure 6.11e and f) were also utilised, and both revealed no significant differences between season and sites sampled. Although no significant correlations could be determined between selected in-stream water quality parameters and both Shannon-Weiner and Margalef's indices, EC (+0.31), DO (+0.13) and temperature (-0.28) were significantly correlated with the number of EPT families recorded ($R^2 = 0.61$).

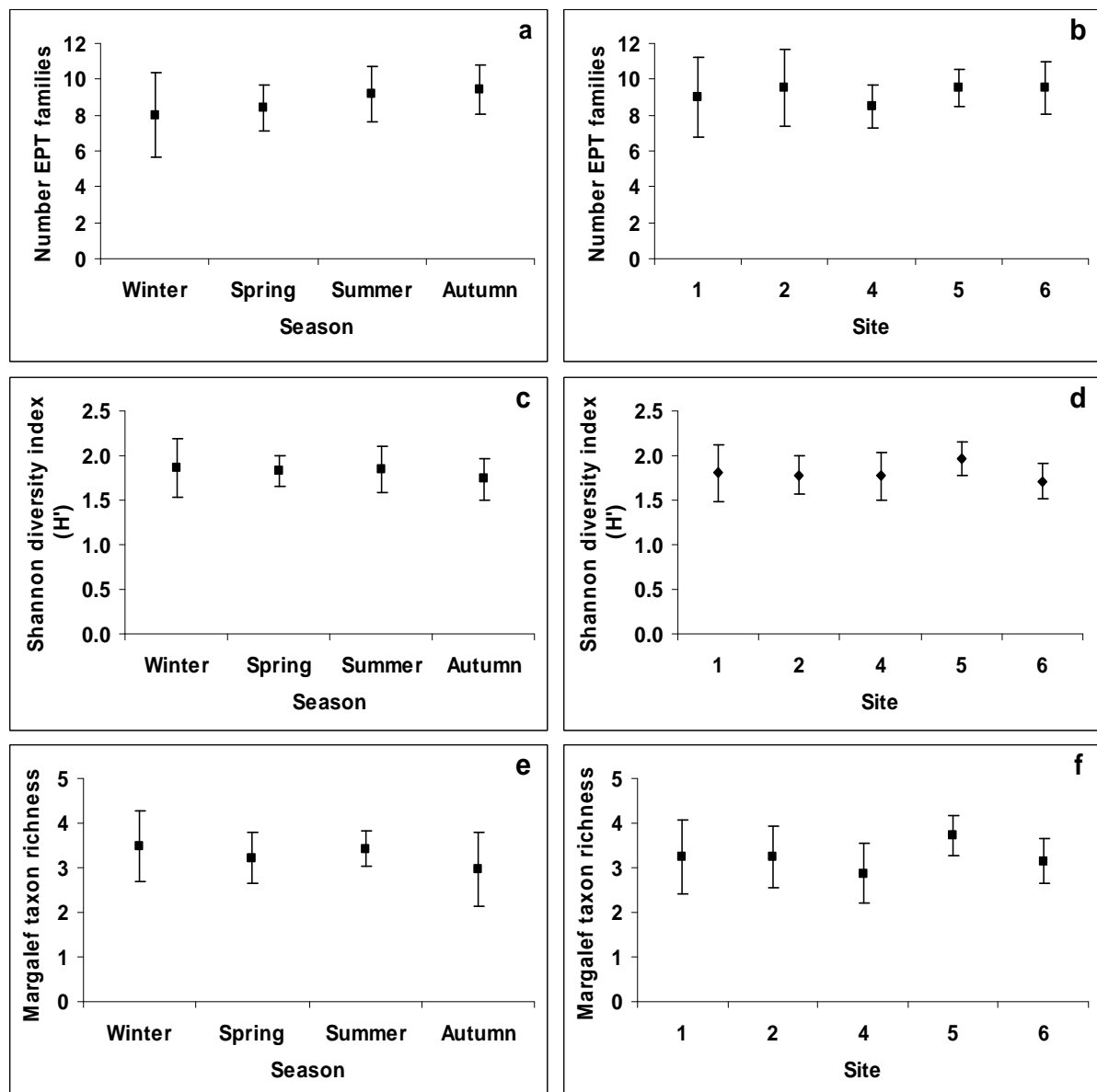


Figure 6.11 The mean (with standard deviation) number of families belonging to the Ephemeroptera, Plecoptera and Trichoptera orders are presented by season (Graph a) and site (Graph b). These graphs, together with those depicting the mean (with standard deviation) values of Shannon-Weiner Diversity Index (Graphs c and d) and Margalef's Taxon Richness Index (Graphs e and f), represent measures of macroinvertebrate community richness.

Measures of trophic level representation in macroinvertebrate community

To determine if macroinvertebrates, representing different trophic levels, varied in abundance among the sites sampled, taxa were assigned to known FFGs for analysis. Abundances of FFGs were similar in spring and summer, but were statistically different from each other in all other seasons, although the degree of segregation was not high (Global R = 0.2) (Figure 6.12a). Abundances of functional feeding groups at sites sampled were not found to be statistically different (Figure 6.12b).

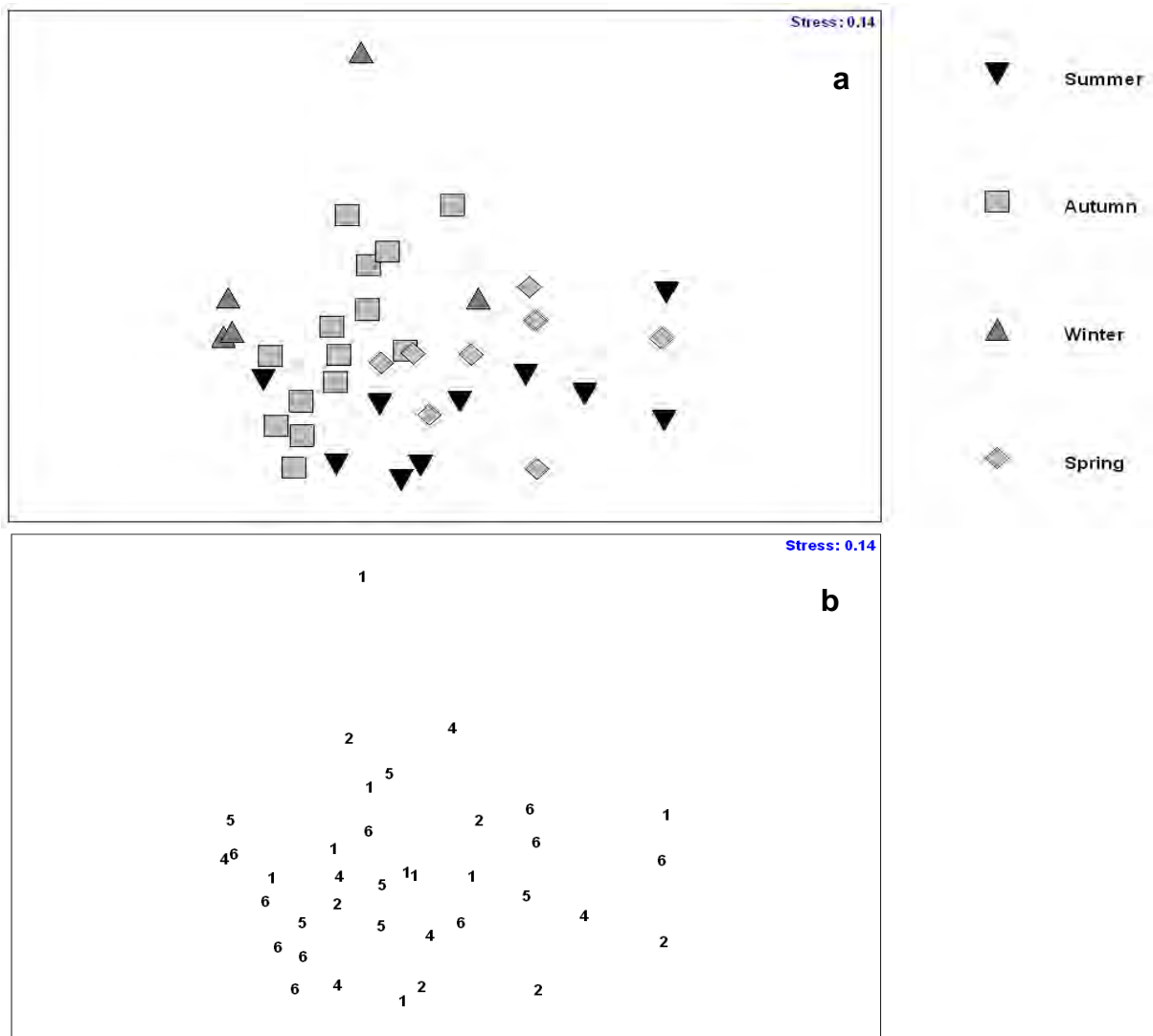


Figure 6.12 NMDS ordinations of enumerated macroinvertebrate functional feeding groups as a measure of trophic level community composition by season (Graph a) and site (Graph b). All seasons, except summer and spring, were statistically different from one another (ANOSIM: Global R = 0.20; $p \leq 0.05$). Sites were not statistically different from one another (ANOSIM: Global R = 0; $p > 0.05$).

6.4 Discussion

6.4.1 Water quality

Water quality data measured during the collection of samples for cellular and sub-cellular stress response analysis, and when macroinvertebrates were sampled to determine effects at higher levels of biological organisation, exhibited similar trends and are discussed together below. The results for parameters such as water temperature and DO were as expected, with higher water temperatures and lower DO concentrations during the warmer summer months and vice versa during the cooler winter months. Electrical conductivity was variable across the four seasons (Figures 6.1d and 6.5d), probably because of the small size of this river and the potential rapid changes in EC during isolated rainfall events. Concentrations of LAS were only measured during the collection of samples for cellular and sub-cellular stress response analysis and only exceeded detection limits during summer. During the summer sampling session, however, a recent rainfall event - that had caused increased surface runoff to the river, thereby increasing the turbidity - may have interfered with the MBAS method (Pedraza et al. 2007) (see Section 2.4 for an explanation). This should be borne in mind when interpreting results. In terms of the variation of the above parameters between sites, water temperature, EC and LAS were lower at Site 1 and increased at Site 2. The EC then remained at similar levels at all downstream sites. Water temperature was similar at Sites 2 to 5 but decreased slightly at Site 6, while LAS concentrations decreased between Site 2 and 6. With regard to EC and LAS, this trend probably relates to impacts from the Balfour Village upstream and adjacent to Site 2, with lower LAS concentrations at Site 6 being due to limited laundry washing along the unpopulated banks of the river upstream of Site 6. In the case of water temperature, lower values at Site 1 and Site 6 were probably in response to increased shading of the river in the upper and lower reaches. Overall, however, the recorded values for temperature, DO, pH and EC can be categorized as having little or no change from estimated default natural conditions used when determining Present Ecological State for Ecological Reserve assessments for South African rivers (DWAF 2008c).

6.4.2 Stress responses measured at lower levels of organization

Cholinesterase activity

There was no consistent pattern in ChE inhibition in sampled organisms by season or site. The lack of significant differences in seasonal ChE activity in *Dugesia* sp. from Site 1, which being upstream of the identified laundry washing activity should not have been impacted by LAS, suggests that ChE activity in this organism is not affected by the varying environmental

variables measured in this study. Seasonal variation in ChE activity was, however, recorded at Site 1 in *T. discolor* and *B. stenochorias*, with ChE in *T. discolor* being significantly inhibited in spring and summer, while *B. stenochorias* ChE was significantly inhibited in winter and summer. Of the measured environmental parameters, DO and pH did not differ markedly between seasons, whereas temperature was considerably lower, and EC higher, in winter. Seasonal variation in the specific ChE enzyme acetylcholinesterase (AChE) has been reported in a number of organisms and in some cases correlated with environmental parameters. Robillard et al. (2003) reported increased AChE inhibition in the freshwater mussel *Anodonta cygnea* with higher water temperatures and when pH increased, or decreased from a pH of approximately 7.9, while DO did not have any effect. Xuereb et al. (2009), however, reported no effect on AChE activity in the fresh water amphipod *Gammarus fossarum* when exposed to water temperatures ranging from 6-24°C. In contrast, Menezes et al. (2006) recorded significant AChE inhibition in the estuarine brown shrimp *Crangon crangon* at lower temperatures, as did Pfeifer et al. (2005) in the marine bivalve *Mytilus* sp. Xuereb et al. (2009), however, suggested that the seasonal patterns observed in AChE activity might be less related to abiotic factors and more to biotic factors. They showed that — depending on gonadal and embryonic development — significant differences in AChE activity exist between different individual females of *G. fossarum* with the enzyme being ‘biologically diluted’ by the presence of mature oocytes (which can account for up to 15% of the female body weight). The mature oocytes constitute an additional protein source in females homogenized for AChE analysis, with a resultant underestimation of AChE activity. The sex and reproductive state of the *Dugesia* sp., *T. discolor* and *B. stenochorias* used in this study could not be determined prior to whole-body maceration and determination of ChE and protein concentration. Furthermore, the sizes of organisms used did not allow for a particular organ (for example, the brain) to be targeted for ChE analysis. Biotic factors could thus have been responsible for the variable results observed in organisms from the three taxa sampled at Site 1.

Although LAS was measured at Site 1 in summer, suggesting a possible link to the ChE inhibition observed in *T. discolor* and *B. stenochorias* (although not in *Dugesia* sp.), high turbidity in the river water at the time of sampling could have interfered with the MBAS method of LAS detection. This could have resulted in an erroneous estimation of the concentration of LAS in the river water. Indeed, the extensive chemical monitoring of river water for LAS (described in Chapter 5), where the more reliable method of liquid chromatography/mass spectrometry was employed, indicated almost insignificant levels of LAS ($< 4 \mu\text{g.L}^{-1}$) at Site 1, over the three seasons sampled.

Due to the potential effect of seasonality on ChE activity, results from *Dugesia* sp., *T. discolor* and *B. stenochorias* sampled from sites downstream of laundry washing (Sites 2 and 6) were compared to the upstream reference site (Site 1) within the same season. The only significant difference in *Dugesia* sp. ChE activity was observed in summer, when there was inhibition at Site 1. There was also significant ChE inhibition at Site 1 in *T. discolor*, but only in spring. ChE activity in *Burnupia stenochorias* was also significantly inhibited in spring, but only at Site 6. Multiple regression analysis of ChE activity of all three organisms from all three sites, against the respective water quality parameters at each site, confirms the lack of a consistent response. While *Dugesia* sp. ChE activity was positively correlated with LAS concentration, *B. stenochorias* ChE was negatively correlated. Regarding water temperature, *Dugesia* sp. ChE activity was positively correlated with water temperature, while the ChE activities of *T. discolor* and *B. stenochorias* were negatively correlated. Correlations between EC and ChE activity were also contradictory, with *Dugesia* sp. having a positive correlation and *T. discolor* a negative correlation. Only for DO was there a consistent response, with the ChE activities of *T. discolor* and *B. stenochorias* being positively correlated to this water quality parameter. Indeed, *Dugesia* sp., *A. auriculata*, *B. stenochorias* and *C. nilotica* exposed to increasing concentrations of LAS in the laboratory (Chapter 3) also showed inconsistent responses, with higher LAS concentrations resulting in ChE inhibition in *Dugesia* sp., but increased activity in *B. stenochorias*, while no change in activity was recorded for mayflies and shrimp. Inconsistent ChE responses in various organisms exposed to LAS have also been reported in the literature. Although Li (2008) measured inhibition of ChE in flatworm *Dugesia japonica*, Jifa et al. (2005) reported increased AChE activity in fish *Lateolabrax japonicus* brain. The use of ChE activity as an indicator of the effect of near-stream laundry washing on in-stream organisms appears to be unreliable, due to variable and unpredictable responses measured in the three organisms. Either the ChE activities are reflecting the impact of some unmeasured variable or the process of analyzing the ChE using whole body homogenisation is causing high variability in results.

Lipid peroxidation

Lipid peroxidation values in all three organisms sampled showed strong seasonal variations, both up- and downstream of laundry washing activity. The highest LPx values were recorded in summer and the lowest in winter. Consequently, water temperature (which was highest in summer) and LAS (which was only measured in summer) had a strong positive correlation with LPx, while DO and pH, being lower during the warmer summer months, were negatively correlated with LPx. It is not possible from these analyses, however, to discern which of the measured physicochemical water quality parameters, either individually or in combination, had the primary influence on LPx in the three organisms sampled.

When comparing LPx values between sites for each season, the highest values in *Dugesia* sp. and *B. stenochorias* were recorded at Site 6, with the highest in *T. discolor* recorded at Site 2. In all cases, Site 1 had the lowest LPx values. Generally, water temperature increased with distance downstream, as did EC, while LAS was higher at Site 2 compared to Site 6. Abiotic factors such as temperature have been shown to affect LPx, with Van der Oost et al. (2003) and Verlecar et al. (2007) reporting a positive correlation between the two. In contrast, however, Malek et al. (2004) reported that decreasing temperatures did not suppress LPx potential in fish *Danio rerio* and, similarly, Sanchez et al. (2007) found no significant seasonal variation in LPx in fish *Gasterosteus aculeatus* sampled from uncontaminated outdoor mesocosms. In addition, LPx responses were not gender dependent in *G. aculeatus* from contaminated sites. During laboratory exposure of *Dugesia* sp., *A. auriculata* and *C. nilotica* to LAS (Chapter 3), abiotic factors were kept constant across the various treatments. Yet increased levels of LPx were measured at the lowest LAS exposure concentrations (500 $\mu\text{g.L}^{-1}$ for *Dugesia* sp. and 1000 $\mu\text{g.L}^{-1}$ for *A. auriculata* and *C. nilotica* (Figures 3.12b-d)) suggesting that LAS may cause some level of oxidative stress. The relationship is complicated however, as LPx values decreased with higher LAS exposure concentrations. In contrast, Li (2008) reports no LPx increase in the flatworm *D. japonica* exposed to LAS concentrations of 10–50000 $\mu\text{g.L}^{-1}$. Consequently, it is possible that the LAS concentrations measured in summer (although the precise concentrations are in doubt due to possible interference with the MBAS method) could be related to increased LPx values measured in the in-stream organisms, although the influence of other abiotic factors cannot be discounted.

6.4.3 Stress responses at higher levels of organization

Measures of tolerance

The three measures of macroinvertebrate tolerance to water quality utilised in this study (the rapid bioassessment index SASS5, and ratios of Baetidae/Ephemeroptera and EPT/Chironomidae) did not differ significantly between the sites sampled, suggesting limited impact from near-stream laundry washing activity. Indeed, the ratios of Baetidae/Ephemeroptera and EPT/Chironomidae suggest more stress at the lower-most sites. The ASPT value and EPT/Chironomidae ratio were both significantly lower in winter. These low values are probably due to seasonal trends in the development of Ephemeroptera taxa, with a slow-growing generation being present during the cooler winter months, followed by a number of rapidly-growing generations during warmer months (Brittain 1982; Barber-James and Lugo-Ortiz 2003), suggesting a greater number of EPT families and individual organisms during summer compared to winter. In addition, the

abundance of Chironomidae was greater during the cooler winter months compared to summer and spring.

Measures of community composition

The composition of the macroinvertebrate community at the unimpacted upstream site was compared to the potentially impacted downstream sites to determine any effects from near-stream laundry washing. Components of the community were assessed separately, with EPT taxa (Ephemeroptera, Plecoptera and Trichoptera) representing organisms sensitive to water quality impairment and Diptera taxa representing tolerance of poor water quality. When assessing all organisms sampled using NDMS, no significant difference in community composition could be determined by site. Although not statistically significant, the relative abundance of the EPT taxa decreased at Site 2, immediately below the location of near-stream laundry washing, but increased again at the downstream sites. The pattern is similar to that observed for ASPT and while high LAS concentrations had been recorded at Site 2 (Chapter 5), the habitat for macroinvertebrates at this site was considered the poorest of all sites sampled (Figure 6.6d) and could potentially be a confounding factor responsible for the lower abundance of EPT taxa. The low relative abundance of Diptera taxa at Site 2 suggests that water quality conditions were not consistently poor enough for these taxa to outcompete more sensitive taxa, i.e. there was sufficient recovery time between LAS inputs to the river to forestall significant effects.

The composition of sampled macroinvertebrates was similar in autumn and spring according to the NMDS ordination, with all other seasons being significantly different from one another. The relative abundance of EPT taxa increased significantly during summer and autumn and was much lower in winter, at which time the relative abundance of Diptera taxa in the community increased significantly. As mentioned previously, these patterns of relative abundance mirror seasonal trends in actual abundances of Ephemeroptera and Diptera. Miserendino et al. (2008) successfully used % EPT abundance to discriminate between urban and rural sites along three streams in Argentina. They, however, report % EPT abundance to be significantly lower in December (summer).

Measures of community richness

None of the measures of community richness were found to be significantly different by site or season sampled. Paul and McDonald (2005) state that reference conditions for macroinvertebrate biomonitoring require a least nine different EPT taxa. The average number of EPT taxa at all sites on the Balfour River was approximately nine, with some seasonal variation, suggesting limited impact from near-stream laundry washing on this particular metric.

Measures of ecosystem function

While there were significant seasonal variations in abundance of FFG, no significant difference could be determined between sites sampled. In a study on the nearby Buffalo River, using the same eight taxa assigned to the FFG used in this study, Palmer et al. (1996) found no response by FFG to changes in in-stream physico-chemical variables (the Buffalo River is significantly impacted by industrial and urban pollution at various points along its length). Yet, the FFG approach has been successfully applied as a metric in various indices designed for ecological assessments (Rawer-Jost et al. 2000; Tomanova et al. 2006) and has been used to successfully identify macroinvertebrate community functional responses to deposited sediment (Rabeni et al. 2005) and general human activities (Charvet et al. 2000).

6.5 Conclusions

None of the stress responses measured at the different levels of biological organisation indicated any significant effects to the aquatic macroinvertebrates inhabiting the Balfour River downstream of laundry washing activity. Although measures of community composition and the ASPT index suggested some impairment at Site 2, immediately below the most popular washing site, the in-stream habitat at this site was poorer than at the other sites sampled. This factor could thus be the cause of results observed.

The ChE inhibition assay does not appear to be a useful biomonitoring tool for indicating LAS exposure in the field. No consistent trend was observed in ChE activity in the three organisms sampled seasonally from sites upstream and downstream of laundry washing activity. Neither was a consistent response observed in the laboratory (Section 3.6.2) or reported in the literature. In contrast, a constant seasonal trend in LPx was observed, with higher levels of LPx recorded in organisms sampled during the warmer seasons. In addition, LPx generally increased with distance downstream from the reference site. Although LAS concentrations measured in the Balfour River were higher in summer and at Site 2, it was not possible to discriminate effects of possible LAS exposure from naturally fluctuating biotic or abiotic factors occurring in the river.

Sanderson et al. (2006) investigated responses of aquatic macroinvertebrates in three American streams impacted by treated effluents from wastewater treatment works (WWTW). Surface water LAS concentrations downstream of the WWTW were low, ranging from 0.2–9.7 $\mu\text{g}\cdot\text{L}^{-1}$. The biological stress responses measured (total number of taxa, total abundance, number of EPT taxa, % EPT taxa, % tolerant taxa and % clingers taxa) showed no significant difference between sites upstream of the WWTW and those downstream. Dyer et

al. (2003) investigated the effects of untreated wastewater on a river in the Philippines, comparing the macroinvertebrate communities at each site using similarity analysis. The highest LAS concentration measured at a site was $122 \mu\text{g.L}^{-1}$ and there were no significant differences in the macroinvertebrate communities sampled from the different sites. In another study investigating direct effluent discharges to the aquatic environment, Whelan et al. (2007) reported LAS concentrations of up to $513 \mu\text{g.L}^{-1}$ in a river in Lao PDR. Qualitative observations of the benthic invertebrate community included dominance of Chironomidae and Oligochaeta at sites with the highest LAS concentrations, and improvement in the diversity of fauna at sites with lower LAS concentrations (Whelan et al. 2007).

Given the above results from the literature and results determined during the biomonitoring of the Balfour River, it appears that the following values are indeed protective of aquatic ecosystems:

- PNECs (of $250\text{--}351 \mu\text{g.L}^{-1}$), determined by Dyer et al. (2003) and Van de Plassche et al. (1999),

and

- WQGs determined for Australia and New Zealand ($280 \mu\text{g.L}^{-1}$) (ANZECC and ARMCANZ 2000) and for South Africa in this thesis ($304 \mu\text{g.L}^{-1}$) (Chapter 5).

Chapter 7 Conclusion: Is near-stream laundry washing affecting the associated in-stream biological community?

7.1 General introduction

This thesis posed two research questions: 1) 'What are the LAS concentrations in a small rural South African river?', and, 2) 'Is the in-stream biological community negatively affected at these concentrations?' The Balfour River in the Eastern Cape Province was identified as a suitable location in which to explore these two research questions. The first question was addressed in two ways: by predicting LAS concentrations in Balfour River water by assessing detergent consumption and laundry washing behaviour of residents living alongside the river and measuring actual in-stream LAS concentrations on different days of the week and during different seasons. Results, outlined in Chapter 5, predicted that, if all laundry washing liquor entered the river (Figure 5.3a), the peak LAS concentration, at a site immediately below the lowermost washing site, would be $414 \mu\text{g.L}^{-1}$. This peak concentration exceeds the LAS water quality guideline (WQG) of $304 \mu\text{g.L}^{-1}$, determined in Chapter 4. However, the predicted exceedence of the WQG for LAS was infrequent and would occur only on Saturdays during July and August and on Fridays, Saturdays and Mondays during October (Figure 5.3a). If the more realistic assumption is made (i.e. that only 30% laundry washing liquor enters the river), then peak concentrations of LAS are predicted to remain below the WQG (Figure 5.3b). Measured LAS concentrations in the Balfour River peaked at $342 \mu\text{g.L}^{-1}$ immediately below the lowermost washing site between 08h00 and 12h00 on a Saturday in summer, but averaged only $21 \mu\text{g.L}^{-1}$ over the whole sampling period (Table 5.2). Concentrations of LAS decreased rapidly downstream, with the highest concentration measured at Site 3 (only 49 meters downstream) being $11 \mu\text{g.L}^{-1}$ (Table 5.2). These data form part of the evidence used to answer the second research question posed in this study.

The second research question is explicitly addressed in this chapter, utilising an integrated environmental assessment approach proposed by Cormier and Suter (2008) (Figure 1.1). This approach begins with a condition assessment to determine if there is an impact on the aquatic biological community of the Balfour River at monitoring sites downstream of the lowermost laundry washing site (Figure 2.1). Biological responses to LAS exposure in this thesis are measured at all levels of biological organisation but the impacts at population and community levels are considered particularly important. This relates to the legislative goals for water quality management in South Africa, which stipulate the protection of the aquatic ecosystem as a whole rather than individual organisms or particular species (National Water

Act No. 36 of 1998). If an impact on the aquatic biological community is determined, then a causal assessment is undertaken to ascertain the likely cause, followed — if necessary — by predictive and outcome assessments.

7.2 Condition assessment

7.2.1 Introduction

The condition assessment process utilises the three steps proposed for all environmental assessments: plan; analyse; and synthesise (Cormier and Suter 2008) (Table 1.1). In this thesis, the various activities involved in the planning step are reported in Chapter 1 Sections 1.2–1.5. The aim of the analysis step is to characterise biological and chemical data relevant to the research question. Consequently disparate types of biological and water chemistry data were collected. Biological response data consisted of two types: laboratory toxicity tests (Chapter 3), used to derive a WQG for LAS specifically for South African fresh waters (Chapter 4), and stress response data of biota resident in the Balfour River downstream of laundry washing activities. These data were compared to responses of organisms upstream (Chapter 6). These biological responses to LAS exposure were measured at levels of organisation ranging from sub-cellular and cellular to organism to population and to community. Water chemistry data from the Balfour River consisted of predicted and measured concentrations of LAS (Chapter 5) and measured concentrations/values of the selected system variables: pH, electrical conductivity (EC), dissolved oxygen (DO) and water temperature. As the Balfour River is within a rural catchment with no evident input of anthropogenic chemicals (besides LAS) into the water course, comprehensive water chemistry sampling for metals, pesticides, hydrocarbons etc. was not undertaken. There was no evidence of sediment input along the extent of the river either. The third step of the condition assessment is synthesis. This involves the integration of the above-mentioned data utilising a weight of evidence approach (WoE, described below) to determine if there is an impact on the in-stream biota of the Balfour River at sites downstream of laundry washing activity.

Until recently, there was no standardised method or regulatory guidance on how to conduct WoE studies (Burton et al. 2002). Instead, the steps undertaken as part of original WoE approaches such as the sediment quality triad (Chapman et al. 2002), were implicit. Consequently, Burton et al. (2002) developed a conceptual framework for undertaking stand-alone WoE assessments which included eight 'critical elements' that needed to be

addressed. More recently, Suter and Cormier (2011) have defined a simplified framework for undertaking a WoE for all types of environmental assessment:

- assemble evidence;
- weight the evidence and;
- weigh the body of evidence.

The methods used to apply the WoE approach to the condition assessment are described below, in Section 7.2.2. After this, in Section 7.2.3, the Suter and Cormier (2011) framework is applied to the data, or evidence, from Chapters 3-6, in order to address the second research question of this thesis.

7.2.2 Methods

Methods of assembling evidence

The term 'evidence' refers to the chemical exposure data and measured biological stress responses that will be used to answer the research question formulated during the planning phase of an assessment. In this study, the evidence includes both predicted and measured LAS concentrations, selected WQ system variables and numerous biological responses at various levels of organisation (Figure 7.1). As the Integrated Habitat Assessment System (IHAS) used in this study was developed particularly to aid the interpretation of biological data from the South African Scoring System Version 5 (SASS5) bioassessment tool (McMillan 1998), it could not be used as independent evidence of habitat quality. The nature of the data used as chemical evidence (i.e. predicted or measured concentrations/values of WQ parameters that are compared to a relevant WQG or reference condition), means that the chemical evidence makes predictions regarding possible biological effects to the aquatic community. The biological evidence, however, consists of biological stress measurements from organisms in the Balfour River and thus represents actual biological effects to the aquatic community. Nevertheless, the use of measured and predictive chemical evidence is still important because if the wrong measures of biological stress are investigated a situation may arise where a biological response or effect may not be recorded but is, in fact, occurring.

Assessors often combine all available pieces of evidence as one body. For example, Sanderson et al. (2006) — in a WoE assessment of the risk of LAS in water and sediment to sediment-associated organisms in three North American streams — combined two chemical exposure, one habitat, and six biological response datasets. However, Suter and Cormier (2011) believe that if distinct categories of evidence are discernable, then pieces of evidence

should be combined into these distinct categories. Thus, in the case of Sanderson et al. (2006), three categories of evidence would be distinguished: chemical; habitat; and, biological. In this thesis two categories of evidence were available: chemical and biological (Figure 7.1).

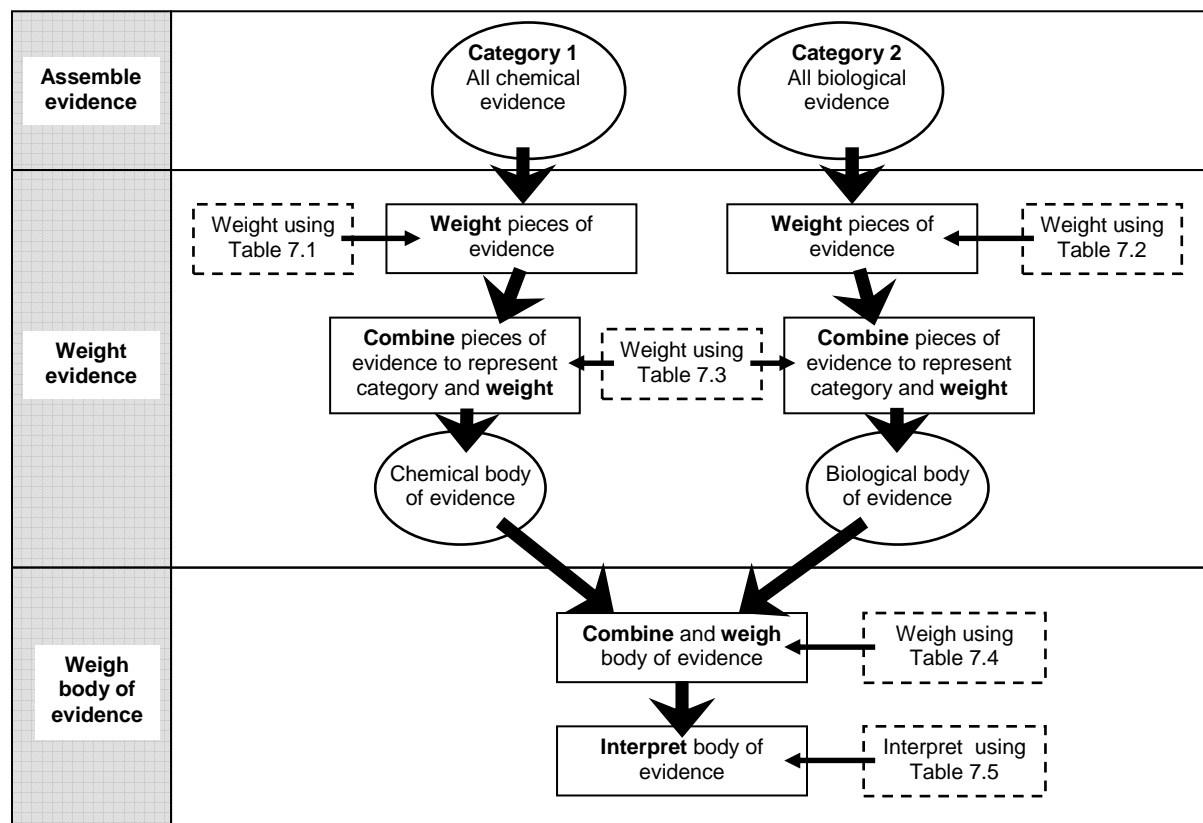


Figure 7.1 Weight of evidence framework applied in this thesis. The three stages of assembling evidence, weighting evidence, and weighing the body of evidence are depicted along with associated activities (following the methodology of Suter and Cormier 2011).

Methods of weighting pieces of evidence

Evidence is weighted or scored, depending on strength and quality. Strength depends on whether the evidence shows a clear distinction from reference or control conditions or violates some WQG. The quality of the evidence is more subjective and relates to confidence in how the data were generated. In this regard, aspects such as the study's design, high variability of results, spatial and temporal sampling regime of chemical and biological evidence, high mortalities in the biological control, and problematical statistical analysis are considered (Suter and Cormier 2011).

The weights or scores assigned are usually qualitative and can comprise symbols (+, – or ○, ■, ●) or numerals. Suter and Cormier (2011) guard against the use of numbers, as they

impart a sense of being a quantitative score when, in fact, they are qualitative (since they are unit-less). Quantitative approaches involve the application of statistical scoring methods (Linkov et al. 2009). They rely on the data being expressed in statistical terms (e.g. probabilities), which the biological data generated in this study are not.

The different methods of how to apply weights are explained in detail in Chapman et al. (2002), Weed (2005) and Linkov et al. (2009). Weights can be applied according to expert judgement — which, although quick and flexible for use in all types of assessments, is not a transparent decision-making process since it requires faith in assessors (Suter and Cormier 2011). Methods get increasingly more prescriptive and rigid, from using simple checklists, to logic tables, to statistical weighting approaches. The approach utilised in this thesis — criteria-guided judgement — is more transparent than relying solely on expert judgement. Although expert judgement is required to a certain extent, criteria (or rules) are developed by which the strength and quality of the evidence are assessed. Two sets of judgement criteria were developed for weighting the pieces of evidence (Figure 7.1): one for weighting the chemical evidence (Table 7.1); and, another for weighting the various biological stress responses of in-stream biota at each site (Table 7.2). In weighting the chemical evidence, a distinction is drawn between xenobiotics or anthropogenic chemicals (e.g. LAS) and system variables (e.g. EC or pH etc.). In the case of LAS assessments, a WQG was used to guide the assignment of a weighting, while the measured values of system variables from downstream sites were compared to those from the upstream reference site (Table 7.1). The predicted LAS concentrations were used as part of the body of evidence despite having a comprehensive in-stream sampling programme to determine actual LAS concentrations. This approach was necessary because of the highly variable nature of laundry washing alongside the river, which may have meant that the water sampling programme did not cover the full range of potential LAS concentrations.

Regarding the application of weights to chemical evidence, a '+' symbol was utilised if the WQG of a xenobiotic was exceeded or if the value/concentration of a system variable was significantly different (statistically) from a reference condition. The WQG utilised was the one derived in Chapter 4 for a Class A South African water resource (Table 4.8). Conversely, a weight of '-' symbol was applied to chemical evidence when the xenobiotic was not measured or if the value/concentration of the system variable was similar to that of the reference site (Table 7.1). The application of a + weight was assigned to biological evidence if the measured biological stress response was significantly different (statistically) from the reference condition. A weight of '-' was assigned to biological evidence when there was no statistically significant difference from the reference condition (Table 7.2). It is, however,

important to acknowledge that the specific statistical p value of 0.05 used for assigning the + or – weightings to the biological evidence does not definitively indicate the difference between the occurrence of a biological stress response and no effect because, in reality, biological responses to stress occur along a continuum. Similarly, a WQG value used for assigning the + or – weightings to the chemical evidence does not definitively indicate the difference between a biological stress occurring and no biological stress. In an attempt to account, to some extent, for the uncertainty associated with making point estimates along a biological response continuum a weight of 'o' was therefore employed in the following situations: whenever substantial quantities of the xenobiotic were measured but the respective WQG was not exceeded; whenever a substantial, although not statistically significant, difference in a system variable was measured; or whenever a substantial biological stress response was observed but not judged to be statistically significant (Tables 7.1 and 7.2). Assigning the o weight is unavoidably a subjective process.

Table 7.1 Judgement criteria for weighting chemical evidence (xenobiotic LAS and the system variables of pH, electrical conductivity, dissolved oxygen and water temperature).

Weight	Explanation of judgment criteria
+	Xenobiotic WQG for Class A water resource exceeded. Concentrations/values of system variables are statistically different from those measured at the reference site.
o	Xenobiotic does not exceed WQG for Class A water resource but is found in substantial concentrations. Concentrations/values of system variables show some difference from those recorded at the reference site but this difference is not statistically significant.
–	Xenobiotic concentration low or not present at site. Concentrations/values of system variables are not different from those recorded at the reference site.

Note: LAS WQG = 304 µg.L⁻¹

Table 7.2 Judgement criteria for weighting biological evidence.

Weight	Explanation of judgment criteria
+	Statistically significant difference from reference condition observed.
o	A substantial effect is observed but it is not statistically significant.
–	No difference from reference condition observed.

The process of combining the weighted pieces of evidence to represent the category of evidence is then undertaken for both chemical and biological evidence. During this process there is often the need to make a judgement of the final weight that will represent this category of evidence. For example, if four pieces of evidence within a category are weighted + o – –, one could say that one + and one – cancel each other out, but then should the category be weighted o or – ? It is thus useful, at this juncture, to have some guidance to help decide the weight of each category of chemical or biological evidence (Table 7.3).

Using the criteria or rules outlined in Table 7.3 in effect means that pieces of evidence do not have equal weighting when the weight of the category is determined.

Table 7.3 Guidance to help decide the weight of each category of evidence (Suter and Cormier et al. 2011).

Guide	Explanation
Significance or relevance of the evidence	Different types or categories of evidence are more important in terms of ecological relevance or significance. McPherson et al. (2008) suggest that field-based biological monitoring evidence is given more weight than laboratory-based toxicity tests. In most circumstances measured chemical concentrations are more relevant than predicted concentrations.
Aggregate strength and quality of evidence	The strength and quality associated with individual pieces of evidence making up the category should be considered.
Quantity of evidence	More pieces of evidence within a category increase the weighting.

Method for weighing (assigning a level of confidence to) the body of evidence

This method involves listing the different categories of evidence together (combined) in a matrix or table, to be considered as one body of evidence (Figure 7.1). At this stage the confidence or uncertainty in the body of evidence is estimated by weighing each category of evidence according to the quality attributes listed in Table 7.4. Following this, the body of evidence is interpreted.

Table 7.4 Qualities that help provide an estimate of confidence in the evidence of each category (Cormier et al. 2010).

Quality	Explanation
Credibility	The evidence was produced in a reliable manner (water quality sampling, toxicity test, biological monitoring and biochemical assay methodologies were of high quality).
Coherence	The pieces of evidence provide a consistent explanation. They reflect the scientific knowledge and theory that govern that type of data.
Strength	The evidence is logically or statistically compelling (e.g. concentration-response relationships and clear statistical trends were observed).
Diversity	There is a variety of different types of evidence represented by the category.

Method for interpreting the body of evidence

In an attempt to limit the exclusive use of expert judgment when assessing the evidence presented in the matrix, a set of criteria for interpreting the body of evidence was developed for this study (Table 7.5). The criteria are based on the weightings assigned to the chemical and biological categories of evidence, providing an interpretation of whether an effect on the in-stream biological community is occurring downstream of laundry washing sites on the Balfour River. As a large and varied body of biological evidence was collected, a + weight for this evidence could immediately initiate a causal assessment, regardless of the weight associated with the chemical evidence (Table 7.5). For the same reason, the assessment

process was terminated and the research question deemed 'answered' if the biological evidence was given a – weight (Table 7.5).

Table 7.5 Guide for interpreting whether chemical and biological evidence indicate impacts to the aquatic biological community of the Balfour River downstream of laundry washing sites (see Tables 7.1 and 7.2 for an explanation of +, – and ○ symbols).

Chemical evidence	Biological evidence	Interpretation
+	+	Chemical evidence predicts an effect on the in-stream biological community. Biological evidence indicates effect on in-stream biological community is occurring. Undertake a causal assessment.
+	○	Chemical evidence predicts an effect on the in-stream biological community. However, the biological evidence is inconclusive. Undertake a causal assessment.
+	–	Chemical evidence predicts an effect on the in-stream biological community. Biological evidence suggests no effect is occurring. The measured WQ variables could be transient in nature and thus present a lower hazard to biota. Alternatively, the WQG could be too conservative or inappropriate for the aquatic community being assessed. Research question deemed answered, end assessment process.
○	+	Chemical evidence is inconclusive; however, the biological evidence indicates an effect on the in-stream biological community is occurring. The stressor might be a WQ variable not measured by the study, or a physical stressor affecting habitat. Undertake a causal assessment.
○	○	Chemical and biological evidence are inconclusive. Further monitoring is required to resolve the research question. Continue the condition assessment.
○	–	Chemical evidence is inconclusive but biological evidence suggests no effect on the in-stream biological community. Research question deemed answered, end assessment process.
–	+	Chemical evidence predicts no effect on the in-stream biological community. However, the biological evidence shows an effect is occurring. The stressor might be a WQ variable not measured by the study, or a physical stressor affecting habitat. Undertake a causal assessment.
–	○	Chemical evidence predicts no effect on the in-stream biological community and the biological evidence is inconclusive. Research question deemed answered; end assessment process.
–	–	Chemical evidence predicts no effect on the in-stream biological community and biological evidence confirms this. Research question deemed answered; end assessment process.

7.2.3 Results

The structure of this section is as follows: the relevant chemical and biological data generated in this study are identified and assembled into categories of evidence; a summary of this evidence is provided in order to facilitate its weighting. The evidence is then combined, weighed and interpreted according to the methods described in Section 7.2.2.

Assembling evidence

The evidence generated in this thesis can be categorised as chemical or biological in nature. The various pieces of evidence belonging to each category are presented in Table 7.6. The level of organisation increases down the list of biological evidence, as does the ecological relevance of the evidence (Clements 2000). The results, or data associated with each piece of evidence, are summarised in the text below.

Table 7.6 Categories of evidence to be utilised in the weight of evidence process.

Categories of evidence	
Chemical evidence	Biological evidence of impairment
Predicted concentrations of LAS in Balfour River	Cholinesterase activity (ChE)
Measured concentrations of LAS in Balfour River	Lipid peroxidation (LPx)
Measured concentrations of pH in Balfour River	SASS5
Measured concentrations of EC in Balfour River	Baetidae / Ephemeroptera
Measured concentrations of DO in Balfour River	EPT / Chironomidae
Measured water temperature in Balfour River	% EPT abundance
	% Ephemeroptera abundance
	% Trichoptera abundance
	% Diptera abundance
	NMDS of all macroinvertebrate taxa
	Number of EPT families
	Shannon-Weiner Diversity Index
	Margalef's Taxon Richness Index
NMDS of functional feeding groups	

Notes: SASS5 = South African Scoring System version 5

EPT = Ephemeroptera, Plecoptera and Trichoptera.

NMDS = non-metric multi-dimensional scaling

EC = electrical conductivity ($\text{mS}\cdot\text{m}^{-1}$)

DO = dissolved oxygen ($\text{mg}\cdot\text{L}^{-1}$)

Predicted LAS concentrations in the Balfour River

In-stream LAS concentrations were predicted from detergent consumption and laundry washing behaviour of residents living alongside the Balfour River. LAS concentrations were only predicted for Site 2, immediately below the main washing area. LAS concentrations were not predicted for the remaining downstream sites as the variable dispersion and degradation of LAS with increasing distance downstream could not be accurately estimated.

Under the assumption that 100% of the detergent used for near-stream laundry washing entered the river, the predicted LAS exposure concentrations at Site 2 peaked at 414 $\mu\text{g.L}^{-1}$ (Figure 5.3a). Exposure was predicted to be transitory however, occurring only on Saturdays during July and August and on Fridays, Saturdays and Mondays during October. These peak concentrations were also only likely to occur during the morning of each day. Observation of laundry washing activity alongside the Balfour River, which is undertaken in plastic containers on the banks of the river, revealed that laundry liquor containing LAS was usually emptied onto the river bank with little flowing directly into the river. As the degradation of LAS is generally high in the natural environment (Fox et al. 2000), the quantity entering the river via baseflow was considered insignificant. Consequently, the assumption that 100% of the laundry liquor enters the river represents a 'worst-case' scenario. If 30% of LAS enters the river, then the potential peak concentration is less than half the LAS WQG (Figure 5.3a).

Measured concentrations/values of selected system variables in the Balfour River

Dissolved oxygen and pH did not exhibit any consistent discernable trend between the upstream reference site (Site 1) and the sites downstream of laundry washing (Sites 2–6) (Figures 6.1b–c; Figure 6.5b–c). However, both EC and water temperature were consistently higher at Sites 2–5 compared to the reference site, although these differences were not significant (Figures 6.1a and d; Figure 6.5 a and d) and are considered within the range of default natural conditions used when determining Present Ecological State for Ecological Reserve assessments for South African rivers (DWA 2008c).

Measured LAS concentrations in the Balfour River

Measured LAS concentrations in Balfour River water confirmed the temporal variability suggested by the predicted water column LAS concentrations (concentrations of LAS in river sediment were insignificant). A peak LAS concentration of 342 $\mu\text{g.L}^{-1}$ was measured at Site 2 on a Saturday in summer, but the average concentration at this site was only 21 $\mu\text{g.L}^{-1}$ (Table 5.2). The LAS appeared to rapidly degrade and disperse as the highest concentration measured at Site 3, only 49 meters downstream, was 11 $\mu\text{g.L}^{-1}$ (Table 5.2). The peak LAS concentrations are used as evidence in the WoE assessment to account for the possibility that the LAS measurement program undertaken at the Balfour, despite being fairly rigorous, is not a continuous record of in-stream LAS concentrations.

Cholinesterase responses of in-stream biota

Although inhibition in cholinesterase activity (ChE) is usually associated with stress exposure (Forget et al. 2003), some researchers have reported increases in ChE activity with stress

exposure (Brown et al. 2004; Jifa et al. 2005; Jifa et al. 2006). Responses of the in-stream organisms sampled in this study were variable. Flatworm *Dugesia* sp. and mayfly *Tricorythus discolor* exhibited an increase in ChE activity at sites downstream of laundry washing during summer and spring, while limpet *Burnupia stenochorias* exhibited a decrease when sampled in spring (Figure 6.2). In contrast, in laboratory toxicity tests *B. stenochorias* exposed to LAS exhibited a significant increase in ChE activity at concentrations above 1500 $\mu\text{g.L}^{-1}$ (Figure 3.11a) and *Dugesia* sp. a significant decrease at concentrations above 1500 $\mu\text{g.L}^{-1}$ (Figure 3.11b). Thus ChE activity in organisms from the Balfour River did not respond in a consistent way or according to the LAS exposure gradient. Nor could ChE activity be consistently correlated with measured physico-chemical variables. It is possible that ChE activity could be reflecting some other unidentified stressor, but it is more likely that the biological variability of the sampled organisms (Olsen et al. 2001; Printes and Callaghan 2003) is confounding results.

Lipid peroxidation responses of in-stream biota

An increase in lipid peroxidation (LPx) is associated with stress exposure (Valavanidis et al. 2006). The LPx responses of sampled organisms from the Balfour River were more consistent than measured ChE activities. Significantly higher LPx was measured in *T. discolor* and *B. stenochorias* sampled at Site 2, immediately downstream of the washing site, in spring and summer (Figure 6.4). However, significantly higher LPx was also measured in *Dugesia* sp. and *B. stenochorias* sampled at Site 6 in spring and summer (Figures 6.3 and 6.4). There was a strong correlation between season and LPx, with LPx being lowest in winter, higher in spring and highest in summer. Although in-stream LAS concentrations are higher during summer, they were always low at Site 6 (Table 5.2). Water temperature, however, was dramatically higher in spring and summer, with temperatures higher at both Sites 2 and 6 during these seasons. Van der Oost et al. (2003) and Verecar et al. (2007) have reported a positive correlation between LPx and temperature, although Malek et al. (2004) and Sanchez et al. (2007) could not concur. In laboratory toxicity tests, *Dugesia* sp. showed increased LPx with increasing LAS exposure concentrations, while *B. stenochorias* showed no response (Figure 3.12a and b). It is not possible to discern whether the LPx measured in biota from the Balfour River can be ascribed to either LAS exposure concentrations or water temperature. At Site 2 LAS seems the more likely stressor, while at Site 6 water temperature does.

SASS5 response: measure of water quality tolerance

The South African Scoring System version 5 (SASS5) is a rapid bioassessment method measuring macroinvertebrate tolerance to water quality impairment (Table 6.1). Three

variables are determined: the SASS score (sum of sensitivity scores of taxa identified at site); number of taxa sampled; and, the average score per taxon (ASPT) score (SASS score divided by number of taxa). All three variables are considered when weighting is assigned to this piece of evidence. Results from the Balfour River did not indicate any difference in the SASS score or number of taxa sampled at sites downstream of laundry washing compared to the upstream reference site (Figure 6.6a and b). There was a slight decrease in the ASPT score at Site 2; however, it increased to reference levels at sites further downstream (Figure 6.6c). The ASPT scores follow a similar trend to the IHAS score (Figure 6.6d) suggesting that the slight decrease at Site 2 may have been related to poorer in-stream habitat at this site.

Baetidae/Ephemeroptera abundance: measure of water quality tolerance

It is thought that increase in the ratio of Baetidae abundance to the abundance of all Ephemeroptera sampled at a site indicates impaired water quality (see Table 6.1). There was no significant difference between the ratio determined at the reference site (Site 1) and Site 2 immediately downstream of the laundry washing area (Figure 6.8b). There was a slight decrease in the ratio at downstream Sites 4-6, although this was not significantly different from that of Site 1. This stress response did not correlate with the measured or predicted in-stream LAS gradient.

EPT/Chironomidae abundance: measure of water quality tolerance

A decrease in the ratio of Ephemeroptera, Plecoptera and Trichoptera (EPT) abundance to Chironomidae abundance is expected at sites with impaired water quality (see Table 6.1). There was no significant difference between the ratio determined for the reference site and sites downstream of laundry washing activity (Figure 6.8d).

% EPT abundance: measure of community composition

As EPT taxa are considered sensitive indicator organisms, a decrease in their relative abundance can suggest water quality impairment (see Table 6.1). Although not significant, there was a large decrease in the % EPT at Site 2 (Figure 6.9b). At sites further downstream the % EPT was the same as that at reference levels. As the IHAS score was also lower at Site 2 (Figure 6.6d), this suggests that habitat may be a confounding factor, accounting for the reduction in relative abundance of EPT taxa at this site.

% Ephemeroptera abundance: measure of community composition

The specific response of Ephemeroptera taxa was also determined. A decrease in their relative abundance suggests water quality impairment (see Table 6.1). As Ephemeroptera

taxa dominated abundance of the EPT grouping, the result was similar to that of % EPT with a decrease in % Ephemeroptera at Site 2 compared to the remaining sites (Figure 6.9d). Poor habitat for these macroinvertebrates at Site 2 may be a confounding factor.

% Trichoptera abundance: measure of community composition

The specific response of Trichoptera taxa was determined. A decrease in their relative abundance suggests water quality impairment (see Table 6.1). There was a slight decrease in % Trichoptera at Site 2 compared to the reference site and sites further downstream (Figure 6.9f). Again, poor habitat for these macroinvertebrates at Site 2 may be a confounding factor.

% Diptera: measure of community composition

Diptera are generally considered to be tolerant of poor water quality conditions; consequently, an increase in the relative abundance of this taxon suggests contaminant exposure (see Table 6.1). There was no significant difference between sites, with the relative abundance of Diptera taxa being low at all sites (Figure 6.9g). The low relative abundance of Diptera taxa at Site 2 suggests water quality conditions were not poor enough for these taxa to outcompete more sensitive taxa.

NMDS of all sampled macroinvertebrate taxa: measure of community composition

A non-metric multi-dimensional scaling ordination of all the sampled macroinvertebrate data was undertaken to discern possible differences in community composition at sampled sites. There was no significant difference in community composition between sites, with seasonal variation appearing to be a better explanation for community composition (Figure 6.10b).

Number of EPT families: measure of macroinvertebrate community richness

High richness is often used to indicate good habitat quality (water physico-chemical, food resources, etc) (see Table 6.1). There were no significant differences in number of EPT families present at sampled sites (Figure 6.11b). Paul and McDonald (2005) state that reference conditions for a macroinvertebrate biomonitoring require a least nine different EPT taxa. The average number of EPT taxa at all sites on the Balfour River was approximately nine, with some seasonal variation, suggesting limited impact from near-stream laundry washing on this particular metric.

Shannon-Weiner Diversity Index: measure of macroinvertebrate community richness

An increase in richness can be used to indicate good habitat quality (water physico-chemical, food resources, etc) (see Table 6.1). The Shannon-Weiner Diversity Index

indicated no significant difference in macroinvertebrate community richness between sites (Figure 6.11d).

Margalef's Taxon Richness Index: measure of macroinvertebrate community richness

An increase in the Margalef Taxon Richness Index can indicate better habitat quality (see Table 6.1). There was, however, no significant difference in taxon richness between sites sampled (Figure 6.11f).

NMDS of functional feeding groups: measure of trophic level dynamics

By simplifying macroinvertebrates into functional feeding groups (FFGs) representing different trophic levels, a change in the trophic dynamics at a site could potentially be measured (see Table 6.1). There was, however, no significant difference in FFGs between sites sampled (Figure 6.12b).

Weighting evidence

Weighting of evidence was undertaken for the sites positioned downstream of near-stream laundry washing activity. Evidence from Site 1 was used as a reference condition against which to compare the biological evidence and some of the chemical evidence (system variables) sampled from sites downstream of laundry washing activity (Sites 2, 4-6). No biological evidence could be collected from Site 3 as this was a hydrological gauging weir. Chemical evidence could be collected from all sites. Measured LAS concentrations at Site 1 were considered low enough to allow this site to be considered a reference site. A predicted LAS concentration was only determined for Site 2.

Weighting chemical evidence

The weighting of the chemical evidence (Table 7.7) involved comparing the predicted and measured WQ variables at each site against the judgment criteria presented in Table 7.1. Despite average measured LAS concentrations at Site 2 being only 21 $\mu\text{g.L}^{-1}$, there was evidence of peak measured concentrations in excess of the WQG for LAS of 304 $\mu\text{g.L}^{-1}$. Predicted peak LAS concentrations under the worst-case scenario of 100% laundry washing liquor input to the river were also in excess of the LAS WQG. However, these predicted and measured peak LAS concentrations were only transitory, occurring for a limited number of hours on selected days during particular months (Figures 5.1c and 5.3a). As the WQG derived for LAS is intended to protect aquatic organisms from indefinite exposure to consistent concentrations of LAS, and the LAS peak concentrations are infrequent, of short duration, and do not greatly exceed the LAS guideline, it was decided to assign a weighting of 0 to the predicted and measured LAS evidence (Table 7.7). Although LAS was measured

at the remaining downstream sites, the peak concentrations were always below 11 µg.L⁻¹ and averaged less than 3 µg.L⁻¹. A weight of – was therefore assigned. Regarding the remaining WQ parameters, pH and DO at downstream sites did not differ substantially from the upstream reference Site 1 and were thus allocated a – weighting. Water temperature and EC at Sites 2–5 differed from the reference site, although not significantly, and were thus allocated a ○ weighting (Table 7.7).

Weighting of the overall chemical evidence category (i.e. combining weights from the pieces of evidence: Table 7.7) involved applying the guidelines presented in Table 7.3. Based on the strength and quantity of evidence it was decided to weight the chemical evidence category at Site 2 as ○ and at Sites 3–6 as –.

Table 7.7 Weighting pieces of chemical evidence and the chemical category.

Evidence	Sites				
	2	3	4	5	6
Predicted LAS concentration	○				
Measured LAS concentration	○	–	–	–	–
Measured pH	–	–	–	–	–
Measured EC concentration	○	○	○	○	–
Measured DO concentration	–	–	–	–	–
Measured water temperature	○	○	○	○	–
Chemical evidence category	○	–	–	–	–

Weighting biological evidence

The weighting of the biological evidence (Table 7.8) involved applying the judgment criteria presented in Table 7.2 to the measured biological stress responses. A weight was not applied to the ChE evidence as it was not found to reliably indicate a consistent response to LAS exposure in the organisms tested in the laboratory and sampled in-stream from the Balfour River. Organisms for LPx analysis were only collected from Sites 1, 2 and 6.

A + weight was assigned to the LPx evidence collected from Sites 2 and 6. The % EPT abundance and % Ephemeroptera abundance evidence received a weight of ○ at Site 2 as these biological stress responses were substantially lower than those measured at the reference site. The remaining evidence at the remaining sampling sites received a – weighting.

Weighting of the overall biological evidence category (i.e. combining weights from the pieces of evidence) involved applying the guidelines presented in Table 7.3. A weight of – was applied to Site 2 (Table 7.8) despite evidence of LPx occurring in selected organisms at this

site, the rationale being that the preponderance of evidence from higher levels of organisation, indicating no significant effect on the in-stream biological community, outweighed the significance of this sub-cellular stress response. Although there was some evidence that the % abundance of EPT individuals was considerably lower at Site 2 compared to the reference site (Figure 6.9b), the number of EPT families was not reduced at this site (Figure 6.11b). A weighting of – was assigned to the remaining downstream sites due to the aggregate strength of the evidence (Table 7.8).

Table 7.8 Weighting pieces of biological evidence and the biological category.

Evidence	Sites				
	2	3	4	5	6
Cholinesterase activity (ChE)					
Lipid peroxidation (LPx)	+				+
SASS5	–		–	–	–
Baetidae / Ephemeroptera	–		–	–	–
EPT / Chironomidae	–		–	–	–
% EPT abundance	○		–	–	–
% Ephemeroptera abundance	○		–	–	–
% Trichoptera abundance	–		–	–	–
% Diptera abundance	–		–	–	–
NMDS of all macroinvertebrate taxa	–		–	–	–
Number of EPT families	–		–	–	–
Shannon-Weiner Diversity Index	–		–	–	–
Margalef's Taxon Richness Index	–		–	–	–
NMDS of functional feeding groups	–		–	–	–
Biological evidence category	–		–	–	–

Weighing (assigning a level of confidence) the body of evidence

The confidence in the body of evidence was estimated by weighing each category of evidence according to the quality attributes listed in Table 7.4, with weights of +, ○ and – applied. Chemical and biological evidence were judged to be credible as the methodologies used to produce the evidence were reliable and properly executed (Table 7.9). Overall, both the chemical and biological evidence provided a consistent explanation (i.e. exhibited the quality attribute of coherence). Only a small proportion of the evidence was contradictory (e.g. it could not be determined whether LAS or water temperature was the cause of increased LPx), although the chemical evidence was often weighted as inconclusive. Regarding the strength of the evidence, the variable LAS input to the Balfour River — as a consequence of intermittent laundry washing — caused difficulty when characterising the exposure of this chemical to in-stream aquatic biota. Consequently, the chemical evidence was rated as moderately compelling. The biological evidence was deemed highly compelling. However, had ChE data been included as a piece of biological evidence, the

quality attributes of coherence and strength would probably have been rated lower. The chemical evidence was less diverse than the biological evidence. Overall confidence in the body of evidence could be described as high.

Table 7.9 An estimate of confidence in the body of evidence.

Evidence	Quality attributes of evidence categories			
	Credibility	Coherence	Strength	Diversity
Chemical	+	○	○	○
Biological	+	○	+	+

Interpreting the body of evidence

The body of evidence (Table 7.10) comprises the chemical category weighting (Table 7.7) and the biological category weighting (Table 7.8), and can be interpreted using the set of guiding criteria detailed in Table 7.5. At Site 2, located immediately below the area where near-stream laundry washing occurs, the chemical evidence was inconclusive, while the preponderance of biological evidence suggested no effect on the in-stream biological community. At sites further downstream both the chemical and biological evidence showed no significant biological effects to the in-stream aquatic community (Table 7.10). According to Table 7.5, the condition assessment process is thus completed and the research question can be answered as follows: the in-stream biological community situated downstream of laundry washing sites is not negatively affected by the LAS concentrations present in the Balfour River. There is thus no need to undertake a causal assessment. Furthermore, as there is no problem to address, predictive and outcome assessments are not necessary.

Table 7.10 The body of evidence comprising chemical and biological category weightings.

Evidence	Sites				
	2	3	4	5	6
Chemical evidence category	○	–	–	–	–
Biological evidence category	–		–	–	–

7.3 Overall conclusion

The research questions posed in this thesis were: 1) 'What are the LAS concentrations in a small rural South African river?' and, 2) 'Is the in-stream biological community negatively affected at these concentrations?'

Predicted and measured LAS concentrations in the Balfour River suggested that exposure of in-stream biological organisms was highly variable temporally and spatially. High peak

concentrations of LAS occurred infrequently and were limited to the immediate vicinity of the near-stream laundry washing activity (Chapter 5). Furthermore, this study showed that predicting LAS concentrations in river water — estimated from detergent consumption data and laundry washing behaviour of residents living alongside the river — was possible. This approach could potentially be used as a screening tool in other rural catchments similar to the Balfour River for potential effects from near-stream laundry washing. Validation using actual in-stream measurements would however be necessary. The accuracy of this method in larger river systems is not known.

The second research question was addressed using a condition assessment approach, which utilised results generated from the first research question. Predicted and measured LAS exposure concentrations from the Balfour River were compared to a WQG for LAS specifically derived for South African fresh waters. The majority of literature-sourced biological responses to LAS exposure utilised in the WQG derivation were no observed effect concentration (NOEC) endpoints. The scientific credibility of NOEC data has been severely criticised (Landis and Chapman 2011; Jager 2012). However, the paucity of alternative statistical endpoint data – e.g. exposure concentration associated with x% effect (ECx) – meant that the guideline for LAS derived in this study could only be accomplished by using NOEC data. This guideline should, therefore, be viewed as preliminary and should be updated once sufficient ECx data become available.

Selected system variables at downstream sites were compared to the concentration/value ranges found at the upstream reference sites. This chemical evidence was integrated with the various types of biological stress responses measured at sites in the Balfour River upstream and downstream of near-stream laundry washing activity. A WoE methodology was utilised to assess, integrate and interpret the evidence with the aim of answering the second research question. The WoE approach was based on a framework proposed by Suter and Cormier (2011), in which the decision processes of integration, assessment/weighting and interpretation was aided by explicit criteria specifically developed during the current study. Expert judgment was still required but there was transparency through the use of these predefined decision criteria. At its conclusion, the WoE process determined no effect to the in-stream biological community of the Balfour River downstream of laundry washing activity. Consequently, with no effect on the in-stream biological community observed, the problem that initiated the condition assessment was considered solved, eliminating the need for a causal assessment or any management intervention (and associated predictive and outcome assessment) (Figure 1.1).

In this thesis, one of the many common activities impacting South African water resources — near-stream laundry washing in rural catchments — was investigated. In the course of the investigation, a method for predicting LAS concentrations in river water was successfully tested. LAS WQGs were derived for the six-resource management class system, utilised in South Africa, according to best international practise. This system is applicable for use in national water quality monitoring programs and implementation of the Ecological Reserve. Alternative biological assessment methods such as cellular and sub-cellular responses and community structure and richness indicators were utilised alongside the standard SASS5 rapid bioassessment approach. The alternative methods were more time consuming and costly to undertake than the SASS5, yet in their application in this thesis, they did not provide better resolution of potential biological impacts due to LAS exposure. The usefulness of cellular and sub-cellular stress responses as bioassessment tools was also called into question as their measured responses were shown to be highly variable and often inconsistent. However, it is possible that increasing the number of samples for the alternative biological assessment methods through more frequent monitoring may have resulted in different conclusions to those mentioned above. Furthermore, notwithstanding the fact that there were high peak concentrations of LAS in the Balfour River, these were infrequent and of short duration with sampled in-stream LAS concentrations most often being low. Consequently, there may have been few to no biological stress responses from in-stream organisms available for measurement. Ultimately, the SASS5 approach appears the most suitable for continued application in national monitoring programs. Finally, due to low population density and relatively high discharge, the Balfour River likely represents the best case scenario for effects of LAS on aquatic biota. The biological effects of LAS input via near-stream laundry washing in higher populated areas or where the water resource has low discharge or no flow (e.g. dam) still need further investigation.

Chapter 8 References

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Appendix 1

Table A Twelve criteria for defining an ideal biomonitoring tool used in the comparative analysis of biomonitoring approaches relying on aquatic insects and other invertebrates (after Bonada et al. 2006).

Criteria	Biomonitoring approach						
	Organism level bioassays	Sub-organism level bioassays	Fluctuating symmetry	Functional feeding groups	Multimetric approaches	Multivariate approaches	
Rationale							
1	Derived from sound theoretical concepts in ecology	+	-	+	+	±	+
2	A priori predictive	±	-	±	+	±	±
3	Potential to assess ecological functions	±	-	-	+	±	?
4	Potential to identify general anthropogenic disturbance	?	±	+	-	+	+
5	Potential to identify specific types of anthropogenic disturbance	+	+	±	±	+	±
Implementation							
6	Low costs for sampling and sorting (field approaches) or for standardised experimentation (laboratory approaches)	±	±	±	+	±	+
7	Simple sampling protocol	+	+	+	?	±	+
8	Low cost for taxa identifications	+	+	+	±	±	±
Performance							
9	Large scale applicability	±	?	?	±	-	-
10	Reliable indication of changes in general human impact	?	?	-	-	±	±
11	Reliable indication of changes in different types of human impact	±	?	-	±	+	?
12	Human impact indication on a linear scale	±	±	?	?	-	±
Total score		10	6	7	8	10	9

Appendix 2

Water quality parameters measured during short-term lethal toxicity tests exposing indigenous macroinvertebrates to linear alkylbenzene sulfonate.

Table A. Daily temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO) measured in vessels exposing *Caridina nilotica* and *Adenophlebia auriculata* to LAS.

Nominal conc. ($\mu\text{g.L}^{-1}$)	Temperature ($^{\circ}\text{C}$)	pH	EC (mS.m^{-1})	DO (mg.L^{-1})
0	18.41 \pm 0.38	7.28 \pm 0.13	30.28 \pm 2.92	8.44 \pm 0.13
1000	18.35 \pm 0.40	7.40 \pm 0.13	30.35 \pm 1.62	8.49 \pm 0.16
2000	18.31 \pm 0.46	7.41 \pm 0.13	30.39 \pm 1.44	8.45 \pm 0.14
4000	18.19 \pm 0.41	7.40 \pm 0.15	30.53 \pm 1.37	8.44 \pm 0.18
8000	18.18 \pm 0.42	7.38 \pm 0.15	30.70 \pm 0.76	8.46 \pm 0.18
16000	18.21 \pm 0.43	7.39 \pm 0.14	30.94 \pm 1.56	8.46 \pm 0.15
32000	18.18 \pm 0.40	7.39 \pm 0.12	31.58 \pm 1.82	8.42 \pm 0.19

Table B. Daily temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO) measured in vessels exposing *Dugesia* sp. and *Burnupia stenochorias* to LAS.

Nominal conc. ($\mu\text{g.L}^{-1}$)	Temperature ($^{\circ}\text{C}$)	pH	EC (mS.m^{-1})	DO (mg.L^{-1})
0	21.65 \pm 0.65	8.80 \pm 0.03	93.57 \pm 1.88	8.50 \pm 0.11
0.5	21.40 \pm 0.69	8.75 \pm 0.16	93.45 \pm 1.75	8.61 \pm 0.14
1.0	21.48 \pm 0.52	8.82 \pm 0.02	93.88 \pm 1.46	8.49 \pm 0.12
1.5	21.47 \pm 0.53	8.80 \pm 0.02	93.73 \pm 1.45	8.50 \pm 0.08
2.0	21.43 \pm 0.39	8.80 \pm 0.03	93.93 \pm 1.53	8.47 \pm 0.08
4.0	21.33 \pm 0.50	8.78 \pm 0.0	93.70 \pm 1.56	8.48 \pm 0.04
8.0	21.35 \pm 0.57	8.95 \pm 0.41	94.13 \pm 1.94	8.49 \pm 0.05

Appendix 3

The quality of the toxicological data presented in Tables 3.2, 3.3 and 3.4 was assessed using criteria by Hobbs et al. (2005) (unless already validated by the OECD 2005). The results of the assessment are presented in the table below as validation scores (percentage): High quality $\geq 80\%$, acceptable quality 51-79% and unacceptable $\leq 50\%$.

Scientific source	Validation score (%)
Belanger et al. (2002)	79
Blake (2001)	73
Canton and Slooff (1982)	69
Da Silva Coelho and Rocha (2010)	91
Holman and Macek (1980)	82
Iannoccone and Alvario (2002)	66
Kimerle and Swisher (1977)	80
Lewis and Suprenant (1983)	84
Lewis et al. (1993)	57
Li et al. (2008)	68
McLoughlin et al. (2000)	90
Verge et al. (2001)	79
Versteeg and Rawlings (2003)	87
Warne and Shifko (1999)	93
Wu et al. (2010)	81

Appendix 4

SDS-PAGE gel for *Burnupia stenochorias* reveals significant quantities of protein within the ≈ 200 , ≈ 60 , ≈ 45 and ≈ 40 kDa areas, although there appeared to be no specific induction of stress proteins with increasing concentrations of LAS (Figure A).

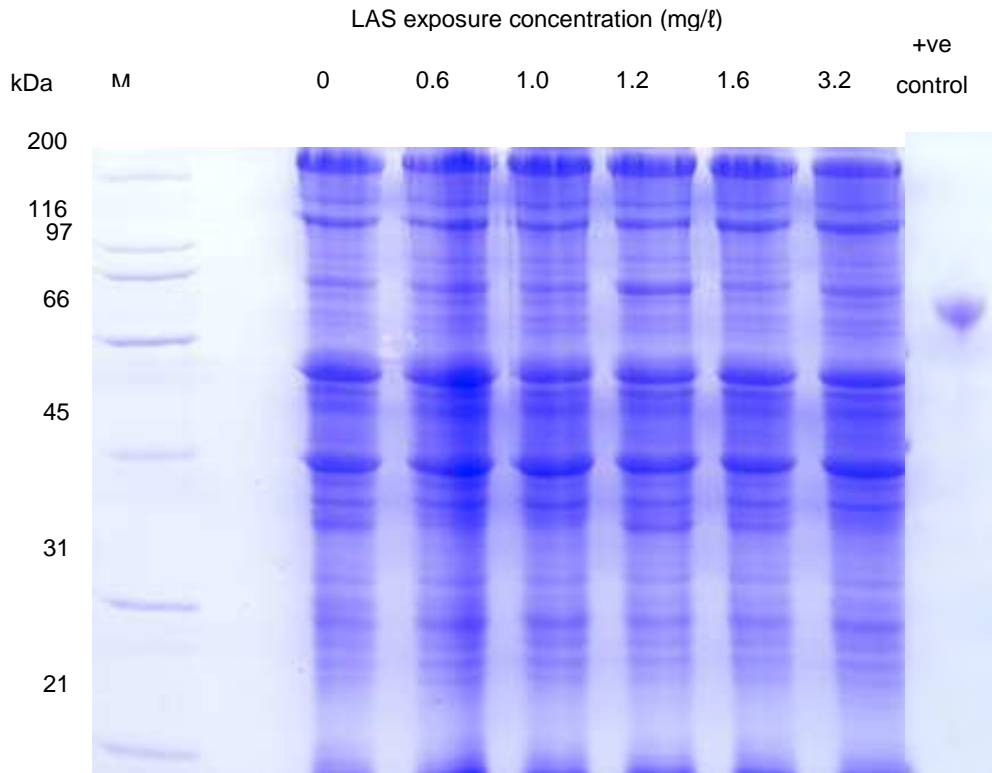


Figure A SDS-PAGE gel of *Burnupia stenochorias* exposed to increasing concentrations of LAS (protein content per well = 22.4 μ g). Legend: M = molecular marker; +ve control = DNA K (Hsp70).

Appendix 5

The anti-Hsp40 antibody only weakly detected Hsp40 proteins in heat-shocked *Caridina nilotica* (in contrast to limpet *Burnupia stenochorias* where Hsp40 proteins were strongly detected) suggesting there may be a problem with the efficacy of the antibody when probing this *C. nilotica*.

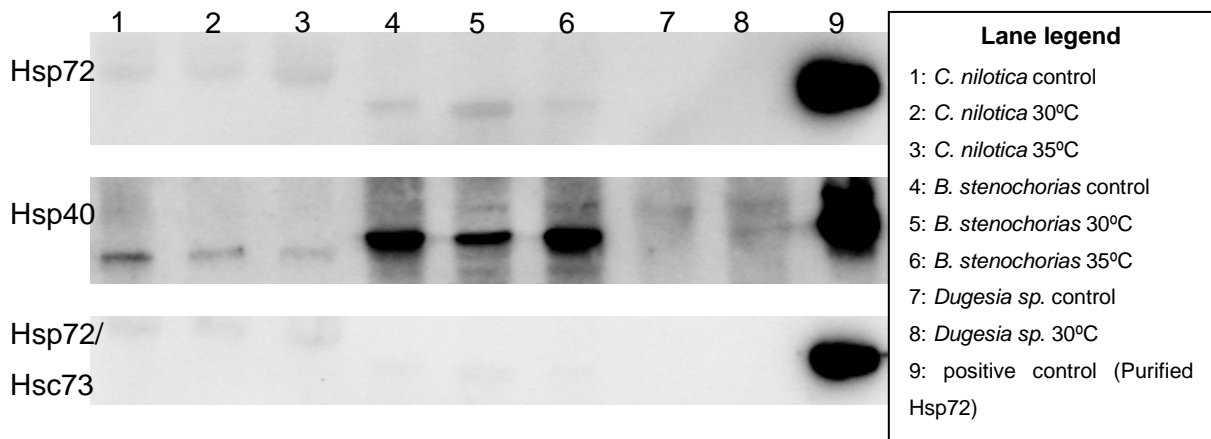


Figure A Western blots of *C. nilotica* and *B. stenochorias* probed with Hsp72, Hsp40, and Hsp72/hsc73 antibodies. Hsp40 was detected in *B. stenochorias*, but only slightly in *C. nilotica* (Exposure times: 150, 12 and 40 secs, respectively; protein contents per well: 16.7 μ g).

Appendix 6

The table below lists taxa that were utilised in the derivation of a WQG for LAS for: Australia and New Zealand (ANZECC and ARMCANZ 2000); for predicted no effect concentrations for two risk assessments (Van de Plaasche et al. 1999; Dyer et al. 2003); and for the LAS WQG derived in this thesis. Taxa used for the respective guideline are indicated by the shaded blocks).

Taxon	ANZECC and ARMCANZ (2000)	Van de Plaasche et al. (1999)	Dyer et al. (2003)	This study
Alga / macrophyte				
<i>Chlamydomonas reinhardi</i>				
<i>Chlorella kessleri</i>				
<i>Hydrocharis dubis</i>				
<i>Microcystus</i> sp.				
<i>Plectonema boryanum</i>				
<i>Scenedesmus subspicatus</i>				
<i>Selenastrum</i> sp.				
Bacterium				
<i>Pseudomonas</i> sp.				
Fish				
<i>Brachydanio rerio</i>				
<i>Lepomis macrochirus</i>				
<i>Limanda yokohamae</i> ^a				
<i>Oncorhynchus mykiss</i>				
<i>Poecilia reticulata</i>				
<i>Pimephales promelas</i>				
<i>Tilapia mossambica</i>				
Crustacean				
<i>Brachionus</i> sp.				
<i>Caridina nilotica</i>				
<i>Ceriodaphnia</i> sp.				
<i>Ceriodaphnia dubia</i>				
<i>Ceriodaphnia silvestrii</i>				
<i>Daphnia magna</i>				
<i>Mysidopsis bahia</i> ^a				
Insect				
<i>Chironomus riparius</i>				
<i>Paratanytarsus parthenogenica</i>				
Mollusc				
<i>Corbicula fluminea</i>				
<i>Crassostrea virginica</i> ^a				
<i>Mytilus edulis</i> ^a				

Note: ^a indicates marine species

Appendix 7

Washing practises recorded from the rural community living alongside the Balfour River in the Eastern Cape, South Africa.

Category		Responses of households (n = 40) * Reported figures are percentages, with number of respondents per category option in brackets, unless otherwise indicated	
Washing frequency (number of times per week)	0.5 (Fortnightly)	2.5 (1)	
	1	35 (14)	
	2	42.5 (17)	
	3	17.5 (7)	
	Unsure	2.5 (1)	
Popular washing days (because each respondent washes more than once – no.s in brackets are not respondents but no. washes ie 70.5)	Monday	19.9 (14)	
	Tuesday	4.3 (3)	
	Wednesday	15.5 (11)	
	Thursday	7.1 (5)	
	Friday	18.4 (13)	
	Saturday	27 (19)	
	Sunday	0 (0)	
	Whenever water available (Rain or fetched from river)	7.8 (5.5)	
Duration of washing activity (hours)	1	20 (8)	
	1.5	2.5 (1)	
	2	27.5 (11)	
	2.5	5 (2)	
	3	22.5 (9)	
	4	15 (6)	
	5	2.5 (1)	
	8 (multitasks)	2.5 (1)	
	Uncertain	2.5 (1)	
Frequency of soap purchasing (per month)	1	57.5 (23)	
	2	30 (12)	
	3	7.5 (3)	
	4	5 (2)	
Washing product as a percentage of the mass of all washing products used by households. And average monthly quantity per household (grams)	Sunlight bar	37.5	550g
	Omo powder	29.4	431g
	Sunlight powder	18.7	275g
	Any soap powder	10.2	150g
	Surf powder	4.3	62.5g

Category		Responses of households (n = 40) * Reported figures are percentages, with number of respondents per category option in brackets, unless otherwise indicated	
Types and combinations of washing products used by respondents	Omo powder and Sunlight bar	42.5 (17)	
	Sunlight powder and Sunlight bar	22.5 (9)	
	Surf powder and Sunlight bar	7.5 (3)	
	Any soap powder and Sunlight bar	2.5 (1)	
	Omo powder only	10 (4)	
	Sunlight powder only	5 (2)	
	Any soap powder only	4 (10)	
	Sunlight bar only	0 (0)	
Laundry washing location	Summer	River	25 (10)
		Homestead	75 (30)
	Winter	River	7.5 (3)
		Homestead	92.5 (37)
Source of water if washing undertaken at homestead	River	55.0 (22)	
	Tap (from borehole)	32.5 (13)	
	Rain tank	12.5 (5)	
Fate of dirty washing water	At River	Poured on river banks. At some washing sites washing water may flow into river	
	At Homestead	Poured on the ground	
Persons undertaking laundry washing	Senior female household member and young girls. Occasionally young males. Undertaken in groups.		