BIOMARKERS IN EEL TO EVALUATE EFFECTS OF DIQUAT IN A CHRISTCHURCH RIVER

L A Tremblay
Landcare Research, CENTOX, PO Box 69, Lincoln 8152, New Zealand.
E-mail: TremblayL@landcareresearch.co.nz

Manuscript received, 30/5/03; resubmitted, 4/12/03; accepted, 8/12/03.

ABSTRACT

Christchurch City Council uses herbicides for the control of exotic plant species in and around the waterways throughout Christchurch City. This study evaluated the acute effects on fish associated with the use of the herbicide diquat to control the water weed *Egeria densa*. Shortfin eels (*Anguilla australis*) were caged at locations in the Avon River that received diquat treatments. Following a three-week exposure, a series of physiological responses (biomarkers) was measured and compared to responses in eels caged upstream from the treatment areas. The biomarkers measured included hepatic mixed-function oxygenase, plasma lysozyme, and vitellogenin. Results showed the diquat treatments caused no observable signs of acute toxicity in the shortfin eel and did not significantly alter biomarker responses.

Key words: biomarkers, herbicide, shortfin eel, vitellogenin, cytochrome P450.

INTRODUCTION

There is concern over the effects on non-target species in New Zealand where herbicides are being used in large quantities to control the growth of exotic plant species in and around waterways. Diquat (1,1'-ethylene-2, 2'-dipyridine, CAS registry number 2764-72-9), molecular formula $C_{12}H_{14}N_2$, has a molecular weight of 186.26 (Howard 1991; Worthing and Hance 1991). It is a quick-acting herbicide and plant desiccant with some translocation properties and little residual activity. It is used to control floating and submerged weeds in water. Diquat has been shown to be moderately toxic via ingestion in mammals (Stevens and Sumner 1991) and to have very little to no effects on fish and invertebrates (Campbell *et al.* 2000). In Christchurch, diquat treatments are used in the Avon River, a spring-fed stream, to control the growth of *Egeria densa*, a water weed that rapidly forms dense strands crowding out other plants and fish. The treated area of the Avon River is particularly used by a rowing club, where the high density of *Egeria* interferes with the club's normal operations. The treated area is lightly under tidal influence.

The *in situ* approach, whereby fish are confined to treated areas by placing them in cages, was taken to ensure exposure to diquat under normal application (treatment) conditions. The fish can be exposed to the waterborne diquat and also through limited contact with sediment. Following a series of public consultations by the Christchurch City Council, concerned Maori (the New Zealand native people) and other interest groups have identified several potential adverse effects on the environment resulting from the herbicide use. To address these concerns, the council initiated a monitoring programme including measurements of diquat levels in water and sediments (R. Vaughan, pers. comm.), effects on invertebrates (McMurtrie 2001), and toxicity to fish. The current research evaluated the potential of diquat to cause adverse effects to the shortfin eel (*Anguilla australis*), a New Zealand native fish species. Eels are resident in the Avon River and therefore likely to be exposed to diquat treatments.

Previous studies have reported that diquat has moderate to practically non-toxic effects on fish and aquatic invertebrates (see Campbell *et al.* 2000). Due to the low toxicity of diquat to fish and the lack of knowledge about any potential mechanism of toxicity, a variety of specific and non-specific biomarkers were measured.

MATERIAL AND METHODS

Fish exposures

All manipulations were approved by the Landcare Research Animal Ethics Committee. An *in situ* protocol using shortfin eel has been developed and used to evaluate the effects of diquat. The use of the eel in this study was warranted by concerns over the potential adverse effects of diquat on native fish. The eels were obtained from Lake Ellesmere, located south of Christchurch and where eels are harvested commercially, and acclimatised in the Lincoln University aquatic laboratory facility for 21 days. The fish were held in groups of 10 in 350-L black polyethylene tanks with constant aerated freshwater flow at 18°C. They were fed fresh veal liver every two days. Fish weights and lengths were recorded before the experiment began. During the exposure, the fish were not fed as they could obtain sufficient food from the environment.

The Avon River was treated with diquat (Reglone, Zeneca Ltd, UK) on 15 January 2001. The area treated was a 2.0-km length at Kerr's Reach with an average width of 50 m. The formulation was applied at an application rate of 30 L/hectare as recommended by the manufacturer. The formulation contained 200 g/L of diquat as the bromide salt in the form of a soluble concentrate. Council staff were responsible for the treatments at two locations, which were carried out from boats and lasted a few hours. Stainless steel cages with a nylon mesh insert containing 10 fish were deployed at four locations on the Avon River on 12 January 2001, three days prior to diquat treatment, for a 3-week period. Sites for placement of the cages were chosen to reflect the condition of the river. The Avon River is an urban stream that receives contamination from a series of point and non-point sources. Therefore, it was important to include a site that was located near the source of the Avon River, to differentiate effects related to diquat from those of contaminants

arising from other sources. Two cages were placed downstream from the diquat treatment sites (one was lost) and two reference cages were placed upstream from the treatment sites (1 km upstream from treatment area) and close to the source of the Avon River (Table 1). Cages were monitored daily for fish mortality and to clear debris from around the cage. Dissolved oxygen levels were measured twice a week using a 6920 multi-parameter data sonde (underwater probe) (YSI, USA).

Fish sampling

All chemicals used were from Sigma. Fish were collected from the cages and transported to the Landcare Research laboratory in iced water to minimise handling and transport stress. At the laboratory, fish were anaesthetised by immersion in a solution of ice and benzocaine (250 mg L^{-1}, ethyl p-aminobenzoate) as modified from Arukwe *et al.* (1997). The fish weights and lengths were recorded and 1-mL blood samples taken from the caudal vein, using a heparinised syringe. The plasma was collected by centrifugation at 4°C for 10 min at 1000 x g, and stored at -80∞C until analysed for vitellogenin content and lysozyme activity. Spleen and liver weights were recorded and livers stored at -80∞C until analysed for hepatic enzyme activity.

Body indices

The hepatosomatic index (HSI) was calculated by the following equation: (liver weight (g)/body weight (g)) x 100. The condition factor (K) of each fish was calculated by the following equation: (weight (g) x 100)/(length (cm))^3. This index provides an overall indication of the fish's health.

Liver mixed-function oxygenases

The induction of hepatic mixed-function oxygenases (MFO) was evaluated by measuring the enzyme cytochrome P450 (CYP1A) activity by the ethoxyresorufin-*O*-deethylase (EROD) assay. Activity was measured in the S-9 fraction using a fluorometric technique described by Burke and Mayer (1974), as adapted for 96-well plate format and simultaneous protein measurement by Kennedy and Jones (1994). Protein concentrations were evaluated by the fluorescamine method of Bridges *et al.* (1986) using bovine serum albumin (BSA) as a standard.

Lysozyme activity

Plasma lysozyme activity was determined by a method adapted from Lie *et al.* (1986). Briefly, heat-killed *Micrococcus lysodeikticus* was dispersed into 1% agar made up in Phosphate Buffer Salt (PBS) buffer (0.13 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 2 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, pH = 6.3) at a concentration of 1 mg *M. lysodeikticus*/mL. Aliquots of 25 mL were poured into 90-mm Petri dishes and allowed to set overnight at 4°C. Small regular holes were made in the agar using a plastic straw. The wells were filled with 20-µL aliquots of diluted eel plasma or hen egg white (HEW) lysozyme standards (0.001 to 10 mg mL^{-1}). Plates were incubated for 24 h at 37°C. Lysozyme concentration was quantified by measuring the diameter of lysis around the wells and comparing it to the HEW standard curve.

Table 1. *Location of eel cages on the Avon River, Christchurch, New Zealand.*

Cage	Site description
Site 1	Reference 1, near source of Avon River
Site 2	Reference 2, upstream from the diquat-treated area
Site 3	Exposed to diquat treatment

Plasma vitellogenin

The plasma egg yolk protein precursor vitellogenin was measured by a specific enzyme-linked immunosorbent assay (ELISA) following the methodology described in Ataria *et al*. (in print).

Statistical analyses

Group means were compared using a one-way analysis of variance with *P*-values for the hypothesis of no difference calculated using an *F*-test. Although some measurements had rather skewed distributions, transformations had little effect on *P*-values. The least significant difference (LSD) needed for significance with $P = 0.05$ was also calculated from the analysis of variance to illustrate whether the experiment was precise enough to detect biologically important differences.

Because there was no replication of sites, it must be assumed that the cause of differences between the measurements was exposure to diquat or other contaminants present in the river, and not other conditions related to site.

RESULTS AND DISCUSSION

Chemical analyses revealed that the river water contained a peak concentration of 3.51 mg L^{-1} of diquat dibromide that rapidly decreased after 1 h to undetectable levels (R. Vaughan, Christchurch City Council, pers. comm.). Three of the four cages were successfully deployed on the Avon River. Unfortunately, one of the cages located downstream from the diquat treatment area was lost. At the end of the 3-week exposure period no fish had died but one fish escaped from Reference 1 (site 1). Observations of fish morphology showed no sign of common external stress such as fin rot or lesions. However, all fish, independently of site, showed slight loss of body weight after three weeks, resulting in a lower condition factor (K) (not statistically significant among sites; before $P = 0.2$, after $P = 0.3$, Figure 1). K is an indication of the overall health of the fish as calculated by a ratio between length and weight. Drop in body weight is a normal response in fish being caged, as they had to adapt to a new feeding regime different from that under laboratory conditions. Measurement of dissolved oxygen indicated that the water was saturated at near maximum at all sites throughout the exposure period.

Following the 3-week exposure, biomarkers were measured in the fish to evaluate the biological effects of contaminants. Biomarkers can be described as functional measures of exposure to stressors usually expressed at the suborganism level of biological organisation, and the information generated can then be used in ecological risk assessment of diquat (Adams *et al.* 2001). Some biomarkers are specific while others are less specific in their power

to establish cause-and-effect relationships between an exposure to contaminants and biological responses.

The liver, the main detoxifying organ involved in eliminating contaminants from the body, will often increase in mass when challenged with heavy contamination. There was no difference in liver weights as indicated by HSI (data not shown, $P = 0.6$). The level of the hepatic detoxifying enzyme biomarker cytochrome P450 1A (CYP1A) activity was measured by the EROD assay (Figure 2). This biomarker has previously been used successfully in eel caging studies to measure the effects of pulp mill effluents (Jones *et al.* 1995). The induction of P450 enzymes is modulated by a variety of chemicals including dioxins, PAHs, and PCBs. The fish caged downstream (Site 3) and at one site upstream (Site 2) from the treatment area had levels of CYP1A activity that were significantly higher than site 1 ($P = 0.04$). Site 2 was not exposed to diquat, this strongly suggests that the increase in P450 activity was caused by contaminants from other sources. The Avon River drains an urban catchment, therefore there are many sources that may be contributing to chemical contamination and other types of stressors.

Lysozyme is an enzyme with bacteriolytic activity that acts as a non-specific component of innate immunity. It is present in serum and within cells with immune function. Lower plasma lysozyme activities were present in fish from Sites 2 and 3 compared to fish from Site 1, and the difference in activity between Site 1 and 2 exceeded the calculated LSD for this parameter (Figure 3). However, the three groups were not significantly different ($P = 0.1$.). A similar profile was also seen with the spleen weights (data not shown) although these trends were also not significantly different ($P = 0.09$). Further investigations should look at the effects of diquat and other contaminants present in the Avon River on other components of the fish immune system.

Vitellogenin is a specific biomarker of exposure to contaminants with oestrogenic activities, a family of endocrine-disrupting chemicals (Jones *et al.* 2000). Plasma vitellogenin was measured by a specific eel ELISA, but no trace was detected in the exposed fish, suggesting that if compounds with oestrogenic activity are present in the Avon River, they would be at concentrations unlikely to modulate vitellogenin production in the eel (data not shown).

The current study demonstrates the low acute toxicity of diquat to the shortfin eel. Similarly, a study conducted concurrently on aquatic invertebrates also found no adverse effects arising from diquat treatment (McMurtrie 2001). These results support conclusions from overseas studies that found diquat was successful in killing weeds without causing any fish mortality (Olaleye *et al.* 1993) and that diquat posed minimal risk to aquatic systems (Bartell *et al.* 2000; Campbell *et al.* 2000; Ritter *et al.* 2000).

CONCLUSIONS

Diquat had no significant effects on the physiological endpoints measured in eels caged downstream from a treated area on the Avon River. The higher activities of P450 detoxifying enzymes appear to be at least in part due to other stressors present in the Avon River. Overall, diquat had no statistically significant toxicity to the short-finned eel under the application conditions used to control *Egeria* in the Avon River.

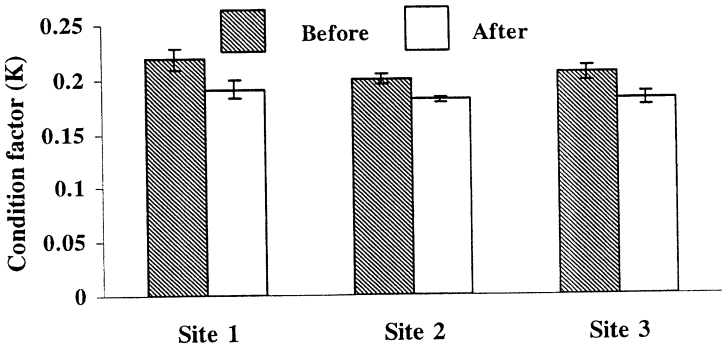

Figure 1. *Average condition factors (K) of the fish at the beginning of the exposure and on sampling day. Sites are described in Table 1. Each bar represents the mean ± standard errors (S.E.M.). LSD = 0.017.*

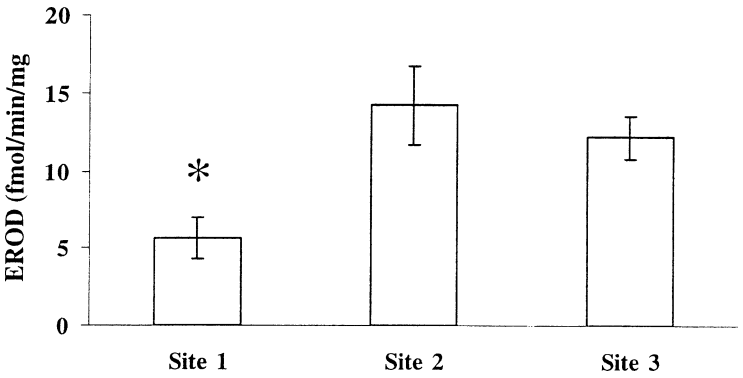

Figure 2. *Hepatic ethoxyresorufin-O-deethylase (EROD) activity in fish following a 3-week exposure in the Avon River. Sites are described in Table 1. Each bar represents the mean ± standard errors (S.E.M.). * indicates a significant difference (P = 0.05). LSD = 5.63*

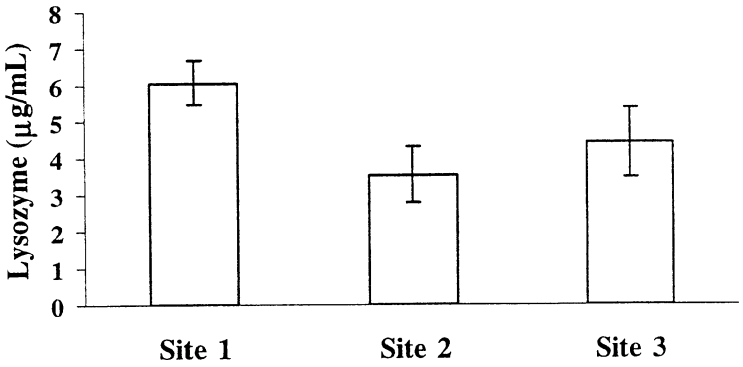

Figure 3. *Average plasma lysozyme concentrations in fish following the 3-week exposure in the Avon River. Sites are described in Table 1. Each bar represents the mean ± standard errors (S.E.M.). LSD = 0.340.*

Although diquat exposures are relatively transient for fish, potential diquat accumulation in the sediments should be monitored, as it binds tightly to upper layers of soil where it may remain for extended periods of time (Howard 1991). Investigations of the sensitivity of other fish species both in the field and under controlled laboratory conditions should be undertaken, as eel might not be representative of all fish species.

ACKNOWLEDGEMENTS

This work was funded by the Christchurch City Council and the Foundation for Research, Science and Technology, New Zealand. The author thanks Greg Arnold, Christopher Bailey, Leslie Brown, Vanessa Hepplethwaite, and Anjoeka Pronk for their technical assistance; Rachel Vaughan, City Solution, for her assistance with the work planning and providing diquat data; Clem Smith for providing the eels; Ngai Tahu for approving the eel work; Mike van den Heuvel, Forest Research, for his help with the EROD assay; James Ataria, Jo Cavanagh, and Kathryn O'Halloran for comments on early drafts; Christine Bezar for editorial comments; and Wendy Weller for the final formatting.

REFERENCES

Ataria J, Gooneratne R, and Tremblay LA. 2004. Development of a specific vitellogenin ELISA for the shortfin eel (*Anguilla australis*). *Australasian J. Ecotox.* **10**, 43-52.

Adams SM, Giesy JP, Tremblay LA and Eason CT. 2001. The use of biomarkers in ecological risk assessment: recommendations from the Christchurch conference on biomarkers in ecotoxicology. *Biomarkers* **6**, 1–6.

Arukwe A, Förlin L and Goksøyr A. 1997. Xenobiotic and steroid biotransformation enzymes in Atlantic salmon (*Salmo salar*) liver treated with an estrogenic compound, 4-nonylphenol. *Environ. Toxicol. Chem.* **16**, 2576–2583.

Bartell SM, Campbell KR, Lovelock CM, Nair SK and Shaw JL. 2000. Characterizing aquatic ecological risks from pesticides using a diquat dibromide case study. III. Ecological process models. *Environ. Toxicol. Chem.* **19**, 1441–1453.

Bridges MA, McErlane KM, Kwong E, Katz S and Applegarth DA. 1986. Fluorometric determination of nanogram quantities of protein in small samples: Application to calcium-transport adenosine triphosphatase. *Clin. Chim. Acta* **157**, 73–80.

Burke MD and Mayer RT. 1974. Ethoxyresorufin: direct fluorometric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methycholanthrene. *Drug Metab. Dispos.* **2**, 583–588.

Campbell KR, Bartell SM and Shaw JL. 2000. Characterizing aquatic ecological risks from pesticides using a diquat dibromide case study. II. Approaches using quotients and distributions. *Environ. Toxicol. Chem.* **19**, 760–774.

Howard PH. 1991. In *Handbook of environmental fate and exposure data for organic chemicals*. Vol. 3 Pesticides, Howard PH (Ed.), Lewis Publishers, Chelsea, MI, pp 303–308.

Jones PD, Hannah DJ, Buckland SJ, Power FM, Gardner AR and Randall CJ. 1995. The induction of EROD activity in New Zealand freshwater fish species as an indicator of environmental contamination. *Australasian J. Ecotox.* **1**, 99–105.

Jones PD, Tremblay LA, De Coen WM and Giesy JP. 2000. Vitellogenin as a biomarker for environmental estrogens. *Australasian J. Ecotox.* **6**, 45–58.

Kennedy SW and Jones SP. 1994. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. *Anal. Biochem.* **222**, 217–223.

Lie O, Syed M and Solbu H. 1986. Improved agar plate assays of bovine lysozyme and haemolytic complement activity. *Acta Vet. Scand.* **27**, 23–32.

McMurtrie S. 2001. *Assessment of the effects of diquat on invertebrates in the Avon River*. A report prepared for the Water Services Unit, Christchurch City Council, Christchurch, New Zealand.

Olaleye VF, Akintunde EA and Akinyemiju OA. 1993. Effect of a herbicidal control of water hyacinth (*Eichhornia crassipes* Mart.) on fish composition and abundance in the Kofawei Creek, Ondo State, Nigeria. *J. Environ. Manage.* **38**, 85–97.

Ritter AM, Shaw JL, Williams WM and Travis KZ. 2000. Characterizing aquatic ecological risks from pesticides using a diquat dibromide case study. I. Probabilistic exposure estimates. *Environ. Toxicol. Chem.* **19**, 749–759.

Stevens JT and Sumner DD. 1991. In *Handbook of pesticide toxicology*, Hayes WJ Jr and Laws ER Jr (Eds), Academic Press, New York, pp 10–88.

Worthing CR and Hance RJ. 1991. *The pesticide manual*. 9th Edition. The British Crop Protection Council, UK.