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Application of a direct toxicity assessment approach to assess the hazard of potential pesticide exposure at selected sites on the Crocodile and Magalies rivers, South Africa

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The potentially hazardous effects of agricultural pesticide usage in the Crocodile (west) Marico catchment were evaluated using the *Danio rerio* and *Daphnia pulex* lethality, *Selenastrum capricornutum* growth inhibition and the Ames mutagenicity plate incorporation assays. Hazard assessment categories are proposed to standardise the output of the different toxicity assessments. Sites were categorised according to the toxicity hazard indicated and were related to the gradients of agricultural land-use intensity at each site. Intensive agricultural sites showed the highest effects to all tested biota. Receiving water at urban sites associated with increased nutrients and lowest pesticide usage showed few adverse effects, while the relatively unimpacted site indicated no hazard to any organism, and only a slight stimulation to algal growth. Weighted hazard scores indicated that the unimpacted sites were least hazardous, falling within a B category, the urban sites were moderately hazardous (C category), and the agricultural sites (D category) had the highest potential impacts on aquatic organisms. This study demonstrated the usefulness of using the hazard assessment approach and the role it could play in assessing site-specific potential toxicity hazards of river water impacted by agrochemicals. It can be used together with other assessment methods, such as biological indices, in a tiered approach.

Keywords: agricultural impacts, algal growth inhibition, classification, fish, hazard assessment, invertebrates, mutagenicity test

Introduction

A large amount of pesticides is used annually in agriculture. Whilst pesticides are of irrefutable value to agriculture, they are also significant causes of environmental impact (Campagna et al. 2006). Consequently, surface water runoff and spray drift from agricultural land may cause contamination of surrounding aquatic systems by pesticides and their synergists. As a result, streams and rivers become the collection points for a range of pollutants (Rand et al. 1995). In some instances, carelessness or deliberate discharge of pesticides directly into these aquatic ecosystems may occur (Campagna et al. 2006) which can cause toxic effects to aquatic flora and fauna (Forney and Davis 1981, Mulla and Mian 1981) and further indirectly affect human health (Faria et al. 2007). Concern about the presence and detection of toxicants, especially in aquatic ecosystems, has increased dramatically in recent years (Martins et al. 2007). It is therefore important to elucidate the effects of these chemicals on aquatic organisms.

Pollutants in aquatic ecosystems, in South Africa and elsewhere, have been managed mainly through assessing specific single-substance components using chemical-based analyses or through substance-specific ecological risk assessments (DWAF 2003, Smolders et al. 2003, Muller and Palmer 2004). Most techniques used in detecting specific chemicals in surface waters are, however, difficult from a toxicological, environmental and analytical point of view (Jooste and Herbst 2004), especially in developing countries which lack resources. Experience has highlighted the inadequacy of using a single-substance approach to assess fully the direct ecotoxicological hazard that may be posed by complex mixtures containing more than one substance (DWAF 2003, Slabbert 2004). The main disadvantages of using these analytical techniques are: the cost, time constraints, expertise and the equipment required to carry out such analysis; it is often difficult to prioritise and select which chemical components should be assessed, especially if there are many to select from; concentrations may be too low to detect yet may still have an negative effect; and new chemical mixtures can be formed which can have different environmental effects compared to the

aggregation of individual substances (Sarakinos et al. 2000, Muller and Palmer 2004). These techniques detect the effects on target chemicals only and cannot fully ascertain the potential ecological effect or biological impairment of complex mixtures on non-target organisms (Slabbert 1994), thus rendering them of low ecological relevance.

Laboratory toxicity tests are used worldwide to manage environmental resources such as water quality and are considered to be the first step in a tiered approach in establishing guidelines for setting maximum acceptable concentrations of specific pollutants (Kimball and Levin 1985, Chapman 1995, Muller and Palmer 2004). The use of bioassays incorporating complex mixtures has proven to be a relevant and complementary tool in evaluating adverse toxic effects of effluents and receiving water bodies (Rand et al. 1995, Grothe et al. 1996). This effect-based approach overcomes the above-mentioned limitations and is relatively rapid, simple and, in most cases, cost-effective (Martins et al. 2007). The techniques are site-specific, account for both point and diffuse sources of aquatic contamination and uncharacteristic sources of toxicity, and can detect their potentially unknown interactions (e.g. synergistic, antagonistic and additive effects) (Dorn 1996, Sarakinos et al. 2000, Smolders et al. 2003, Martins et al. 2007). However, no single toxicity test has proven to be suitable to assess all adverse ecological effects because individual organisms differ in susceptibility to different chemicals (Rand et al. 1995, Chapman 2000, Fernandez-Alba et al. 2001, DWAF 2003). Consequently, several different bioassays at different levels of biological complexity and trophic levels need to be used simultaneously to adequately assess if a potential hazard is posed (Jergentz et al. 2004).

Such approaches used internationally in water quality monitoring programmes include the whole effluent toxicity (WET), used in the USA, the direct toxicity assessment (DTA), used in the UK, and the 'totale effluent milieu' hygiene (TEM) or 'whole effluent environmental risk' approach, used in the Netherlands (DWAF 2003). These approaches are based on similar fundamental concepts by using an array of acute and chronic toxicity test endpoints and, in certain instances such as TEM, include in addition other 'indirect' hazard parameters such as oxygen depletion potential, bioaccumulation and mutagenicity to ascertain ecological effects of pollutants.

In South Africa the Department of Water Affairs and Forestry (DWAF) have adopted a multifaceted approach referred to as the 'direct estimation of ecological effect potential' (DEEEP) which includes these international approaches (using TEM as a foundation) and has become an integral first-tier tool used in the ecological hazard assessment of complex waste discharges (DWAF 2003). The practical implementation of DEEEP methodology in collaboration with industry, scientists, government departments and other role players has already been established (Jooste and Herbst 2004). The DEEEP approach uses representative organisms from different trophic levels of the food chain (fish, invertebrates, algae and bacteria) and endpoints (both lethal and sublethal) to reflect the overall impact of toxicants, with provision to define acceptable ecological hazards providing protection to aquatic ecosystems (Jooste and Herbst 2004, Liu and Dutka 1999). More recently the same suite of hazard assessment methods was selected for inclusion in the National Toxicity Monitoring Programme (NTMP) for surface waters (DWAF 2005). The main objectives of the NTMP are to monitor South African water resources in terms of (a) the toxic effects to selected organisms and (b) selected potentially toxic substances.

The suite of complementary toxicity parameters incorporated in the DEEEP approach and the NTMP are given in Table 1. Not all the tests listed are currently at a level where implementation would be viable (Jooste and Herbst 2004). Therefore, four rapid and relatively inexpensive internationally standardised toxicity bioassays that form part of the DEEEP and NTMP were adapted and incorporated into the present study. These were (1) the freshwater fish (*Danio rerio*) lethality test; (2) the water flea (*Daphnia pulex*) lethality test; (3) the algal (*Selenastrum capricornutum* Printz) growth inhibition test; and (4) the Ames *Salmonella typhimurium* mutagenicity test. Differential responses of organisms such as these represent diverse physiological capabilities and niches in aquatic systems that can help focus field studies where non-target effects due to off-site movement, for example of pesticides, are suspected (Moore et al. 1998). Fish and water fleas (*Daphnia* spp.) are routinely employed to detect chemical pollution of river water on the basis of their toxicity (Elnabawary et al. 1986, US EPA 1993, Rand et al. 1995). The fish lethality test described in Slabbert (2004) uses the guppy *Poecilia reticulata*. In this study the species has been replaced with the zebrafish *D. rerio* for practicable reasons highlighted by Ross (2004). *Danio rerio* and *Daphnia* sp. have been used for many years as 'standard' aquatic test species in toxicity testing (OECD 1992, Roex et al. 2001, Martins et al. 2007). Unicellular algae form the basis of the energy flow in the aquatic trophic chain and show great advantage for use in estimating the toxicity of effluents and receiving waters (Joy 1990, Galassi et al. 1993, Weyers and Vollmer 2000, Sbrilli et al. 2005). They are relatively sensitive to a variety of chemicals especially herbicides, and are easy to handle and have a short life cycle (Hörnström 1990, Rioboo et al. 2001). The Ames mutagenicity test, although not commonly used in South Africa, is based on the method developed by Maron and Ames (1983) and the US EPA (1983) and is relevant for assessing a compound's impact on the microbial ecology of biological systems. Substances within mixtures can induce or facilitate mutagenicity of bacterial cells. Bacteria are therefore important in the most basic ecosystem processes such as release of nutrients and detoxification of natural toxins such as ammonia among others, and are thus important to human and aquatic ecosystem health (DWAF 2003).

The extent of an ecological hazard for each individual series of toxicity test parameters may vary. The need for approaches in aggregating the individual parameters within the hazard assessment has been identified (DWAF 2003). Jooste and Herbst (2004) recognised that criteria for ecological hazard assessments need to be validated locally before finally adopting them. By assessing each parameter according to a certain criterion that determines a specific rating, an overall ecological hazard can be predicted,

Table 1: Tests and proposed rationale applied in the DEEEP protocol and the NTMP (from DWAF 2003)

Effect parameter	Ecological toxicity hazard represented by the parameter
Oxygen demand (oxygen depletion potential)	The extent to which the substance or mixture serves as a nutrient to ubiquitous bacteria in the water that will cause depletion of the oxygen in the water to the detriment of larger organisms.
Acute toxicity*	Specific effects that occur within a relatively short period of exposure to a substance or mixture. Often the effect referred to is death of the organisms used in the selected tests (e.g. water fleas or fish).
Chronic toxicity*	Specific effects that occur within a relatively long period of exposure to a substance or mixture. Often the effect referred to is sublethal (such as growth or fertility).
Bioaccumulation#	The net accumulation of substances in an organism because of the combined exposure via water, sediment and food. This characterisation may have a severe impact on ecosystems over the long term.
Mutagenicity*	The introduction of hereditary changes in living organisms. Mutagenic substances do not necessarily cause cancer in humans but may do so. This may however be implicated in defects in any organism.
Persistence potential#	A property of substances that indicate how long they remain in a specific environment before they are converted to other substances.

* Parameters usually measured by direct toxicity assessment (DTA)
# Not currently available or being carried out in South Africa

i.e. no, slight, moderate, high and very high hazards. The currently applied assessment methods are sufficiently flexible to allow preliminary criteria to be used as a first-tiered approach, to be refined and validated over time with further biological assessments and chemical analysis (DWAF 2003). With the implementation of the NTMP a relatively simple classification system based on the occurrence or absence of toxicity was proposed (DWAF 2005). However, in this study we propose a broader set of hazard assessment categories that could be clearly linked to and integrated with other aspects of the regulatory process, such as the development of resource water quality objectives and river health monitoring with interpretation within the South African water resources management classification context (Jooste and Herbst 2004).

This study evaluates the potential toxic effects of agricultural pesticide usage on nearby receiving waters in a section of the Crocodile (west) Marico catchment, using a combination of toxicological bioassays that allows for a comprehensive and comparative assessment of spatial and temporal variability of effects and that can be used together with other assessment endpoints such as biological indices in a tiered 'weight-of-evidence' approach. Sites on the Crocodile and Magalies rivers and the associated irrigation canal were categorised according to the toxic hazard indicated by the selected bioassays and were related to agricultural pesticide usage intensity.

Materials and methods

Study sites were selected in association with high agricultural activities and pesticide applications adjacent to sections along the Crocodile and Magalies rivers in the Crocodile (west) Marico catchment (Figure 1). Toxicity determinations were carried out on surface water samples collected from seven river sampling sites and on one sample from an irrigation canal system. A representative unimpacted site was selected for comparison to heavy pesticide usage sites that were expected to exhibit a gradient in pesticide contamination, based on data given by farmers during the situation analysis phase of the project. The sites designated as highly impacted by pesticides (C4, C5, Cn1 and M2) were located directly adjacent to agricultural lands, while the unimpacted site (M1) or low-contaminated sites (C1, C2, C3) were located in the upper reaches of the river where no, or very little, pesticides were applied and where agricultural activities take place at quite a distance from sampling localities. The field studies were conducted in late summer (February 2007). This period coincided with the high-rainfall period as well as with the estimated period of high pesticide usage by local farmers (Ansara-Ross et al. 2008). Contamination of the aquatic system through runoff was therefore regarded as being highly likely. Water samples were taken from each of the sites using pre-cleaned sterile glass amber bottles and kept at 4 °C. Water samples were also collected in glass bottles and were used for subsequent pesticide analyses following the methodology given in Schulz (2001). Three pesticides were selected for analysis, based on data given in Ansara-Ross et al. (2008): deltamethrin, dichlorvos and endosulfan. Water samples used for the Ames mutagenicity test were concentrated on arrival at the laboratory and the extracted sample was frozen at −20 °C before further analysis took place. Toxicity tests using fish, daphnia and algae were conducted within 24 hours of sampling.

Water quality parameters

Physico-chemical *in situ* water quality parameters were measured on site and again immediately before sample preparation took place. Parameters measured at the time of sampling included temperature, pH, dissolved oxygen (DO; both percentage saturation and content), total dissolved solids (TDS) and electrical conductivity (EC) using calibrated handheld field meters (Eutech pH 110 RS232C meter; Eutech DO6 dissolved oxygen and temperature meter; and Eutech CON 110 RS232C conductivity and TDS meter). Two litres of subsurface river water were also collected from each of the seven river sites during this sampling, placed on ice and transported to the laboratory for quality analysis. Turbidity, concentrations of ammonium (NH_4), ammonia (NH_3), nitrate (NO_3^-), nitrite (NO_2^-), orthophosphate (PO_4), total phosphate (TP), calcium (Ca),

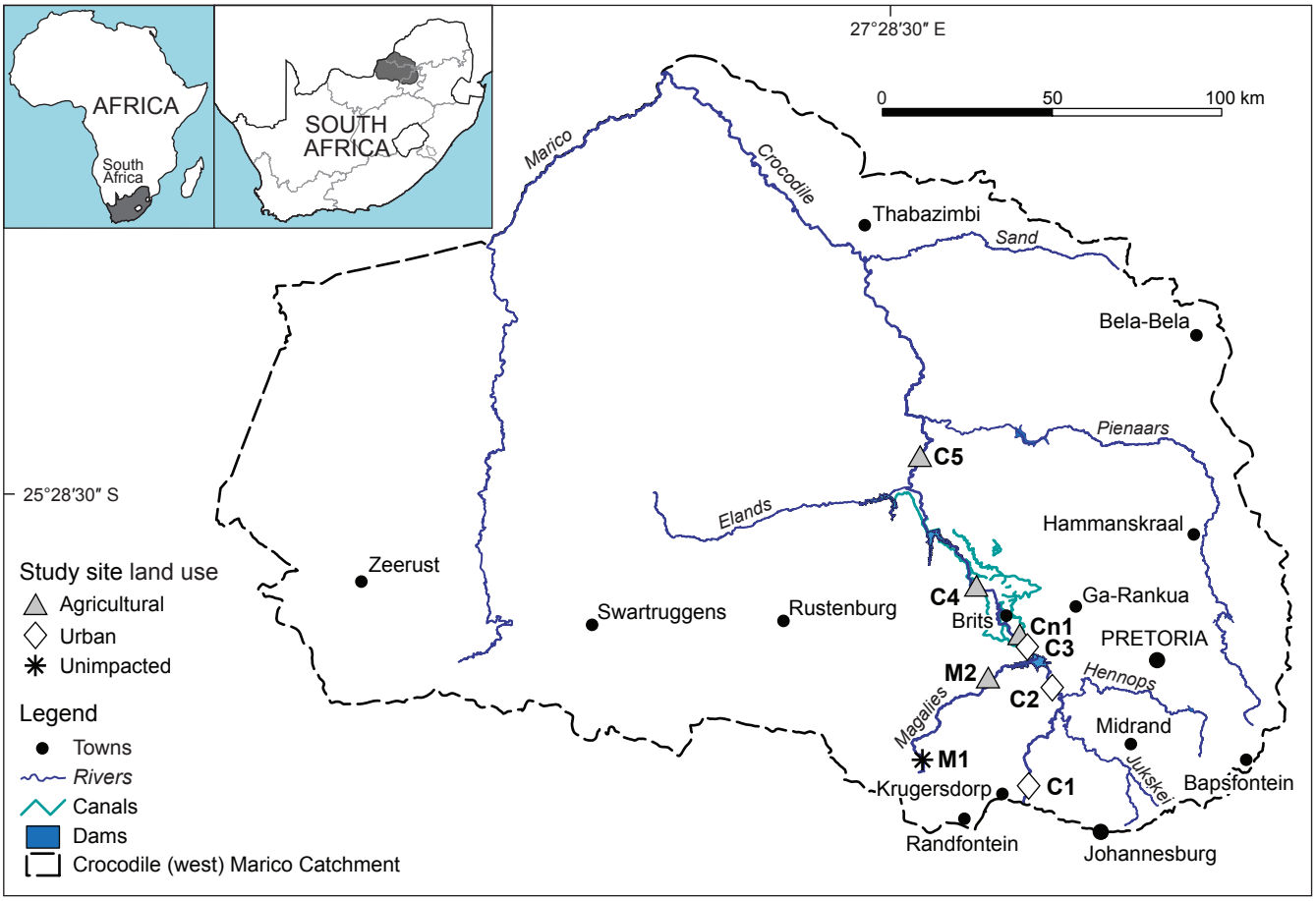

Figure 1: Location of sampling sites in the Crocodile (west) Marico catchment, South Africa

soluble chloride (Cl) and sulphate (SO_4) were determined using a photometer (Merck, photometer SQ 118). The chemical oxygen demand (COD) was determined within 24 hours of sampling following the method of Slabbert (2004) and APHA-AWWA-WEF (1992). The mass concentration of suspended solids (SS) in water was measured by filtering one litre of water samples through pre-weighed, pre-dried nitrocellulose filter membrane (47 mm/0.45 µm pore size) and calculating the mass difference (kg l^{-1}) after drying in a oven at 60 °C for 48 hours. The dried filter paper with SS were placed in pre-weighed porcelain crucibles and placed in an incinerator (Labcon, RM4) for eight hours at 600 °C. The mass fraction organic matter in suspended solids (M_{om}) and mass fraction inorganic matter (M_{im}) were calculated by taking the mass difference (mg l^{-1}) of the crucible and filter membrane before and after incineration and are presented in g l^{-1}.

Pesticide analysis was performed at the Hearshaw and Kinnes Analytical Laboratory, Cape Town, following the methods of Schulz (2001). Water samples were extracted by solid-phase extracted (SPE) using C18 columns previously prepared with 6 ml of each of methanol and water and eluted with 2 ml hexane and then 2 ml of dichloromethane. The extracts were dried in a stream of nitrogen and taken up by 1 ml of hexane before being analysed using gas chromatography (HP 5890) fitted with electron capture, nitrogen-phosphorus detectors.

Toxicity assessment of river water

The following screening toxicity tests of receiving water from sample sites were included: a 96-hour freshwater fish (*D. rerio*) short-term (acute) lethality test; a 48-hour freshwater water flea (*D. pulex*) short-term (acute) lethality test; a 72-hour unicellular algal (*S. capricornutum* = *Pseudokirchneriella subcapitata*) growth inhibition test (sublethal); and a 72-hour chronic toxicity established by means of the Ames *S. typhimurium* mutagenicity test with plate incorporation assay. Water quality parameters (temperature, pH, dissolved oxygen and conductivity) were measured and recorded during the fish, water flea and algal exposures. If the pH and/or oxygen values were outside the optimum range (i.e. if the pH was less than 6 or greater than 8.5 and the dissolved oxygen was less than 40% of saturation) the sample was adjusted accordingly and parallel tests carried out in conjunction. If free chlorines were >0.2 mg l^{-1} during algal tests, samples were neutralised with sodium thiosulphate to a final concentration of 20 mg l^{-1}. All tests were conducted according to the standard whole effluent toxicity testing procedures and recommendations given in the DEEEP toxicity testing protocols (Slabbert 2004).

Test organisms and test protocols

*Fish (*D. rerio*) and water flea (*D. pulex*) lethality bioassay*

Fish and water flea bioassays were carried out using juvenile *D. rerio* (14 days old) and first instar (<24 hours old) *D. pulex*. Organisms were acclimated to experimental conditions for 24 hours prior to the start of the test, at a temperature of 26 ± 2 °C for fish and 22 ± 2 °C for water flea exposures. Groups of 20 experimental organisms (four replicates of five organisms per treatment and negative controls) were selected and used in static exposure tests with the total test volume being made up to 400 ml and 40 ml for fish and water fleas respectively. Each test series consisted of a dilution series of 50% and 100% (undiluted sample) of the receiving water and an untreated (negative) control containing only reconstituted water. The dilution and control water consisted of synthetic moderately hard water, prepared according to the US EPA (1985a, 1993) methods. The endpoints were recorded as percentage lethality. The criterion for mortality was a lack of response to a stimulus by gentle agitation. Toxicity was indicated if lethality was greater than or equal to 10%. A test was regarded as valid if control vessels exhibited <10% mortality and water quality parameters remained within acceptable limits.

*Algal (*S. capricornutum *Printz) growth inhibition assay*

Tests using *Selenastrum capricornutum* (sourced from CCAP 27/4 Cambridge, UK, batch no. SC251108) were carried out in environmentally controlled rooms (21–25 °C) following standard techniques (OECD 2006) using an AlgalTox kit. In all experiments, control cultures without receiving water were included. Experiments were carried out in duplicate, and the results are expressed as the average of the replicates. Optical density (OD) using a Jenway 6300 spectrophotometer was used to establish growth inhibition or growth stimulation relative to a control. Percentage growth inhibition or stimulation was measured respectively as a reduction or increase in growth rate relative to a control carried out under identical conditions. Growth was determined in terms of optical density (OD). Growth inhibition ≥20% indicated toxicity and growth enhancement ≥20% usually indicated excess nutrients.

*Ames (*S. typhimurium*) mutagenicity plate incorporation assay*

The Ames mutagenicity test, using two histodine-requiring strains of the bacterium: TA98 and TA100, was used to assess the mutagenic potential in receiving water samples following plate incorporation methods (Maron and Ames 1983, US EPA 1983, Slabbert 2004). Each of the two tester strains carries a variation of mutation within the operon coding for biosynthesis of histidine (Ames 1983, Slabbert 2004), making it unable to synthesise the amino acid histidine (auxotrophic) from the ingredients in its culture medium. Selected mutagens can, however, be reversed (i.e. back mutation) to prototrophy, whereby the gene regains its function, and are indicated as revertant colonies that grow on a plate without histidine. The rate of reversion caused by a mixture of chemicals compared to a control was measured in this way. TA98 (*hisD3052* mutation) detects frameshift mutagens (which restores correct reading frame for histidine synthesis) and is reverted by mutagens such as 2-aminofluorene and benzo(a)pyrene and the TA100 (*hisG46* mutagen) detects base-pair substitution mutagens (which substitutes praline for leucine in the wild-type organism), e.g. sodium azine or ethyl methane sulphonate (Slabbert 2004). Cultures were obtained from MolTox™ (Boone NC, USA) in the form of lyophilised (freeze-dried) and stabilised disc cultures that were initially cultured from master cultures from Dr Bruce Ames (TA98 lot no. 4457D and TA100 lot no. 4451D). Cultures were grown in nutrient broth to a density of $1–2 \times 10^9$ cfu ml^{-1} OD (approximately 0.4 at 600 nm; 1 cm cuvette) with the addition of a cryoprotective agent (DMSO). Four litres of receiving water samples were concentrated and eluted with acetone to 2 ml (i.e. 2 000 times concentrated) in an initial step by means of solid-phase adsorption techniques using amberlite XAD-7 (Röhm and Haas, Sigma) as described in Slabbert (2004) and US EPA (1985b) and stored in a freezer at −20 °C until analysis. Samples were subsequently tested by combining the extracted sample together with one of the bacteria subcultures (TA98 or TA100), phosphate buffer solution and top agar (with histidine-biotin solution) onto a minimal agar Petri dish as the test vessel. Sterility checks, negative controls (containing acetone instead of extracted sample) and positive controls (containing either sodium azide or 2-aminofluorene for the TA100 and TA98 respectively) were run concurrently with samples. Assays were done in triplicate per sample site for each tester strain and negative control plates. After incubation for up to 72 hours at 36 ± 1 °C, mutagenicity, expressed as a mutation ratio (MR), was determined by comparison of the number of revertant colonies (revertant to histidine prototrophy) on sample plates relative to that on negative control plates for each bacterial tester strain. An MR greater than or equal to two indicated mutagenicity. The number of colonies on negative control plates should be between 20 and 50 in the case of TA98 and between 100 and 250 in the case of TA100. If the number of revertants was less than 100 the sample was regarded as not, or slightly, mutagenic; between 100 and 500 revertants the sample was regarded to be moderately mutagenic and >500 revertants indicated strong mutagenicity in the sample. A background lawn, which appeared on the plate as thin or granular compared to the negative control plate, indicated bacterial toxicity. Colonies where no or very little background lawn occurred were not revertants and were not scored.

Hazard assessment categories for receiving waters

The hazard assessment criterion described by Persoone et al. (2003) was adapted for the DEEEP approach and was applied to this study. The scoring system comprises five ranking classes that range from ‘not acutely hazardous or toxic’ to ‘extremely acutely hazardous or toxic’. Once the effect for each test series was determined, the sample was ranked in one of the five classes on the basis of highest toxic response shown by at least one of the tests applied. The effect results of each test series were then given a weight hazard score (WHS) as indicated in Table 2 to indicate the quantitative importance of the effects in that hazard class. A cumulative WHS for all tests was then calculated for each sample by summation of the individual

weighted hazard scores. A water resource category for each site was subsequently given to the cumulative WHS for each site as indicated in Table 3. This hazard category can then be assessed in terms of ecological and management viewpoint.

Results

Water quality data and system variables

Water quality parameters (temperature, oxygen concentration and saturation, TDS, conductivity and pH) of receiving water samples from the different sites were monitored at the start and again at the completion of the laboratory bioassays. These were found to be within the optimum range (Slabbert 2004). Total residual chlorines measured during algal tests were found to be present at C4, C3, C2 and C1 and absent from C5, Cn1, M2 and M1 sites. Water quality parameters of the receiving water, taken at the sites, as well as nutrients and other system variables, are presented in Table 4. Levels of deltamethrin, dichlorvos and endosulfan concentrations measured in water samples collected from each site were below detection limits (<0.01 µg l^{-1}). This may be attributed to using a once-off sample of receiving water during a high-flow period where dilution factors play a part.

The COD values determined for all sites were regarded as 'acceptable' (according to the DWAF [2003] acceptable limit of 75 mg l^{-1}). Samples of receiving water from sites C4 and

Table 2: Criteria for ecological hazard assessment for discharges/receiving water proposed for the DEEEP method

Parameter	Effect endpoint	Effect values	Hazard description	Weighted hazard score (WHS) per test
Fish (*Danio rerio*)	Percentage lethality at 100% sample	<10%	No toxic effect	0
		10%	Negligibly acutely toxic	0
		20%	Slightly acutely toxic	1
		30%	Moderately acutely toxic	2
		>30%	Highly acutely toxic	3
Water flea (*Daphnia pulex*)	Percentage lethality at 100% sample	<10%	No toxic effect	0
		10%	Negligibly acutely toxic	0
		20%	Slightly acutely toxic	1
		30%	Acutely toxic	2
		>30%	Highly acutely toxic	3
Algae (*S. capricornutum*)	Percentage inhibition or stimulation at 100% sample	<10%	No inhibition or stimulation	0
		10–20%	Negligible stimulation or inhibition	0
		>(+)20%	Moderate to high stimulation	1
		(–)20–30%	Slight inhibition	1
		(–)30–40%	Moderate inhibition	2
		>(–)40%	High inhibition	3
Mutagenicity (*S. typhimurium*) TA98 and TA100	Mutagenic ratio at 100% sample	MR < 1:1 <100 revertants	Non-mutagenic	0
		MR = 1:1 <100 revertants	Slightly mutagenic	0
		MR = 1–2 100–500 revertants	Moderately mutagenic	1
		MR = 2 >500 revertants	Highly mutagenic	2
		MR > 2 >500 revertants	Very highly mutagenic	3
Oxygen demand	Concentration	COD < 75 mg l^{-1}	Acceptable	0
		COD > 75 mg l^{-1}	Unacceptable	2

Table 3: Hazard assessment categories for the various toxicity test endpoints

Hazard category	Hazard description	Result	Cumulative hazard score
A	No hazard due to toxicity	None of the test show a toxic effect	0
B	Slight hazard due to toxicity	The cumulative hazard score of one of the toxicity tests was 1	1
C	Moderate hazard due to toxicity	The cumulative hazard score of one or more of the toxicity tests was between 2 and 5	2–5
D	High hazard due to toxicity	The cumulative hazard score of one or more of the toxicity tests was between 6 and 10	6–10
E/F	Extreme hazard due to toxicity	The cumulative hazard score of one or more of the toxicity tests was greater than 10	>10

C3 showed the highest COD values of 5 mg l^{-1} and 3 mg l^{-1} respectively, while the rest of the sites indicated COD concentrations below detection limits. The South African water quality guidelines for aquatic ecosystems indicate that the use of biochemical oxygen demand (BOD) and COD is inappropriate for determining the overall health of an aquatic ecosystem, but is useful for determining water quality of effluents discharged into aquatic systems, in order to limit their impact (DWAF 1996). Dissolved oxygen concentrations provide a useful measure of health of a system. The oxygen availability of the receiving water was within the target water quality range (TWQR) of between 80% and 120% (DWAF 1996) indicating that oxygen is available for uptake by organisms at all study sites.

The water quality data indicated that the two intensive agricultural sites (C4 and C5) had the highest sulphate and chloride concentrations relative to other sites (Table 4). The C4 and C2 sites also indicated higher values for nitrates. Wastewater treatment works located in Brits upstream of C4 and the sewage treatment works for the Johannesburg and Pretoria areas upstream of C2 may be responsible for the elevated levels of nitrates. Phosphorous values were also relatively high at the C2 site possibly due to sewage effluent and the urban runoff.

The adjacent land use at the C1 site was predominately recreational and urban; however, this site is located within a South African National Botanical garden and is protected to an extent, except for isolated pesticide spraying for weed control within the gardens and effluent discharges emanating from the catchment areas upstream of this site. This site is fed predominantly by groundwater (A Hankey, Walter Sisulu National Botanical Gardens, Ruimsig) which may dilute the surface water, thereby reducing the impacts of water quality at this site. The unimpacted site, located at the headwaters of the Magalies River (M1), receives water directly from the source at Maloney's Eye. The system at this site is an oligotrophic system, with the water quality variables found to be within all total water quality guide values.

Fish (*D. rerio*) and the water flea (*D. pulex*) lethality test

The percentage lethality for each site, including the control exposure after 96 hours for *D. rerio* and 48 hours for *D. pulex*, is given in Figure 2a and 2b. No mortalities occurred in the control exposures for both *D. rerio* and *D. pulex*. Fish lethalities greater than 10% were recorded for all sites except the relatively unimpacted site (M1), where no mortalities were recorded with both the 50% dilution and 100% of this receiving water sample. C4, C5, Cn1 and M2 sites indicated the highest toxicity to fish in the 100% and 50% receiving water samples. Percentage fish lethality for these sites ranged from slightly (20%) to highly (40%) acutely toxic. For the exposures to *D. pulex* once again the unimpacted site (M1) did not show any lethal responses with 100% and 50% receiving water. Receiving water from C4 had the highest acute toxicity to *D. pulex* at 100% and 50% of the receiving water. The C5, Cn1 and M2 sites were moderately toxic at the 100% receiving water exposure.

Algal (*Selenastrum capricornutum *Printz) growth inhibition test

Percentage inhibition or stimulation for each sample site is presented in Figure 2c. Growth inhibition or stimulation

Table 4: Physico-chemical water quality for once-off sampling of receiving water from the Crocodile and Magalies rivers used in the hazard assessment methods: temp. (temperature); pH; DO (dissolved oxygen content); O_2 (dissolved oxygen saturation); EC (conductivity); TDS (total dissolved solids); turb. (turbidity); NO_2^- (nitrite); NO_3^- (nitrate); TP (total phosphate); PO_4 (orthophosphate); Ca (calcium); Cl (chloride); COD (chemical oxygen demand); SO_4 (sulphate); NH_4 (ammonium); mass fraction organic matter in suspended solids (M_{om}); mass fraction inorganic matter (M_{im}); and suspended solids (SS)

Site	Temp. (°C)	pH	O_2 (mg l^{-1})	O_2 (%)	EC (µS cm^{-1})	TDS (mg l^{-1})	Turb. (NTU)	NO_2^- (mg l^{-1})	NO_3^- (mg l^{-1})	TP (mg l^{-1})
C1	22.9	7.6	7.19	97.9	143	72	5	0.02	7.3	0.03
C2	23.1	8.0	7.08	96.1	536	268	24	0.11	24.8*	0.55
C3	22.9	8.4	7.14	95.5	545	270	24	0.02	5.6	0.59
C4	23.0	8.0	7.06	95.6	812	408	30	0.31	19.5*	0.30
C5	22.8	7.9	7.21	96.5	654	328	18	0.72*	3.5	0.06
Cn1	23.0	8.1	7.04	97.0	540	270	Nd	Nd	Nd	Nd
M1	22.1	7.8	7.10	97.5	272	136	11	<0.01	3.1	0.01
M2	23.1	8.2	7.05	96.6	500	249	20	0.02	<0.01	0.05

	PO_4 (mg l^{-1})	Ca (mg l^{-1})	Cl (mg l^{-1})	COD (mg l^{-1})	SO_4 (mg l^{-1})	NH_4 (mg l^{-1})	M_{om} (g l^{-1})	M_{im} (g l^{-1})	SS (g l^{-1})
C1	0.10	7	120	<0.01	<5	0.03	0.0004	0.02	0.02
C2	1.68	25	148	<0.01	36	0.01	0.010	0.03	0.04
C3	1.78	33	186	3	34	0.72	0.003	0.01	0.01
C4	0.91	44	348*	5	84	0.19	0.030	0.02	0.05
C5	0.20	37	310*	<0.01	69	0.02	0.002	0.01	0.01
Cn1	Nd	Nd	Nd	Nd	<5	Nd	0.030	0.02	0.04
M1	0.03	14	23	<0.01	<5	0.05	0.020	0.01	0.03
M2	0.16	19	133	<0.01	7	0.03	0.020	0.02	0.04

* = Above TWQR (target water quality range)
Nd = Not determined

greater than 20% indicated toxicity or excessive nutrients respectively. Only one site, namely C5, indicated growth inhibition above 20% indicating toxicity although this value is quite low (23%). The remaining sites indicated (moderate to high) growth stimulation to some extent with C1 indicating the lowest growth stimulation (23%) and the canal site (Cn1) the highest (69%) resulting in excessive nutrient loads at this site. The high green algal stimulation at all sites except C5 may be a result of high nutrient contamination within the study area from several upstream sewage treatment works that discharge into the two rivers, as well as from the return flows from agriculture resulting in eutrophication of this system. At the time of sampling, excessive amounts of filamentous algae were observed at sites C3, Cn1 and M2. The release of high amounts of fertilisers (phosphorus and nitrogen) at M2 may be the cause of the high stimulation at this site. The C3 site is directly below Hartbeespoort Dam which is known to be a highly eutrophic system (Owuor et al. 2007). The irrigation canal water also comes directly from Hartbeespoort Dam water and can be the cause of the high stimulation at these sites. Upstream of site C5, the Crocodile River flows into the Roodekopjes Dam and the endpoint of the canal irrigation scheme enters this river at this point. This may indicate why C5 had inhibition in algal tests. Herbicides are used more extensively within the surrounding areas of this site (A van der Merwe, Laeveld Agrochem, Brits, pers. comm.), which may also result in the inhibition of algae. The slight stimulation at the unimpacted site (M1) may be a result of very low concentrations of ammonium and nitrates at this site. Ammonium has little or no toxicity to aquatic biota but contributes to eutrophication (DWAF 1996).

*Ames (*S. typhimurium*) mutagenicity plate incorporation assay*

Sterility checks indicated no contamination occurred during the test procedure. Positive controls indicated clear halos and rings around the respective chemicals for TA98 and TA100 tester strains, indicating induced frameshift and base-pair mutations, respectively. The numbers of colonies on the negative control plates were within the stated range of expected revertant bacterial colonies. Figure 2d shows that a mutagenic ratio of 1 was indicated for C1, C2, C3, C4 and C5 for the TA98 bacteria, and for C3, Cn1 and M2 for the TA100 bacterial strand. Mutagenicity was, however, only clearly evident (MR ≥ 2) at the Cn1 and M2 sites for the TA98 culture and at C4 and C5 for the TA100 culture. Revertant colonies for the Cn1 and M2 sample plates for TA98 were below 100 and are regarded as slightly mutagenic, indicating only slight frameshift mutagens in the

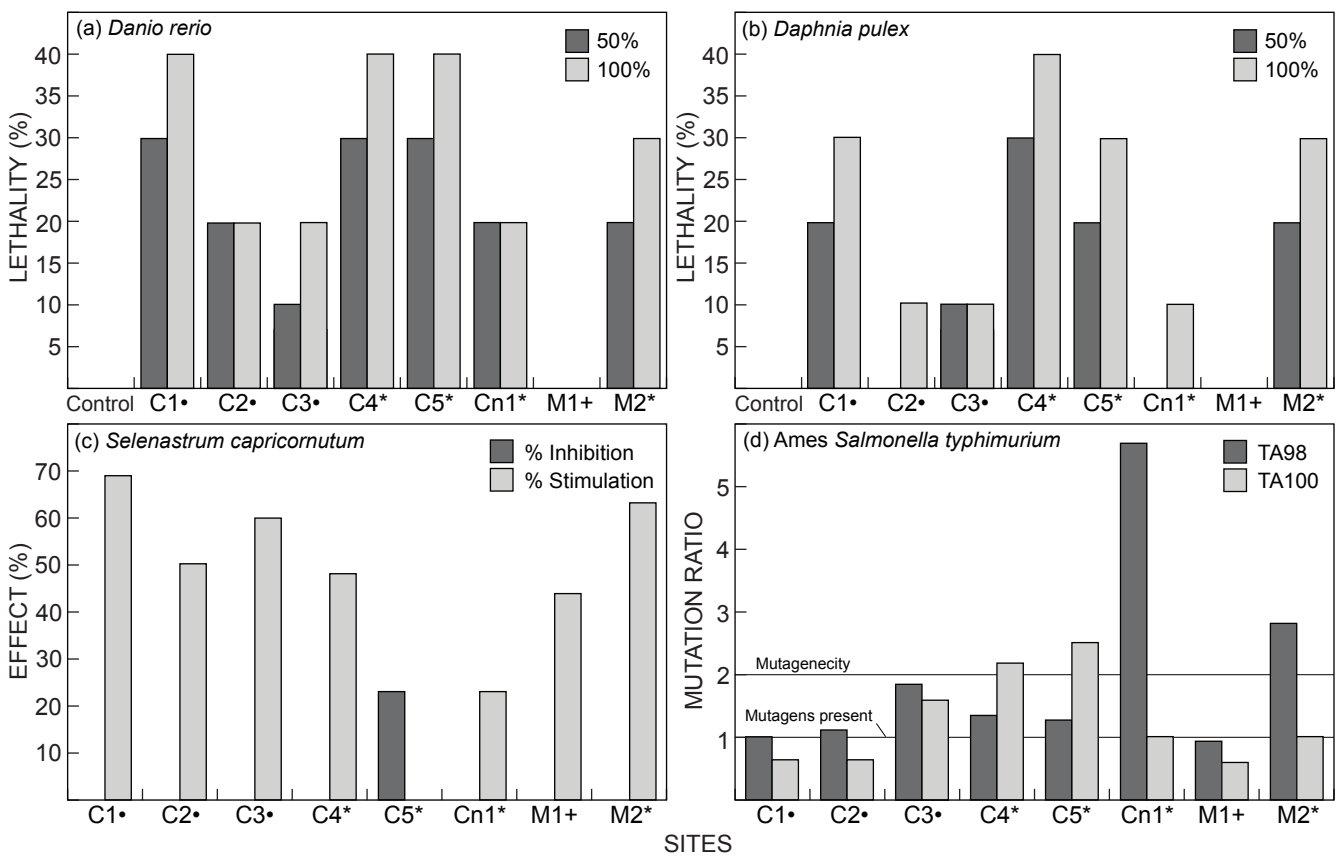

Figure 2: Effect data for (a) *Danio rerio* lethality assay, (b) *Daphnia pulex* lethality assay, (c) *Selenastrum capricornutum* growth or inhibition assay and (d) Ames *Salmonella typhimurium* mutagenicity assay for agricultural (*****), urban(**•**) and unimpacted (**+**) sites located along the Crocodile and Magalies rivers including an irrigation canal site

bacteria. Revertant colonies determined on the C4 and C5 plates for TA100 ranged between 100 and 500 and thus indicated a moderate mutagenicity in the form of base-pair substitution. No samples indicated toxicity. All sites except for the unimpacted site (M1) showed the presence of mutagens (either with TA98 or TA100) with the number of colonies on test plates exceeding the 1:1 ratio to that of the negative controls. No site, however, indicated strong mutagenicity for either the TA98 or TA100 bacterial strands. Sampling sites indicating mutagenicity with the TA100 or TA98 bacterial strands (namely C4, C5, Cn1 and M2) have the highest agricultural land use with intensive pesticide-spraying occurring in these regions.

Hazard assessment

By applying weight hazard scores for each site, the large array of data can be simplified and an overall ecological hazard class per site can be calculated (Table 5). A summary of the four assays including COD results indicated that the Magalies River unimpacted site (M1) and C1 receiving water were least hazardous, falling within the B category based on a cumulative WHS score of 1. Receiving water from sites C4, C5, Cn1 and M2 fell within the D hazard category, which showed the highest potential to elicit harmful impacts on the aquatic organisms in the Crocodile and Magalies rivers. Receiving water from the two urban sites C2 and C3 indicated a moderate hazard with a cumulative WHS score of 2, falling within the C category for these sites.

The results obtained in this study indicated that water samples collected from intensive agricultural sites (C4, C5, Cn1 and M2) with high pesticide usage showed the highest effects to all tested biota used in the bioassays. In particular, the agricultural site (C5) located at the lowest reach of the Crocodile River sites with presumed high insecticide and herbicide usage exhibited severe algal growth inhibition. The highest cumulative hazard score was calculated for the agricultural site C4 located in the middle section of the Crocodile River reflecting the highest intensity of agricultural activities and highest pesticide usage taking place close to the water course. The highest WHS scores for these sites were calculated for fish and water flea exposures. Receiving water at urban sites associated with increased nutrients and little pesticide usage showed little adverse effects on test organisms while the relatively unimpacted site indicated no hazard to any organism, with moderate stimulation to algae during exposures, resulting in it being classified in a B category instead of an A category. The primary pesticide group predicted to have a potential risk on aquatic ecosystems from the agricultural study sites was the insecticides (Ansara-Ross et al. 2008). It would thus be expected that the fish and water flea tests would show a greater response than that of the algal exposures. The herbicide usage was highest in the C5 area, which may have lead to algal inhibition exclusively at this site.

The ecological and water use categories proposed for the NTMP were applied to the data from this study. This classification system allocates the following scores: 1 = no toxicity of any kind; 2 = no short- or long-term lethality; 3 = lethality. Based on these criteria all the sites, with the exception of M1, would have exhibited toxicity and would fall under the 'unacceptably degraded' management class. However, the NTMP does not regard algal stimulation as a negative effect. In the classification system provided here we regard algal stimulation as a biological response to environmental change. Thus, from an ecosystem point of view, the approach followed in the NTMP does not provide meaningful information on the degree of toxicity hazard at each site. The authors are of the opinion that the hazard approach proposed in this paper is therefore better suited for implementation in higher tier ecological risk assessments.

Conclusions

This study demonstrated the usefulness of combining a series of toxicity tests and the role that this approach can potentially play in assessing the potential ecological impacts in a river that receives agricultural inputs. It is important to include organisms from different trophic levels when the primary aim is the protection of aquatic life as a whole, especially when pesticides that involve different modes of action that are applied in various mixtures end up in aquatic ecosystems affecting non-target groups of organisms. Based on their sensitivity, the fish and water flea lethality tests, and

Table 5: Effect class categories for *D. rerio*, *D. pulex*, *S. capricornutum* and *S. typhimurium* toxicity exposures, as well as COD, with associated weighted hazard scores (WHS) using 100% receiving water from the Crocodile and Magalies rivers and irrigation canal water

Site	Fish (*D. rerio*)		Water flea (*D. pulex*)		Algae (*S. capricornutum*)		Mutagenicity (*S. typhimurium*)				COD		Hazard status	
							TA98		TA100					
	% effect	WHS	% effect	WHS	% effect	WHS	MR	WHS	MR	WHS	Effect	WHS	Cumulative WHS	Hazard category
C1	20	1	10	0	+17	0	1.03	0	0.65	0	0	0	1	B
C2	20	1	10	0	+50	1	1.12	0	0.66	0	0	0	2	C
C3	20	1	10	0	+60	1	1.85	0	1.61	0	2	0	2	C
C4	40	3	40	3	+48	1	1.35	0	2.18	1	1	0	8	D
C5	40	3	30	2	–23	1	1.29	0	2.51	1	2	0	7	D
Cn1	40	3	30	2	+69	1	5.69	1	1.01	0	Nd	0	7	D
M1	0	0	0	0	+44	1	0.94	0	0.59	0	0.01	0	1	B
M2	30	2	30	2	+63	1	2.81	1	1.03	0	0.50	0	6	D

Nd = Not determined

the algal growth inhibition tests, have for many years been recommended for regulatory and management purposes (Martins et al. 2007, Sandbacka et al. 2000). There remains a need to include other chronic tests or sublethal endpoints especially where chemicals may occur in very low concentrations. *Daphnia pulex* reproductive tests at present do form part of the accepted hazard assessment methodologies in South Africa and should be included in the future to give information on chronic exposures as well. It has also been suggested that if toxic responses based on a specific form of contamination, e.g. pesticides, is suspected then higher-tier studies that incorporate biomarker responses to pesticide exposures should be carried out (Wepener 2008).

It should be noted that the pesticide residue analyses in the current study were limited to deltamethrin, endosulfan and dichlorvos (i.e. only three insecticides) and could not explain all sources for biological effects observed within tests. Other compounds that were not tested for could have been present in these samples at the time of sampling. These results, however, clearly show that the use of toxicity tests, using receiving water, produces additional information when considering the relative health of a system under stress. If a sample is indicated as having a high hazard score and its toxicity is assessed as being at an unacceptable level, it is recommended that higher-tiered assessments such as further chemical analysis be conducted for this sample, especially for a range of pollutants such as pesticides. It would, however, be valuable if the same temporal sample is available for analysis. This study indicated that the use of toxicity assessment methods in a holistic manner is applicable and appropriate for assessing site-specific potential toxicity hazards of receiving water impacted by agrochemicals and provides a means of protecting the ecological integrity of aquatic ecosystems. It is recommended that the toxicity tests and hazardous effect categories used in this study be aligned within the River Health Classification Scheme. This study can be regarded as an initial step on which other case studies could follow to determine its applicability in other systems.

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