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Freshwater biological indicators of pesticide contamination

Science Report – SC030189/SR3

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Steve Killeen

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Executive summary

Pesticides are commonly detected in freshwater ecosystems, yet there is considerable uncertainty over whether they are having any adverse impacts on aquatic communities. The Environment Agency would like to establish the need, cost or benefits of further measures to control pesticides, and thus requires further field-based evidence on the potential impacts of pesticide contamination in aquatic ecosystems.

The aim of this project was to find one or more biological indicators that could be used by the Environment Agency to identify pesticide effects and responsible contaminants. Potential end users from different Environment Agency teams (Science, Conservation and Ecology, Policy, Operations - Ecological Appraisal) and the Catchment Sensitive Farming initiative were asked about their expectations for a diagnostic biological indicator. The response was that it should provide information about temporal trends in pesticide contamination as well as trends across sites on a national scale, to help target water quality monitoring programmes as well as risk management measures. The indicator should be sensitive to pesticide-induced changes in aquatic macroinvertebrate communities, and be able to identify the type of pesticide and the magnitude of contamination. It should be reliable, easy to deploy and interpret and easy to communicate to external audiences. Further, the indicator should run on macroinvertebrate data from the General Quality Assessment programme to minimise costs, and should be rapid and usable in the field to give a first indication of potential pesticide contamination.

These requirements were used in a critical assessment and comparison of approaches to diagnose pesticide contamination from biological data. The review included methods potentially specific to pesticides (biochemical and molecular biomarkers) as well as diagnostic approaches at the population to community level, either taxonomy or trait-based. In addition, the study reviewed prognostic techniques that could be used with a diagnostic tool (RIVPCAS, PSYM) or that were potentially useful for diagnostic purposes (PERPEST).

The assessment showed that currently, no single approach meets all the expectations specified by potential end users. However, three approaches showed promise as diagnostic tools. The SPEAR indicator and the Pesticide Index were found to be quick and cheap, making them suitable for screening at the landscape level. The acetylcholinesterase (AChE) assay has the potential to identify or exclude organophosphate or carbamate insecticides as contaminants responsible for observed biological impairment. Finally, the software tools RPDS and RPBBN, as although they are not yet pesticide-specific, they do not rely on a priori knowledge but rather on self-organisation of data.

The different strengths of these methods are best combined in a tiered approach, with the first tier using community-based methods to screen for pesticide contamination at larger scales. The mechanistic and empirical knowledge on pesticide-sensitive species incorporated in the SPEAR indicator and the Pesticide Index could be combined into a hybrid approach that compares expected community composition (based on RIVPACS predictions) and observed community composition, to indicate the level of contamination.

As well as the hybrid approach, the first tier could make use of RPDS/RPBBN software. The results of first tier assessment would have more weight if two diagnostic tools pointed in the same direction. To use the RPDS/RPBBN software, however, requires collation of monitoring data on pesticide contamination and macroinvertebrate community composition, in order to update both systems so that they could indicate pesticide contamination. Should both the hybrid and software approaches indicate

contamination, this would trigger the second tier of a refined diagnosis using sub-organism methods to identify the type of pesticide responsible. Currently, the only suitable method is the AChE assay, which can identify organophosphates or carbamates as the contaminants potentially responsible for the observed impairment. However, this would require the validation and linking of biomarker responses to exposure in the field.

Such a tiered approach would require a dataset of pesticide and biological field data to establish exposure-response relationships. With these data it would be possible for England and Wales to test the SPEAR index, to relate the absence of species to observed pesticide contamination (Pesticide Index), to revise the RPDS/RPBBN software and to link biomarker responses to exposure conditions in the field. If these major gaps can be addressed, the tiered approach could prove a suitable indicator of pesticide contamination in freshwater habitats.

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1 Introduction

Pesticides are commonly detected in freshwater ecosystems, yet there is considerable uncertainty over whether they are having any adverse impacts on aquatic communities.

The Environment Agency has decided that more field-based evidence needs to be collected on the impacts of pesticides on aquatic ecosystems, to complement the current regulatory model in which the effects of pesticides are assessed by extrapolating the results of laboratory tests on single aquatic species and (micro)mesocosm studies of aquatic communities under semi-field conditions.

To date, few studies have directly investigated the effects of pesticide contamination in the field. In a recent EU workshop, *Effects of pesticides in the field* (EPiF), European and North American pesticide scientists from government, industry and academia concluded that to link pesticide exposure to biological impairment remained a major problem, particularly because of the need to separate pesticide effects from confounding environmental factors (Liess *et al*., 2005). To date, the number of field studies reporting a clear relationship between measured pesticide exposure and observed community response is very small. Where effects have been described, studies have usually been concerned only with a small number of water bodies.

Is the lack of identified exposure-response relationships in the field due to pesticide effects being exceptional, or do effects occur more frequently but remain largely undetected because of shortcomings in the number and design of studies? To answer this question, more field-based information is required on the link between pesticide exposure and patterns in aquatic community composition.

Detecting the effects of pesticides in the aquatic environment is technically difficult and often costly. Identifying such effects requires aquatic community descriptors that are sensitive to changes in exposure and can indicate the level of exposure in a complex environment with different factors/stressors acting on communities. This study aimed to identify one or more biological indicators that could be used to characterise the temporal and spatial heterogeneity of pesticide contamination at larger scales.

Special attention was paid to the requirements of users of a diagnostic pesticide indicator. Potential end users from different teams within the Environment Agency and the Catchment Sensitive Farming initiative were interviewed about their expectations of a biological indicator of pesticide contamination.

The study then reviewed current biological indicators of pesticide contamination and approaches not specifically designed for pesticides, but which could be adjusted to diagnose pesticide contamination.

A critical assessment and comparison of the reviewed approaches was carried out based on their major features, the expectations of potential end users and compatibility with current Environment Agency procedures.

Finally, the report offers conclusions and recommendations on suitable biological indicators. Further advice is given on potential future research to test these approaches.

2 A suitable indicator - expectations of end users

2.1 Characterising expectations

To identify a suitable indicator, one must first understand what potential end users would expect from such a tool. This requires exploring how such an indicator would be used and especially the types of questions to be answered by the indicator. The requirements might well be different for different users, making it necessary to identify a set of indicators rather than a single one to meet the needs of all groups.

Eleven potential end users from different Environment Agency teams were asked to fill in a questionnaire on their expectations of a biological indicator of pesticide contamination. The questionnaire was designed to gain information on the type of questions to be answered by a diagnostic indicator, the importance of special features (such as specificity, sensitivity, reliability, input data) and experiences with existing indicators in terms of practicality. A copy of the questionnaire is shown in Appendix I. Interviewees were contacted by phone or email when clarification of the responses was necessary.

2.2 Summary of questionnaire responses

Potential end users were members of the Environment Agency Science Group, the Conservation and Ecology team, the Air and Chemicals Policy team and the Operations function. Further, Officers of the Catchment Sensitive Farming initiative were interviewed.

Data used to identify biological impairment differ between Environment Agency teams, but mostly include biological data collected as part of the General Quality Assessment (GQA) programme. Officers from the Catchment Sensitive Farming initiative use these data exclusively. Members of the Operations team and Conservation and Ecology team use biological GQA data and additional data from other routine biological monitoring programmes (such as Catchment Abstraction Management Strategies (CAMS) or drought monitoring) and ad hoc survey programmes (single survey or short-term repeat monitoring to identify sources of impairment and track mitigation processes) where taxa may be identified to higher taxonomic resolution than normal.

Current approaches to indicate biological impairment, including impairment due to pesticides, are based on macroinvertebrate communities. Tiered approaches are applied by members of the Operations and the Conservation and Ecology team that start with the interpretation of Biological Monitoring Working Party (BMWP) scores, Average Score Per Taxon (ASPT), the number of taxa or the Lincoln Quality Index (LQI: Extence *et al*., 1987) scores based on expert knowledge. Methods applied for the second tier vary between teams. Ecological Appraisal Officers rely on expert analysis of data at species to order level (such as number of Ephemeroptera, Plecoptera and Trichoptera) or of the Lotic-invertebrate Index for Flow Evaluation (Extence *et al*., 1999) (LIFE) scores. Software tools such as PRIMER (Plymouth Routines In Multivariate Ecological Research) or RIVPACS (River Invertebrate Prediction and Classification System) are used to further interpret data. The Conservation and Ecology team also use artificial intelligence software, specifically the River Pollution Diagnostic System

(RPDS, see Section 3.4.1.3) and the River Pollution Bayesian Belief Network (RPBBN, see Section 3.4.1.4), for second tier analysis. Some members of the Operations team also envisage the use of RPDS or RPBBN.

The advantage of BMWP or LQI scores lies in their rapid and simple metrics. The disadvantage is that they are not designed for specific stressors and can only indicate deviation from good biological quality. Expert analysis of data is deemed beneficial because it is unlikely to lead to many false classifications, but it requires a pool of experienced staff and is subjective. The advantage of artificial intelligence software lies in the self-organisation of data rather than a priori knowledge. The current drawback of both RPDS and RPBBN is that no pesticide data are included; that is, the systems cannot indicate biological impairment due to pesticides at present.

Besides the above-mentioned methods, members of the Operations team also use two biological indicators developed for specific stressors. The indicator for organic pollution from farm wastes (Rutt *et al*., 1993) was developed using Two-way Indicator Species Analysis (TWINSPAN) and requires assessment of the abundance of four macroinvertebrate taxa (oligochaetes, heptageniids, *Gammarus* sp. and *Baetis* sp.) and of the extent of 'sewage-fungus' (microbial heterotrophic) growth. The key can separate sites into three groups of impact (unpolluted, mild or historic organic pollution, gross organic pollution). The indicator for surface water acidity (Rutt *et al*., 1990) was developed using TWINSPAN and multiple discriminant analysis (MDA) and is based on the presence or absence of macroinvertebrate taxa (Baetidae, Hydropsychidae, Heptageniidae, Hydraenidae, Goeridae, Platyhelminthes, Limnephilidae, Sericostomatidae, Gammaridae, Taeniopterygidae, Perlodidae, Elminthidae). Both keys were developed to aid interpretation of fauna from kick samples.

The keys offer a simple summary of information gathered from sampling and provide consistency in the interpretation of samples. They are deemed fairly objective and can be used by non-specialist staff with little training. The disadvantage is that the keys can only be applied where the test habitat is consistent with site types used to develop them. If they are used to interpret samples from a large number of sites, there should be a good overall statistical match between interpretations and other measures such as water quality. However, there will inevitably be miss-classifications for individual sites due other influences (pollutants, drought and so on) and the normal variability in biological data.

Four interviewees said they use special approaches to indicate biological impairment from pesticides at a local level. Three members of the Operations team use their own indicators, including the Pesticide Index (Humpheryes, personal communication) and a flow chart of sheep dip impacts that lists organisms decreasing in sensitivity to sheep dip pesticides. The Pesticide Index (see Section 3.3.1.1) was developed for streams in Kent and is based on the absence of expected pesticide-sensitive taxa.

The flow chart for sheep dip (Rutt, personal communication) was developed to guide interpretation of kick samples. *Gammarus* sp. is listed as the most sensitive taxon, followed by stoneflies and Heptageniid mayflies. Caddisflies and other mayflies tend to be less sensitive with Hemiptera, snails, flatworms and oligochaetes being the most resistant. The flow chart is considered fairly objective; however, there is concern that its use by non-specialist staff may lead to false results. Another way to investigate the impacts of sheep dip, is by analysing the results of biological monitoring with taxa identified to a higher taxonomic resolution than normal. The advantage of this is that it can identify the type of toxicant that might be involved, and that it adds to the burden of proof. Recognised disadvantages are the resource intensiveness of the procedure and its possible lack of sensitivity.

Members of the Science Group do not current use a biological indicator to indicate pesticide impacts at the national level. Instead, data from chemical monitoring of

various pesticides are compared against Environmental Quality Standards (EQS) or used to comment on the levels of pesticide exposure in surface waters.

From the interview responses, users consider approaches to be beneficial if they:

- are based on macroinvertebrates;
- are transparent;
- are based on adequate reference databases;
- are objective and can be used by non-specialist staff;
- allow for processing large sample numbers;
- give a good summary of the situation;
- use information on faunal composition and environmental parameters at sites to assess the present biological status;
- are designed so that they are compatible with other Environment Agency software.

All end user groups agreed that a biological indicator of pesticide contamination would be useful for their work. The indicator would ideally be used to:

- identify impacts of pesticides in the aquatic environment;
- interpret monitoring data and provide scientific evidence;
- locate hot spots on a national scale;
- identify responsible compounds;
- indicate trends over time;
- target monitoring programmes;
- target risk management measures;
- provide information to advise farmers.

Potential users were asked which biological endpoints they were interested in. Several interviewees said that any endpoint with ecological significance would be suitable if it could:

- show negative impacts of pesticides on the whole ecology of river systems;
- demonstrate cause and effect;
- be easily communicated and understandable (with respect to different groups of stakeholders).

However, overall, macroinvertebrate community composition was considered the most useful due to the abundance of monitoring data on this group of organisms. Some interviewees were reluctant to consider endpoints at the sub-organism level; for example, genetic structure was considered impractical and liable to generate erroneous conclusions because of low signal-to-noise ratios in genetic assay techniques. Further concern was expressed over whether effects at the sub-organism level affect the survival of populations and communities, in other words, whether effects propagate to higher levels of biological organisation. Impacts at the sub-organism level

were also regarded as less easy to communicate and less persuasive than impacts at community level.

When the end user groups were asked to rank six abilities of a potential pesticide indicator, the most important abilities (Rank 1 to 3) were to:

- identify locations of pesticide contamination, by distinguishing the effects of pesticides from other stressors (ranked first in unison);
- indicate trends over time;
- indicate the relative order of magnitude of pesticide contamination.

Considered less important (Rank 4 to 6) were the abilities to:

- indicate the time period of contamination;
- compare levels of pesticide contamination between different regions;
- give an early warning.

If there was the possibility of designing a new biological indicator of pesticide contamination to meet end users' requirements, the specifications were that it should:

- be specific to the impacts of pesticides;
- indicate the magnitude of impact;
- show the magnitude of contamination;
- detect the active ingredient;
- indicate trends over time;
- show trends across sites;
- be pattern-based, considering the presence and abundance of a combination of taxa that together indicate pesticide contamination;
- be able to detect subtle changes and indicate recovery.

A biological indicator of pesticide contamination would have to be:

- cheap, requiring no additional biological sampling;
- scientifically reliable, where it could be used by non-specialist staff and the results used for policy decision-making or convincing farmers;
- quick, rapid in the field with detailed information obtained from lab-sorting of samples;
- transparent, that is, easy to calculate, deploy, interpret and understand and thus easy to accept for external groups;
- applicable at national scale, applicable to small headwaters and large rivers and capable of processing large sample numbers.

2.3 Expectations of potential end users - synopsis

Evaluation of the questionnaires showed that a freshwater biological indicator of pesticide contamination would be a useful diagnostic tool for all end user groups. Potential users would employ the indicator to explore temporal trends in pesticide contamination and trends across sites on a national scale. The indicator should be applicable to small streams and large rivers and could be used to screen for sites where pesticides affect the aquatic environment. Screening is considered valuable to target monitoring programmes of water quality, as well as risk management measures, to areas where pesticides adversely affect the quality of surface waters.

Therefore, the indicator should incorporate the following major abilities:

- It should be sensitive to pesticide-induced changes in aquatic invertebrate communities, but insensitive to effects of other environmental contaminants and factors such as morphology and general water quality.
- It should identify the type of pesticide that acts or acted on invertebrate communities. It should distinguish between pesticide types (such as herbicides, insecticides, fungicides) or even more usefully, between active ingredients.
- The indicator should identify the magnitude of contamination from large impacts to subtle changes, where pesticide-induced shifts in community composition do not necessarily induce substantial decreases in biodiversity at impacted sites.
- It should be transparent and robust and give an objective summary of the observed situation at sites. The tool should be easy to deploy and interpret and yield results on water quality in terms of pesticide contamination that potential end users could easily communicate to external audiences.
- An indicator should be based on the type of macroinvertebrate data already available from GQA biomonitoring procedures, to help minimise costs. Further, an indicator should have the ability to be applied quickly in the field to give a first indication of potential pesticide contamination.

3 Current biological indicators of pesticide contamination

3.1 Available indicators to diagnose pesticide contamination

A review was undertaken to identify current indicators of pesticide contamination based on macroinvertebrates. Questionnaire responses showed that macroinvertebrates (and community composition in particular) are considered the most relevant endpoint in terms of pesticide effects. In the following chapters, the term pesticide generally stands for insecticides (and fungicides to some extent), because these compounds have a much higher potential to act on macroinvertebrates than herbicides.

The review included indicators of pesticide contamination ranging from sub-organism to community level, as well as techniques currently used to establish non-pesticide related biological impairment or environmental gradients, but which could be adapted for pesticide contamination.

The results of the review are presented in the following sections. The first two sections describe (potentially) pesticide-specific approaches that operate at the sub-organism (Section 3.2) and community level (Section 3.3). Section 3.3 is split into two subsections on taxonomy-based and trait-based approaches. In a third section (Section 3.4), approaches are presented that are not designed for pesticides, but that may be adapted to diagnose pesticide contamination with additional future work.

3.2 Pesticide-specific approaches at the sub-organism level

Biomarkers can be defined as biological responses to an environmental chemical at either the individual or cellular level, which indicate a departure from the “normal” status (Walker *et al*., 2006). Biomarker assays can indicate sub-lethal responses to toxicants at the molecular, cellular or tissue level and have been classified broadly as biomarkers either of exposure or of effect. Biomarkers of exposure will indicate that an organism has been exposed, but cannot offer information on the impact of this exposure, whereas toxic effect biomarkers will show what systems have been affected and may indicate modes of action. Ideally, biomarker responses will reflect higher levels of organization where, for example, population growth rate is impacted with a subsequent impact on communities or ecosystems. If this is achieved, biomarkers can be predictive, but this is often not the case. Biomarkers more frequently operate at the individual level and are diagnostic of exposure, and sometimes effect. Widely used in fish, biomarkers are becoming increasingly available for aquatic invertebrates.

Most biomarkers have been developed on the basis of correlations and laboratory tests and currently cannot indicate ecological functions. In the field, they have mainly been deployed in marine systems, where the development of biomarkers is arguably well ahead of freshwater systems. However, most research has focused on fish, and the invertebrate organisms used are often bivalve molluscs, which prevents direct predictions from that body of work. An important impact of pollutants on marine and estuarine organisms, that is not relevant to freshwater systems, is on osmoregulation.

A review of molecular biomarkers was undertaken to explore the use of different methods and their capacity to measure the impact of pesticide contamination in freshwater systems. It was not within the scope of the review to produce a detailed overview of all biomarkers developed, since many reviews are already available (such as Mitchelmore and Chipman, 1998; Snyder, 2000; Lesser, 2006; Monserrat *et al*., 2006). Instead, the review focused on freshwater macroinvertebrates, with some reference to work with vertebrates and marine invertebrates, since some of the more recent developments in DNA microarrays have been with fish. Many studies have concentrated on proof of concept in developing and testing biochemical systems, but such biomarkers have often not been tested under field conditions.

3.2.1 Biochemical biomarkers

The biochemical biomarkers considered are summarised in Table 3.1, which shows their main use, specificity and if they have elicited a response with pesticides. Mixed-function oxidases, glutathione S-transferases and heat shock proteins all have low specificity and are suited as biomarkers of general cellular stress. All respond to pesticide exposure in some instances. Catalase and other markers of oxidative stress, carboxylesterases and steroid metabolism are more specific in the stress they respond to and in some cases can point to the toxicant, which could be a pesticide.

Table 3.1: Biochemical biomarkers, their main use, specificity to pollutant type, and published freshwater macroinvertebrate responses to pesticides

Biomarker	Main use	Specificity	Response of freshwater macroinvertebrates to pesticides
Mixed-function oxidase	Indicates exposure to organic chemicals, PAHs, PCBs	Low	Organochlorines, pyrethroids
Glutathione S-transferases	Indicates exposure to pesticides and metals	Low	Organophosphates, organochlorines
Acetylcholinesterase	Exposure to organophosphates and carbamates	High	Organophosphates, carbamates
Catalase	Indicates oxidative stress	Medium	Pyrethroids
Heat shock proteins	Indicates general stress	Low	Organochlorines, herbicide atrazine
Carboxylesterase	Exposure to pyrethroids and carbamates	Medium	Organophosphates, carbamates
Steroid metabolism	Exposure to endocrine disruptors	Medium	Fungicide, growth regulator

Acetylcholinesterase (AChE) inhibition is fairly specific to organophosphate and carbamate pesticides, although some inhibition has been recorded with heavy metals and detergents. None of the biochemical biomarkers (apart from AChE) could identify a specific pesticide class, and there have been only a few examples of large-scale testing of biochemical biomarkers with a wide group of chemicals to determine specificity or measure responses in the field.

Some of the biochemical biomarkers considered in this review could be used in an integrated system incorporating the simultaneous use of multiple molecular biomarkers designed to quantify known physiological responses to stressors. Integrated assessment systems could reveal whether an organism is physiologically stressed, has physiologically acclimatised or has evolutionarily adapted to a chronic stress, and would establish the physiological impact of the stress (Downs *et al*., 2001).

3.2.1.1 Mixed function oxidases (cytochrome P450 monooxygenases) - Phase I responses

Cytochrome P450-dependent monooxygenases (also known as mixed-function oxidases) are an extremely important metabolic system and are involved in the metabolism of many foreign compounds (Scott, 1999). They have a broad spectrum of substrates, preferring fat-soluble compounds including many pesticides. The activity of CYP450s in insects and crustaceans has been extensively studied because they have a role in pesticide resistance (James and Boyle, 1998; Snyder, 2000; Scott and Wen, 2001). Pesticides are often metabolised by cytochrome P450 monooxygenases (CYP450) as the first step in their breakdown, which in the case of organophosphates activates them (for example, malathion to malaoxon).

There have been a number of attempts to measure CYP450 activity in invertebrates in response to specific xenobiotic exposure. However, most successes have been with detecting polyaromatic hydrocarbons (PAHs). Of the studies investigating CYP450 activity in freshwater macroinvertebrates exposed to insecticides, one found no effect of parathion in *C. riparius*, (Sturm and Hansen, 1999) whereas in the same organism, another measured a nearly three-fold induction by permethrin (Fisher *et al*., 2003). In *C. tentans*, CYP450 activity was induced slightly (1.5 times) after exposure to atrazine. Induction of CYP450 by atrazine increases the production of organophosphate oxons and hence increases the inhibition of acetylcholinesterases (Miota *et al*., 2000). The low response of CYP450 enzymes in freshwater macroinvertebrates is not promising as a tool for monitoring. The lack of substrate specificity for individual CYP450 enzymes means that the biochemical method could not be used to identify specific pesticides. However, recent developments to isolate and clone the multitude of CYP450 genes offers the possibility of studying individual responses in genes which react specifically to particular pesticides.

3.2.1.2 Glutathione S-transferases – Phase II reactions

Glutathione S-transferases (GSTs) belong to a large family of phase II detoxification enzymes in insects and crustaceans (Livingstone, 1998; Vontas *et al*., 2000). They catalyse the conjugation of phase I metabolites (reduced glutathione, product of CYP450 metabolism) and compounds such as pesticides that have electrophilic centres (such as PAHs, organophosphates and organochlorines) (Callaghan *et al*., 2002). This, in general, reduces toxicity and increases solubility so that these compounds can be more readily excreted.

GSTs are a diverse group of enzymes with widely differing specificities and are well characterised in insects, because they have been shown to be responsible for insecticide resistance (Wang *et al*., 1991; Lagadic *et al*., 1993; Usui *et al*., 1997; Willoughby *et al*., 2006). GSTs seem to have some utility as biomarkers of pesticides in crustaceans (Table 3.2). In the freshwater crustacean *Gammarus pulex*, the use of a GST biomarker was found to be as sensitive to pesticide exposure as the standard feeding inhibition assay (McLoughlin *et al*., 2000). GST induction in response to fenitrothion and endosulfan was observed in the Signal Crayfish (*Procambarus clarkia)* (Blat *et al*., 1988). However, variable results were obtained with the non-biting midge *Chironomus riparius* when measuring GST activity in response to pesticide exposure (Callaghan *et al*., 2001; Hirthe *et al*., 2001; Forcella *et al*., 2007). Similar results were obtained in *C. riparius* exposed to the organophosphates chlorpyrifos (Callaghan *et al*., 2001) and pirimiphos methyl (Crane *et al*., 2002), whereas Forcella *et al.* (2007) found inhibition of GST activity in *C. riparius* following exposure to the organophosphate fenitrothion, but no change with carbamates. In another study, a significant increase in GST activity at 10 $\mu g\ l^{-1}$ pirimiphos methyl was observed in *C. riparius* at 3°C, but not at

12°C or 22°C, suggesting that GST induction in chironomid larvae may be influenced by environmental parameters such as temperature (Callaghan *et al*., 2002).

Table 3.2: Summary of GST biomarker studies for pesticides using freshwater macroinvertebrates

Species	Pesticide	Response	Reference
Chironomus riparius	Lindane	Negative	Hirthe *et al*., 2001
	Chlorpyrifos	Negative	Callaghan *et al*., 2001
	Lindane	Negative	Kheir *et al*., 2001
	Pirimiphos methyl	Negative	Kheir *et al*., 2001
	Permethrin	Negative	Kheir *et al*., 2001
	Zinc	Negative	Kheir *et al*., 2001
	Pirimiphos methyl	Negative	Crane *et al*., 2002
	Fenitrothion	Positive	Forcella *et al*., 2007
	Pirimiphos methyl	Positive	Callaghan *et al*., 2002
Gammarus pulex	Lindane	Positive	McLoughlin *et al*., 2000
Lymnaea (now *Stagnicola*)* *palustris*	Hexachlorobenzene	Negative	Baturo and Lagadic, 1996
	Atrazine	Positive	Baturo and Lagadic, 1996
Procambarus clarkii	Fenitrothion	Positive	Blat *et al*., 1988
	Endosulfan	Positive	Blat *et al*., 1988
Sphaerium corneum	Lindane	negative	Looise *et al*., 1996
	Dieldrin	Negative	Looise *et al*., 1996

Baturo and Lagadic (1996) used freshwater pond mesocosms to validate the use of GST as a biomarker of contamination by atrazine and hexachlorobenzene in the gastropod, *Lymnaea* (now *Stagnicola*) *palustris* (Müller). Whereas hexachlorobenzene had no relevant effects on GST activity, atrazine markedly inhibited GSTs at concentrations which had no effects on growth or reproduction. The response was dose-dependent. In contrast, freshwater bivalve *Sphaerium corneum* exposed to sediments spiked with lindane and dieldrin showed no significant change in GST activity, even at very high concentrations of pesticide (Looise *et al*., 1996).

GSTs clearly have a role in xenobiotic metabolism and excretion in cladocerans and induction of GST activity may help confer resistance to some toxicants (LeBlanc and Cochrane, 1985; Rey *et al*., 2000). Yet GSTs have not been successfully developed as biomarkers in aquatic insects, although this could be due to the dearth of studies in this area, since genomic studies of GSTs in mosquitoes and *Drosophila*, for example, are highly developed (Ranson *et al*., 2005; Willoughby *et al*., 2006).

3.2.1.3 Acetylcholinesterase inhibition

AChE is the recognised target site of organophosphate and carbamate insecticides. This enzyme is important in nerve transmission; nerve impulses travel down the pre-synaptic cholinergic nerve axon, provoking the release of acetylcholine (ACh), which then crosses the synaptic cleft and binds to the acetylcholine receptor (AChR) triggering excitation of the post-synaptic neuron. AChE terminates the process by breaking down ACh into acetate and choline. Most organophosphate and carbamate insecticides mimic ACh but remain irreversibly bound to the enzyme.

The inhibition of AChE activity by insecticides has been investigated in a number of aquatic invertebrate species. In many papers, the authors were testing the use of AChE as a biomarker to detect cholinesterase-inhibiting pesticides. In *Chironomus riparius* a suite of biomarkers, including AChE, were applied along with measurements

of metabolites (including alanine, pyruvate and lactate) (Forcella *et al*., 2007). When midges were exposed to a number of organophosphates and carbamates, AChE was universally inhibited. Metabolic analysis found that alanine metabolites increased following fenitrothion exposure, whereas no change was observed after carbamate exposure. The authors proposed metabolic product accumulation along with AChE activity as a new biomarker to identify organophosphates. Some authors suggest that AChE is also inhibited by heavy metals (such as Lagadic *et al*., 1994). However, not all studies (especially those on freshwater invertebrates) find this result.

AChE is clearly considered to be a good potential biomarker, since it has been tested in a few freshwater field studies. These studies, however, highlight the difficulty in extrapolating results to the field. Although both AChE and carboxylesterase activity in water slaters (*Asellus aquaticus)* deployed at sewage waste treatment outlets were significantly inhibited compared to those from reference sites, the lack of information concerning the actual pesticide present make it difficult to claim success (O'Neill *et al*., 2004). This will always be a problem with the AChE assay and with any other assay, since an assay provides a measure of pesticide effect when no pesticide remains to be measured. A second study used the crayfish *P. clarkia* to survey agricultural runoff into Doñana National Park in Spain (Vioque-Fernandez *et al*., 2007). Although inhibition indicated the presence of organophosphates and carbamates, there was no correlation between pesticide concentrations at different sites and the extent of esterase inhibition.

The AChE assay is relatively easy to use and is extremely sensitive to organophosphate and carbamate exposure. Analysis of the literature reveals AChE as a reliable biomarker of organophosphate and carbamate exposure; non-organophosphate insecticides rarely have an effect. Pesticides such as the herbicide atrazine probably have an indirect effect in low levels of organophosphate pollution by inducing P450 activity, which converts organophosphates to their more toxic oxons. Sibley *et al*. (2000) demonstrated in a microcosm study that AChE activity could be used as a reliable biomarker of exposure and mortality at the individual organism level and had the potential to predict responses at the population level for zooplankton.

Although only freshwater invertebrate data were analysed here, AChE inhibition in both vertebrates and invertebrates is a sensitive assay for detecting cholinesterase inhibitors in the field. There are examples where activity has altered between sites and over seasons in the same organism, but these could be controlled by first studying natural variation in activity. In some instances AChE has responded to heavy metal and surfactant treatment, but these confounding responses could be eliminated by further investigating this in the laboratory if it is deemed a problem.

Table 3.3: Recent publications testing AChE biomarkers in freshwater or estuarine invertebrates.

Species	Chemical		Reference
Chironomus riparius	AChE inhibitor	Carbaryl	Forcella *et al*., 2007
		Carbofuran	Forcella *et al*., 2007
		Chlorpyrifos	Fisher *et al*., 2000, Callaghan *et al*., 2001
		Fenitrothion	Choi *et al*., 2002
		Fenitrothion	Forcella *et al*., 2007
		Pirimiphos methyl	Kheir *et al*., 2001
		Pirimiphos methyl	Callaghan *et al*., 2002, Crane *et al*., 2002
		Pirimiphos methyl	Maycock *et al*., 2003
	Other pesticide	Lindane (no effect)	Kheir *et al*., 2001
		Permethrin (no effect)	Kheir *et al*., 2001
		Lindane (no effect)	Maycock *et al*., 2003
	Non-pesticide	Zinc	Kheir *et al*., 2001
Corbicula fluminea	AChE inhibitor	Acephate	Moulton *et al*., 1996
		Aldicarb	Moulton *et al*., 1996
Daphnia magna	AChE inhibitor	Acephate	Printes and Callaghan, 2004
		Aldicarb	Sturm and Hansen, 1999
		Chlorpyrifos	Printes and Callaghan, 2004
		Dichlorvos	Sturm and Hansen, 1999
		Malathion	Printes and Callaghan, 2004
		Paraoxon	Guilhermino *et al*., 1996a,b
		Parathion	Printes and Callaghan, 2003
		Parathion	Guilhermino *et al*., 1996a,b
		Parathion-methyl	Sturm and Hansen, 1999
		Propoxur	Printes and Callaghan, 2004
	Non-pesticide	Surfactants	Guilhermino *et al*., 2000
		Cadmium (no effect)	Guilhermino *et al*., 1996a,b
Elliptio complanata	AChE inhibitor	Profenofos	Abdullah *et al*., 1994
Gammarus fossarum	AChE inhibitor	Fenitrothion	Kuhn and Streit, 1995
		Parathion-methyl	Kuhn and Streit, 1995
Gammarus pulex	AChE inhibitor	Fenitrothion	Kuhn and Streit, 1995
		Parathion-methyl	Kuhn and Streit, 1995
		Pirimiphos methyl	McLoughlin *et al*., 2000
	Other pesticide	Lindane (no effect)	McLoughlin *et al*., 2000
		Permethrin (no effect)	McLoughlin *et al*., 2000
	Non-pesticide	Zinc (no effect)	McLoughlin *et al*., 2000
Gammarus tigrinus	AChE inhibitor	Fenitrothion	Kuhn and Streit, 1995
		Parathion-methyl	Kuhn and Streit, 1995
Palaemonetes intermedius	AChE inhibitor	Chlorpyrifos	Key *et al*., 2003
		Diazinon	Key *et al*., 2003
		Malathion	Key *et al*., 2003
Paratya australiensis	AChE inhibitor	Profenofos	Abdullah *et al*., 1994
Procamarus clarkii	AChE inhibitor	Various organophosphate/ carbamate mixtures	Vioque-Fernandez *et al* 2007
Tigriopus brevicornis	Other pesticides	Atrazine	Forget *et al*., 2003

3.2.1.4 Catalase

Catalase (CAT) is an enzyme which protects against the damaging effects of oxyradical generation (oxidative stress). Induction of this enzyme is used as a biomarker to indicate oxidative stress. There are few examples of its use in freshwater invertebrates. The use of CAT activity suffers from many of the same problems found with GST biomarkers; there is a seasonal variation related to temperature and oxidative stress (Sheehan and Power, 1999). Seasonal trends in CAT activity were observed in the freshwater clam *Corbicula fluminea* inhabiting a lake (Vidal *et al*., 2002). The usefulness of antioxidant parameters as biomarkers of exposure to pollutants was evaluated in a river receiving domestic and industrial sources of pollution using caged freshwater bivalves (*Unio tumidus)* (Cossu *et al*., 1997). A suite of biochemical enzymes associated with oxidative stress, including CAT, were analysed. After 30 days of exposure, while most enzymes remained inhibited, there was a significant induction of CAT activity. A more direct study of the impact of deltamethrin found a reduction in CAT activity in the freshwater fish, *Channa punctatus* (Sayeed *et al*., 2003). This method shows promise as a biomarker of oxidative stress, but the cause of the stress would be difficult to determine.

3.2.1.5 Steroid metabolism

Testosterone metabolism can be used to detect endocrine-disrupting pesticides. Testosterone undergoes biotransformation to polar and non-polar metabolites, which can be separated and quantified using thin layer chromatography, mass spectrometry (Verslycke *et al*., 2003) or, if the testosterone is radiolabelled, liquid scintillation counting (Baldwin and LeBlanc, 1994). Both phase I and II metabolism in crustaceans is vulnerable to perturbation by pesticides (Baldwin *et al*., 1997; Oberdörster *et al*., 1998; Verslycke *et al*., 2002). A number of pesticides have been shown to alter testosterone metabolism in crustaceans (Parks and LeBlanc, 1996; Verslycke *et al*., 2004). However, the suggested method of measurement involves CYP450s and would therefore be subject to all the issues discussed above.

3.2.1.6 Heat shock proteins

Heat shock proteins (HSP) are also known as molecular chaperones. One of their main functions is to refold proteins that have been denatured by stress (Ashburner, 1982). There are many families of genes that code for HSP but the most important are the HSP70s, which have been found to respond to stress in all the animals tested so far. A number of studies have investigated the use of HSP70 expression as a biomarker of exposure to toxins (Pyza *et al*., 1997; Lee *et al*., 2006). There have been technical problems in using this protein since the immunological methods that detect HSP70 also detect other HSP proteins, which could mask the signal. There is also considerable variation in the expression of the HSP70 protein related to developmental stage, age and other environmental conditions (Pyza *et al*., 1997).

Most attempts to use HSP70 as a biomarker have studied the effect of metals, not pesticides (Karouna-Renier and Zehr, 2003; Arts *et al*., 2004; Piano *et al*., 2004). Although researchers have studied the induction and accumulation of HSP70 following exposure to various pollutants, there has been only limited application of HSP70s to environmental biomonitoring. A recent study by Lee *et al*. (2006) looked at the effect of a large number of stressors, including chloropyriphos, fenitrothion, endosulfan, and paraquat dichloride on HSP70 expression in *C. tentans*. They found evidence of a

significant increase in expression in most instances, but so many of the stressors elicited a response, including heavy metals and carbon tetrachloride, that the test would be little use as a biomarker for a specific chemical.

3.2.2 Molecular (DNA) biomarkers

Molecular techniques such as DNA microarray-based technologies and primer-based reverse transcriptase-polymerase chain reaction (RT-PCR) can be used to characterise the expression of individual genes and/or gene families in populations exposed to pesticides. RT-PCR in 96 well microtitre plates offers the ability to assay several genes at once, whilst microarrays have the potential to measure every gene in the genome.

Stressor-specific signatures in gene expression profiles potentially offer a diagnostic approach to identifying pollutants (Snell *et al*., 2003), assuming that exposure to toxicants will transiently alter gene expression at a detectable level. Interpretation is dependent on prior calibration and an understanding of stress pathways in the test organism. If successful, this approach offers a single assay which, based on unique fingerprints, could identify the toxicant and the stress pathways linked to responses at a higher level of organisation.

The molecular biomarker approach (nucleic acid DNA, RNA) to characterising detoxification enzymes has advantages over biochemical biomarker substrate-based induction methods in that problems associated with isolating and purifying individual enzymes and measuring enzyme activity can be avoided. Where a suite of biochemical biomarkers would require a large amount of material and multiple tests, a single gene expression profile test would be sufficient to determine stressor type and potential impacts on the individual.

A DNA microarray is a glass slide to which a collection of DNA fragments has been attached. Ideally, the DNA microarray has fragments of every gene in the organism's genome. The microarray is used to measure the expression of genes in test organisms. Genes control the production of proteins by generating copies of themselves, called RNA molecules, which move into the cell and make proteins using cellular machinery. At the time of measurement, some genes will be turned on to make RNA copies of themselves and manufacture proteins, and other genes will be turned off. The microarray measures how many of these RNA molecules have bonded to their corresponding genes (DNA fragments) on the microarray. An experiment is carried out with two treatments, where one set of *Daphnia* is exposed to a stressor and the other is not. Following RNA extraction, the RNA molecules are made visible by the attachment of a fluorescent dye. One treatment is labelled with a red dye and the other with a green dye. When the labelled RNA attaches to the corresponding gene on the chip, a coloured dot is seen. If a gene is expressed more in the green treatment, the dot appears to be greener. DNA microarrays can therefore indicate whether a gene is up- or down-regulated in response to the stressor.

DNA microarrays can characterize mechanisms of action of contaminants through the identification of gene expression networks; identify modes of action for previously uncharacterized toxicants based on comparisons with the molecular signatures of well-characterized toxicants; assess toxicant-induced gene expression as a biomarker of exposure; extrapolate effects of toxicants from one animal species to another; characterize the biological effects of complex chemical mixtures; examine the effects of chronic versus acute exposure to chemicals (Neumann and Galvez, 2002); compare stress responses between animal groups (Gracey *et al*., 2001; Williams *et al*., 2003).

DNA microarrays are available to analyse the impact of natural and anthropogenic factors in model organisms such as yeast, for which whole-genome chips are available (Gasch *et al*., 2000; Causton *et al*., 2001; Momose and Iwahashi, 2001). Causton *et al*.

(2001) analyzed the response of yeast to a number of stressors including temperature, pH, oxidation and nutrients. The stress response was dose-dependent and showed an additive effect for multiple stressors. Further studies with yeast demonstrated a global response as well as stressor-specific responses (Gasch *et al*., 2000) and gene expression responses to agricultural fungicides (Kitagawa *et al*., 2003). This suggests that DNA microarrays have the potential to predict chemical structures that cause major environmental toxicity (Kitagawa *et al*., 2003). More recently, gene expression studies in *Chironomus tentans* larvae have shown that different toxicants (DDT, phenanthrene, fluoranthene, Cd, Cu and Zn) could be distinguished from each other by expression patterns (Perkins *et al*., 2004). Studies in rainbow trout (*Oncorhynchus mykiss*) exposed to a variety of toxicants under different experimental conditions also found evidence of specific expression profiles in response to toxicants (Koskinen *et al*., 2004; Krasnov *et al*., 2005).

Williams *et al.* (2003) led a pilot study to determine the feasibility of applying toxicogenomics (here a 160 cDNA microarray) in a field situation with wild European flounder (*Platichthys flesus).* They compared fish from the clean Alde estuary with those from the Tyne estuary, a site highly polluted with PAHs and heavy metals. Although they observed high individual variability in gene expression, especially in female fish, the authors successfully identified a signature profile consisting of 11 genes that were differentially expressed in the male fish from the contaminated site.

Recently, a detox *Drosophila* microarray (including P450s, GSTs and esterases) was used to measure responses to six insecticides (DDT, lufenuron, dicyclanil, spinosad, nitenpyram and diazinon). Surprisingly, the only insecticide to elicit a gene induction response was DDT. Whilst this is disappointing, the fact that a single P450 and GST gene out of many responded specifically to DDT and not to other insecticides supports the idea that toxicant-specific genes can be identified (Willoughby *et al*., 2006). This is further supported by fish microarray work where endocrine-specific responses have been found (Hoyt *et al*., 2003; Larkin *et al*., 2003a; Larkin *et al*., 2003b). A group of genes were found to be up-regulated by all environmental oestrogens tested, while other genes showed differential expression only in response to a specific compound.

Microarray technology is relatively new in the field of freshwater ecology. However, work on model insects offers insight into the possibilities. The recent annotation of the malaria mosquito *Anopheles gambiae* genome has revealed many genes of enzyme systems used as biomarkers. At least 30 CYP450 genes from the CYP1, CYP4 and CYP6 families have been isolated (Holt *et al*., 2002). The expression of these genes has been investigated on a specific *Anopheles* microarray named the detox chip (David *et al*., 2005). This chip contains 230 fragments from genes known to code for proteins involved in the metabolism of pesticides. This includes 103 P450 fragments, 31 carboxylesterases and 35 GSTs. This type of approach allows us not only to investigate the expression of metabolic genes known to be involved in detoxification of the pesticide, but also to identify previously unknown genes.

Ecotox chips for aquatic freshwater invertebrates are now being produced (Soetaert *et al*., 2006). A microarray containing some 16,000 DNA fragments (approximately 3,000 genes) has been developed for *D. magna* at the University of Reading (http://daphnia.cgb.indiana.edu/projects/stressresponses/) and used to investigate responses to six stressors, including heavy metal, pesticide, drug, oxidative and physical stress (Connon *et al*., submitted; Connon *et al*., in preparation). Response patterns varied for each toxicant; no one gene responded to every stressor, which is promising for the future development of toxicant-specific responses. The Reading Daphnia group are now placing genes into stress pathways and are validating responses using quantitative PCR (QTPCR). The results so far are extremely promising (Heckmann *et al*., 2006) and could offer an alternative to testing with fish.

Genomics, which includes the use of DNA microarrays, QTPCR (transcriptomics) and other genomics responses not discussed in this report (such as proteomics and metabolomics), will help to identify the metabolic pathways involved in xenbiotic detoxification. This should lead to the development of novel and more sensitive biomarkers for ecotoxicological studies. The technology is still a few years away from application but promises a future where detection, identification and impact prediction of a pollution event are possible. One important consideration is how data will be analysed. Databases composed of expression data wil be needed, along with sophisticated bioinformatics tools to screen large datasets for expression profile matches. Several databases are already being developed in toxicogenomics, one being the Chemical Effects in Biological Systems (CEBS) knowledge base (http://cebs.niehs.nih.gov/) (Waters *et al*., 2003). This incorporates toxicogenomics information as well as phenotypic response data, to offer an integrated overview of the molecular responses measured by genomics and pathological effects measured using traditional toxicology.

It is still too early to predict whether microarrays will emerge as the molecular tool of choice in monitoring aquatic pollution. However, their ability to determine the mechanisms of toxicity will certainly bring a better understanding of cause and effect. This, in turn, may help develop targeted assays that not only identify the class of contaminant, but also indicate the likely impact on the population.

3.3 Pesticide-specific approaches at community level

Effect concentrations at the sub-organism level can be lower than effect concentrations at the individual level (Duquesne, 2006) and molecular effects of toxicants may propagate to higher levels of biological organisation. At the individual level, potential effects include increased mortality of invertebrates as well as sub-lethal endpoints like reduced growth or fecundity along with behavioural alterations. These toxicant-induced alterations at the individual level may affect the performance of populations (population growth rate) and can propagate from population to community level. As a result, toxicant stress may change the species composition in communities.

The response of aquatic communities to toxicants is mostly influenced by the physiological sensitivity of members of these communities to toxic compounds. Hence, identifying species that are sensitive to particular types of toxicants is a prerequisite for developing biological indicators of toxicant stress. Certain species are more affected by a particular toxicant than others; their decline in abundance compared to other species within a community indicates the nature and strength of the toxicant. Knowledge of the sensitivity of different species to individual toxicants is needed in order to interpret these changes. However, the structure of each invertebrate community in the field is unique and results from a unique combination of different environmental parameters. This needs to be considered carefully when several field communities are compared to identify indicator species for particular stressors. The taxonomic composition of communities may vary naturally between sites and regions and so may the presence of indicator species. Given that the presence of groups at higher taxonomic level (family or order level) is less dependent on natural environmental gradients, these groups can be used as indicator taxa. However, large variability in stress responses within a given taxonomic group may render a taxon less specific.

Since taxonomic structure may vary between sites and regions, less variable descriptors of communities are necessary. There is increasing evidence that species traits (such as life-history characteristics) in communities are less dependent than taxonomic composition on natural environmental gradients, seasonal variation or sampling success (Charvet *et al*., 2000; Bady *et al*., 2005; Doledec *et al*., 2006)

3.3.1 Taxonomy-based approaches

3.3.1.1 Pesticide Index

The Pesticide Index is based on a list of taxa that are potentially sensitive to pesticides. Its aim is to indicate pesticide contamination of sites based on the absence of such expected taxa (Humpheryes, personal communication).

The taxa list was developed from a comparison of control and test sites in Kent. Control sites typical of the geology of Kent and not showing any effects of environmental stress were analysed with RIVPACS. RIVPACS was used to produce a list of expected taxa for each of the sites and from this list, taxa were identified which had a predicted probability of capture above 50 per cent, but which were not present in the respective site sample. The percentage of total sites from which the taxa were missing was calculated and a ranked list of taxa that were predicted with above 50 per cent probability of capture but missing from site samples was produced (list of control sites).

Test sites were selected based on generally poor biological quality and where the potentially pesticide-sensitive taxa Gammaridae and Asellidae were expected, but had not occurred on two occasions between 2000 and 2006. These sites were also analysed with RIVPACS and a ranked list produced of species predicted with above 50 per cent probability of capture but missing from the samples (list of test sites). Although the absence was expected to result from pesticides, a cause-effect relationship was not established, since levels of pesticide contamination at the sites of poor biological quality were not measured.

The ranked lists for control and test sites were compared for each taxon. The difference in percentage of sites from which the taxon was missing was considered to be related to the sensitivity of the taxon to pesticides; the greater the difference between control and test sites, the more sensitive the taxon. The differences were used to rank the taxa in terms of their sensitivity to pesticides. This taxon list was divided into four groups and each group given a score: four, sensitive to pesticide pollution; three and two, sensitive to organic pollution; one, not correlated to pesticide or organic pollution.

For a given site and season, the Pesticide Index is calculated as follows: RIVPACS is used to obtain a list of expected taxa with probability of capture. The probability of capture is banded into five groups: below or equal to 20 per cent, one; between 20 and 40 per cent, two; between 40 and 60 per cent, three; between 60 and 80 per cent, four; above 80 per cent, five. For each expected taxon absent from the sample, the sensitivity score (one to four) is multiplied by the probability score (one to five) to obtain the individual score. The scores of all taxa within each of the sensitivity groups (one to four) are totalled to give the group score and the four group scores are totalled to give the site score. Current development of the Pesticide Index aims to calculate and calibrate a Pesticide Quality Index. The observed pesticide score per site is related to the total possible pesticide score (calculated by assuming all predicted taxa are absent from a sample). This ratio subtracted from one and multiplied by 100 gives the Pesticide Quality Index that ranges from one to 100, with the lower scores indicating pesticide impact. At present, the Pesticide Quality Index is run for the Kent region and a distribution analysis is done to determine the interquartiles and to band the scores into a range of impacts.

3.3.1.2 PERPEST

Expert systems use the knowledge gained from previously experienced problems to solve new problems. A current system uses the results of long-term (micro)mesocosm studies for the Prediction of the Ecological Risks of PESTicides (PERPEST) on freshwater ecosystems (Van den Brink *et al*., 2002). The PERPEST model indicates if specified community endpoints are expected to be affected by pesticide exposure (prognosis), but it cannot conclude from the status of endpoints the likely pesticide contamination (diagnosis). However, the model was included in the review to assess whether the underlying method could be adopted to diagnose pesticide contamination.

PERPEST employs a case-based method using knowledge about the sensitivity of certain species gained through (micro)mesocosm studies. Underlying the model is a case database, with each case relating to one pesticide concentration tested in one study and to the reported biological effects of the concentration. Considered in PERPEST are one functional endpoint (community metabolism) and seven structural endpoints. Four of the structural endpoints refer to different groups of macroinvertebrates (insects, macrocrustaceans, microcrustaceans, and other macro-invertebrates). The effects are grouped into three classes (class one - no effect, class two - slight effect, class three - clear effect) according to their statistical significance and duration (Brock *et al*., 2000).

Cases belong to the ‘no effect’ class when no effects are observed following treatment. Cases belong to the ‘slight effect’ class when effects are observed only for individual samplings, especially shortly after treatment. Cases belong to the ‘clear effects’ class when sensitive endpoints show a clear response to treatment and effects are observed at subsequent sampling dates. Given a question case, PERPEST searches the underlying database for the most similar cases. Similarity is assessed according to pesticide properties, exposure concentration expressed as toxic units (exposure concentration in relation to the acute LC50 for the most sensitive standard test organism, TU), and type of test ecosystem. According to weighted average effects reported in the most relevant cases, PERPEST predicts the probability of different effect classes to occur at the concentration of the question case.

PERPEST predicts the effects of a given pesticide concentration using results of studies in experimental ecosystems. Thus, predicted effects are more realistic than predictions based on standard laboratory tests. Van den Brink *et al*. (2006) suggest that PERPEST can be used to translate both measured and modelled pesticide concentrations into ecological risks, which is exemplified for atrazine. Further, the authors argue that PERPEST is a valuable model for evaluating the outcome of chemical monitoring programmes (such as those under the Water Framework Directive). However, although the model predicts the probability of effects on eight generalised community endpoints, it cannot predict how individual taxa (family to species level) of a given community will react to pesticide exposure.

3.3.1.3 LIMPACT

LIMPACT is a rule-based system that uses data on macroinvertebrate communities to estimate the level of pesticide pollution in flowing waters (Neumann *et al*., 2003). The system distinguishes four types of contamination classes (not detected, low, moderate and high contamination) and uses rules on the abundance of 39 indicator taxa during four time periods to establish or rule out contamination classes. The rules are applied in a heuristic diagnosis score pattern to score diagnoses (of different contamination classes). Scores reflect specified probabilities to confirm (positive scores) or refute (negative scores) a diagnosis. A diagnosis (contamination class) is established

(confirmed) if the sum of the given scores exceeds 40, that is, where the diagnosis is established with a probability of greater than 80 per cent.

Rules were specified from biological and chemical monitoring data for small lowland streams within agricultural catchments in the region of Braunschweig, Lower Saxony, Germany. Although this means that LIMPACT is specific to German lowland streams, the system was included in the review to see if the underlying methodology could be used to set up an indicator system for water bodies in England and Wales. The rules were established from 157 investigations per stream and year produced from several investigations of 104 streams between the years 1992 and 2000.

Analysis of abundance data from these investigations was carried out with respect to nine water-quality and morphological parameters. The 39 indicator taxa represent the most common taxa that make up 90 per cent of the total abundance of all taxa in the investigations and include taxa from the orders Amphipoda, Coleoptera, Diptera, Ephemeroptera, Gastropoda, Isopoda, Megaloptera, Oligochaeta, Plecoptera, Trichoptera and Turbellaria. Thirteen taxa (including those from the orders Amphipoda, Gastropoda, Oligochaeta and Turbellaria) are used as positive indicator taxa, which indicate contamination by high abundance values and positive abundance dynamics. Twenty-four taxa (including those from the orders Ephemeroptera, Isopoda, Megaloptera and Plecoptera) are used as negative indicator taxa, where a high abundance of these rules out contamination and indicates an uncontaminated site. The four time periods considered in the analysis are: March/April; May/June; July/August; September/October. Classification is only possible if abundance data from all these periods are available.

Until now, the LIMPACT system has only been evaluated using the training set of 157 investigations. This means that no independent evaluation results are available. For the training set, the correct diagnosis for the 157 investigations per stream and year is established by LIMPACT in 67 to 85 per cent of the cases, with better results for uncontaminated sites. In most of the remaining cases, no diagnosis is made instead of an incorrect one.

3.3.1.4 Relative sensitivity concept

The aim of the relative sensitivity concept (Von der Ohe and Liess, 2004) is to rank aquatic macroinvertebrate taxa in terms of their physiological sensitivity to organic and metal compounds. The relative sensitivity concept originates from Wogram and Liess (2001), who assessed physiological tolerance of macroinvertebrates by means of acute median lethal and effect concentrations (one to 96 hour LC50 and EC50) of substances using the endpoints immunity, intoxication, mortality or reproduction (AQUIRE database, US Environmental Protection Agency). The E/LC50 for a particular species and substance was related to the E/LC50 for *Daphnia magna* obtained for the respective substance with the same duration of exposure and the same endpoint. When several E/LC50 values were reported for a particular species and substance, the arithmetic mean E/LC50 was related to the respective value for *Daphnia magna*. The logarithm of that ratio obtained for each species was termed the relative physiological tolerance, that is, the physiological tolerance to a particular compound compared to *Daphnia magna*. Negative values of the relative tolerance to a given compound indicate that a species is more sensitive than *Daphnia magna*; positive values indicate that a species is more tolerant than *Daphnia magna*.

The relative physiological tolerance of an invertebrate order to organic or metal compounds was obtained by calculating the arithmetic mean of the respective relative tolerance values of species from that order. Wogram and Liess (2001) found three invertebrate orders to be more sensitive to organic compounds (including pesticides)

than *Daphnia magna*: Cladocera other than *Daphnia magna*, Amphipoda and Plecoptera. Von der Ohe and Liess (2004) refined the rank ordering by limiting the set of considered toxicity data to LC50 values from a 24-48 hours freshwater laboratory test to increase comparability between datasets for different taxa. Further, the tolerance of taxa was identified at the lowest taxonomical level possible in order to assess the variability of tolerance within orders.

Von der Ohe and Liess (2004) described the relative sensitivity of a taxon to be the logarithm of the E/LC50 for *Daphnia magna* related to the E/LC50 for a taxon, that is, as the log-transformed reciprocal ratio of E/LC50 values introduced by Wogram and Liess (2001). Consequently, a positive value of relative sensitivity indicates that a species is more sensitive to a compound than *Daphnia magna*; negative values indicate that a species is more tolerant than *Daphnia magna*. Unlike Wogram and Liess, Von der Ohe and Liess calculated the relative sensitivity for each test value for a particular species and substance. In the case of multiple test values for one combination of species and substance, the arithmetic mean was calculated for each study. The results of different studies were then aggregated by taking the mean yielding the relative sensitivity to one compound. Per taxon, the values of relative sensitivity to different compounds were aggregated to obtain the overall relative sensitivity to organic or metal compounds. Values of relative sensitivity for higher taxonomic level were obtained by calculating the mean of the relative sensitivity values to particular compounds of taxa below this level. Von der Ohe and Liess (2004) identified similar invertebrate orders to be sensitive to organic compounds as Wogram and Liess (2001).

3.3.2 Trait-based approaches

3.3.2.1 SPEAR

The response of aquatic communities to toxicant exposure is strongly influenced by the physiological sensitivity that members of these communities show to toxic compounds. However, life-history traits also determine how single species, and communities as a consequence, respond to toxicant exposure.

The SPEcies At Risk (SPEAR) concept (Liess and von der Ohe, 2005) combines information on physiological sensitivity to organic compounds according to Wogram and Liess (2001) and Von der Ohe and Liess (2004) with information on life-history traits. This information is used to identify species at risk from pesticides as one group of organic compounds in particular. According to this concept, a species is classified as at risk of being affected by pesticides if it matches all of the following criteria: its physiological sensitivity to organic toxicants including pesticides is equal to or higher than the sensitivity of *Daphnia magna*; it produces two or less generations per year; it is fully aquatic or does not emerge before the main period of agrochemical application in a particular study area (before May as default assumption); and its migration ability is low. If at least one of the criteria is not met, it is assumed that the species can tolerate exposure, can avoid exposure due to early emergence or short-time migration, or can quickly reproduce after exposure. A list of aquatic macroinvertebrate taxa in Central Europe is available in terms of SPEAR (http://www.ufz.de/index.php?en=14348).

A firm link was established between the abundance of SPEAR in relation to overall abundance per site (percentage SPEAR abundance) and measured pesticide levels (insecticides and fungicides) in three field studies conducted in Finland, France and Germany (Liess and von der Ohe, 2005; Schäfer *et al*., 2007). For the studies in Finland and France, the above mentioned species list was updated where ecological

traits of species in Finish or French habitats differed from data collated for Central European conditions. Measured maximum pesticide concentrations expressed in TU (concentration of a compound divided by the related LC50 for *Daphnia magna*) best described the observed variance in percentage SPEAR abundance. Other parameters that contributed slightly to the variability in this abundance were length of forested stream sections, type of stream bed substrate and cover of submerged plants.

An analysis of the pooled data from Finland, France and Germany showed a significant change in community structure at sites characterised by pesticide contamination at a concentration range as low as 1:100 to 1:1,000 of the acute 48 hour LC50 of *Daphnia magna*. Further, a significant decrease in SPEAR from the pre- to main agrochemical application period was observed, while no indication was found that parameters other than pesticides (such as hydrodynamic stress, water quality) might be responsible for the observed short-term reduction in species. This suggested that short-term changes in SPEAR from the pre- to main application period are best attributed to pesticides.

Short-term changes in SPEAR were also observed in a German field study linking governmental inventory monitoring data on stream invertebrate communities to modelled pesticide runoff given by the relative index Runoff Potential (Schriever *et al*., 2007a). The Runoff Potential (RP) is a generic indicator of the magnitude of pesticide inputs into streams via runoff. The underlying model considers key environmental factors affecting runoff (precipitation, topography, land use and soil characteristics), but predicts losses of a generic substance instead of any one pesticide (Schriever *et al*., 2007b). Relative SPEAR abundance decreased significantly during the main period of agrochemical use at sites of high RP, but completely recovered by the following spring. A long-term decrease in percentage SPEAR abundance was also observed at sites of medium to very high runoff inputs; this suggested that long-term alterations in community measures were probably associated with factors related to runoff input. Long-term decrease in other community measures such as diversity or numbers of Ephemoptera, Plecoptera and Trichoptera (EPT) only occurred at high to very high levels of modelled runoff inputs. Therefore, SPEAR appears to be more sensitive than other community metrics.

The relationship between TU and percentage SPEAR abundance observed by Liess and Von der Ohe (2005) in the German field study was tested to predict effects of short-term contamination with the organophosphate parathion-ethyl in an agricultural head water in that region. Based on that exposure-response relationship, the extinction of nearly all SPEAR taxa due to the measured contamination was accurately predicted. The exposure-response relationship was used to predict effects, but could well be applied to diagnose pesticide contamination from biological community data.

3.4 Non-specific approaches

Although the aim of this review was to identify current biological indicators of pesticide contamination, methods not designed to indicate biological impairment due to pesticides were included. These approaches are used to indicate general ecological integrity and impairment, but could be adapted to help diagnose pesticide impacts.

3.4.1 Taxonomy-based approaches to indicate ecological integrity and impairment

3.4.1.1 RIVPACS

RIVPACS, the River Invertebrate Prediction and Classification System, is an empirical model used to predict the expected aquatic macroinvertebrate assemblage assuming that a river site is unimpaired (Wright *et al*., 1998). The predicted assemblage may then be compared with the observed assemblage to derive a variety of biotic indices. Currently, RIVPACS is mainly used to predict values of the BMWP system, specifically the number of expected taxa and ASPT. As the model predicts the likelihood of individual species, the system can be used to predict any biological metric based on the occurrence of specific taxa.

RIVPACS is based on a dataset of approximately 600 minimally impaired river sites in the United Kingdom. These sites were sampled in three seasons of the year (spring, summer, autumn) between about 1978 and 2000. A standard sampling procedure was used to collect all datasets. Samples were processed in the laboratory and identified to the lowest practical level. The RIVPACS database covers all major macroinvertebrate groups including Diptera and oligochaetes.

The RIVPACS model is based on a combination of classification of sites using two-way indicator species analysis (implemented as the programme TWINSPAN) and multiple discriminant analysis to predict the expected probability of invertebrate taxa under specific physical conditions. The prediction of fauna is based on environmental parameters such as slope, discharge, distance from source and substrate particle size which are largely unaffected by water pollution stresses. Sites used to construct the RIVPACS model were chosen to be free from pollution stresses and as physically unmodified as possible, although some were influenced by agriculture. Given a set of environmental parameters from a site under investigation, the model predicts the expected fauna. The model returns a list of expected taxa or predicted BMWP scores (specifically the number of taxa and ASPT score) for a single season or for two or three seasons combined, depending on the needs of the user. These can then be compared to the observed fauna.

RIVPACS is in general use in the United Kingdom as the basis of national river invertebrate monitoring programmes under the General Quality Assessment programme (Wright *et al*., 2000). Similar models have also been developed in Spain and Australia based on the RIVPACS concept (Smith *et al*., 1999; Wright *et al*., 2000).

3.4.1.2 PSYM

PSYM, the Predictive System for Multimetrics, is an empirical model similar to RIVPACS that has been developed by Pond Conservation to assess the ecological quality of ponds and small lakes (Biggs *et al*., 2000). The system predicts the expected macroinvertebrate and wetland macrophyte species of a site and compares this assemblage with the observed fauna and flora. Currently, PSYM is used to calculate an Index of Biotic Integrity based on six biotic metrics: three relate to macroinvertebrates and three to macrophytes. These are combined in a multimetric index to give an overall score. The system currently operates only in England and Wales.

PSYM is based on a dataset of 150 minimally impaired ponds and small lakes in England and Wales and a complementary dataset of 150 variably degraded sites. Minimally impaired sites are used as the basis for predicting expected fauna and flora. All sites (300 minimally impaired and variably degraded sites) were used to develop

biological metrics related to factors known to cause impairment of sites (such as nutrient pollution, intensive land use, heavy metals, overstocking with fish). Currently the method uses only summer season data: this is not particularly disadvantageous because all sites must be visited in summer to obtain plant data. However, later versions may include more seasons of invertebrate data. Sites were originally surveyed between 1990 and 1998. A standard sampling procedure was used to collect all datasets. Invertebrate samples were processed in the laboratory and identified to the species level in major macroinvertebrate groups, with the main exceptions of Diptera and oligochaetes.

The PSYM model links a multimetric approach to the predictive approach originally developed in RIVPACS. Metrics were initially identified using the combined minimally impaired and variably degraded datasets; the prediction stage of the programme is based on a combination of classification of sites using two-way indicator species analysis (implemented as the programme TWINSPAN) and multiple discriminant analysis to predict the expected probability of species under specific physical conditions. The prediction of fauna is based on environmental parameters such as water body location in England and Wales, size, amount of shade and substrate composition and other parameters which are largely unaffected by water pollution stresses. Invertebrate fauna are predicted in terms of species, but converted to family-based metrics to facilitate use of the model. At present it is necessary to include pH as a predictor, although in later implementations it is hoped that pH can be substituted for a non-variant parameter that is not affected by pollution. Sites used to construct the PSYM model were chosen to be as free from pollution stresses as possible, being located in catchments dominated by semi-natural land use, all minimally impaired sites having 80 per cent or more semi-natural land use in a zone 0-100 m from the pond.

To make a PSYM assessment, the user enters physical variables into the model, which predicts the expected fauna and flora. Biological data from the site (such as invertebrate and plant metrics) are then entered and the model returns the PSYM result, including the values of individual metrics. PSYM is publicly available free of charge in the United Kingdom for use in national pond and small lake monitoring programmes. It can be accessed through the National Pond Monitoring Network (www.pondnetwork.org.uk/main/default.aspx) or, for batches, samples are analysed by the Pond Conservation free of charge for non-commercial work. At present, reporting is largely informal because no statutory national monitoring programme is underway. With the current number of sites on which predictions are based (150), the model is probably best regarded as a beta version, which will need further sites added in due course.

3.4.1.3 RPDS

RPDS, the River Pollution Diagnostic System, is a computer-based system for maximising the information gained from existing river invertebrate monitoring data (and associated environmental data) (Walley *et al*., 2002; O'Connor and Walley, 2002). The system currently indicates how similar new or existing sites are, in terms of their invertebrate fauna, to all other sites in the database. The similarity of the assemblage to other sites in terms of specific families can also be visualised. For specific sites or groups of sites, a histogram-based summary of the physicochemical and biological characteristics can be generated. All available information about a specific site can also be visualised. Maps of the site locations can be generated.

RPDS operates by simulating one of the two complementary processes used by specialists in the interpretation of environmental data. These processes are (i) plausible reasoning based on scientific knowledge and (ii) pattern recognition based on past personal experience. RPDS simulates the process of pattern recognition using a process that groups samples into clusters based on similarities in their biological or

physicochemical composition. The pattern recognition process used in the RPDS system is known a Mutual Information maximisation (MI-max), developed by the Centre for Intelligent Environmental Systems (CIES) at University of Staffordshire (http://www.cies.staffs.ac.uk), which is based on mathematical information theory.

RPDS was constructed using family-level invertebrate data from the 1995 UK General Quality Assessment programme that collected data from over 6,000 sites around the UK. Each family was recorded at four abundance levels. From these sites, 250 clusters of biologically similar sites were generated. Limited physicochemical data are available for all of these sites within the model, but no pesticide measurements are included due to lack of data. Clusters can be described by their physicochemical characteristics and the expected environmental characteristics of new sites predicted from biological data alone, based on the similarity of the new site to existing clusters. RPDS can thus predict expected water quality from the observed biological assemblage.

In order to adapt the RPDS for pesticide risk assessment, a 'training dataset' would be required with biological data from sites with concentrations of as many pesticides as possible. Alternatively, the RPDS may have a role in exploratory analysis. For example, the tool could help to identify sites with relatively high BMWP scores, but lacking taxa indicative of pesticide impacts. RPDS is used mainly by invertebrate biologists at the Environment Agency, and is not generally available outside the Environment Agency.

3.4.1.4 RPBBN

RPBBN, the River Pollution Bayesian Belief Network, is a computer-based system to diagnose and predict the invertebrate fauna expected under certain physicochemical conditions (Walley *et al*., 2002). It was developed by CIES at the University of Staffordshire in conjunction with the RPDS. RPBBN is currently based on invertebrate data from the 1995 General Quality Assessment survey in the United Kingdom and associated physical and chemical data. The RPBBN uses input observational data on taxon abundance to predict the level of chemical variables (such as pH, total oxidised nitrogen, oxygen saturation) consistent with the invertebrate data.

RPBBN is a combined Bayesian Belief Network (BBN) and user interface that provides a probabilistic reasoning tool for water managers, especially biologists. BBNs consist of a series of nodes which, given some information, can generate likelihoods of each node having a specific value. Each node in the network is linked to other nodes and the probability of the dependent node having a particular condition is based on (i) input data and (ii) expert experience. Thus, the BBN can answer 'what if' questions about changes in specific nodes under differing conditions. Specifically, the RPBBN can be used to model scenarios such as 'What if the dissolved oxygen concentration in a river is increased?' and 'Which invertebrate taxa will benefit from this?'.

At present, the RPBBN is under evaluation in the Environment Agency. It does not currently include pesticide data, so cannot be used to predict or model the effects of pesticides. In order to adapt the RPBBN for pesticide risk assessment, a 'training dataset' would be required with biological data from sites with associated concentrations of as many pesticides as possible.

4 Critical assessment of reviewed approaches

4.1 Methodology

A critical assessment and comparison of reviewed approaches was carried out.

Firstly, each approach was assessed in terms of its major features (Table 4.1) and its ability to meet the end users' requirements (Table 4.2). The applicability of each approach was further assessed against current Environment Agency procedures, to check whether it was already used, could be easily added to current techniques with minor changes in practice, or could only be adopted with major revision of current practice (Table 4.3). The details of this assessment are given in Appendix II.

Approaches were then divided according to their diagnostic abilities into a group that can or could be used as diagnostic tools, and a group of non-diagnostic approaches (see Figure 4.1).

Thirdly, approaches that can or could be used as diagnostic tools were compared with respect to their ability to meet end users' requirements (Table 4.4). The comparison included a scoring method assigning scores to indicate an ability or inability to meet a particular requirement. Meeting a particular requirement scored one, while not meeting the requirement scored zero; when the approach met the requirement to some extent or had the potential to meet it, it scored 0.5. The sum of scores indicated the overall ability of each approach to comply with the end users' needs.

For biochemical and molecular biomarkers, a score of one was subtracted from the sum of scores, because these approaches still lack field testing. For community-based approaches, a score of 0.5 was subtracted from the sum when the approach had already been applied under field conditions, but needed revision with a training dataset of measured pesticide and biological data in order to be applicable in England and Wales.

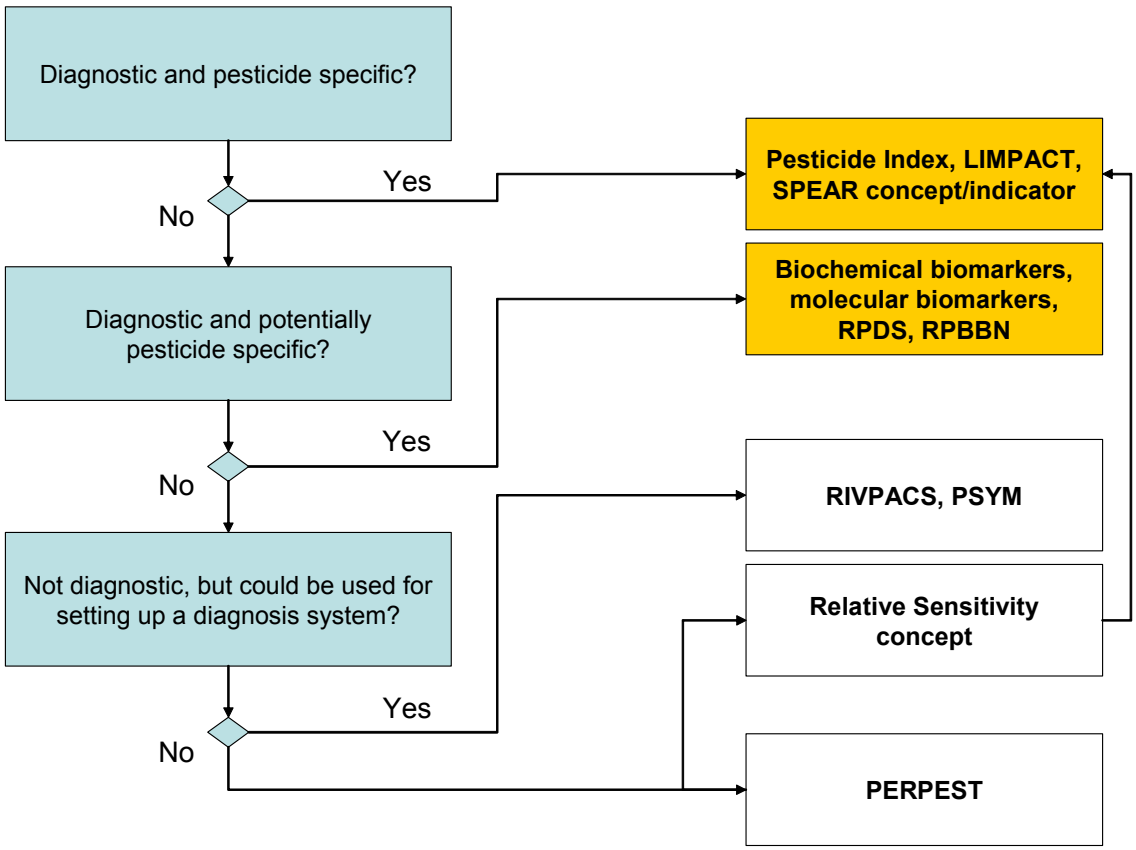

Figure 4.1: Overview of reviewed approaches

Table 4.1: Major features of reviewed approaches

Features		Explanation
General	Category	e.g. community level or taxonomy-based
	Type	e.g. existing indicator of pesticide contamination or classification system
	Aim	e.g. to identify pesticide contamination or pesticide sensitive taxa
	Concept	qualitative or quantitative
	Endpoint 1	type of organism, e.g. freshwater macroinvertebrates
	Endpoint 2	e.g. sub-organism level or community level
	Methodology	e.g. case-based expert system or empirical scoring approach
	Underlying data	e.g. results of standard acute toxicity tests or field-monitoring data
	Input data	data that are required to assess a site (e.g. level of identification)
	Specificity to different modes of actions	e.g. to acetylcholinesterase-inhibiting compounds
	Software required – software existing	—
Successful field evaluation?	Geographical region	—
	Tested water body type	e.g. stream or ditch
	Number of test sites	—
	Sources of pesticide pollution	e.g. field crops
	Type of pesticide pollution	e.g. organophosphates or carbamates
	Reliability	i.e. confidence for diagnosing cause and effect
	Robustness	i.e. the signal to noise ratio
	References	—
General comments		—

Table 4.2: End user requirements for a freshwater biological indicator of pesticide contamination

Requirements	Explanation
Is or can be a diagnostic tool to identify pesticide contamination	—
Can distinguish pesticides from other stressors	e.g. from nutrient enrichment or temporary droughts
Is sensitive to subtle changes	e.g. sensitive to changes in community composition, while diversity is still reasonable
Can indicate trends over time (years)	i.e. shifts in contamination levels over a period of several years e.g. due to changes in agricultural practice or management measures
Can indicate the relative order of magnitude of pesticide contamination	—
Can indicate the time period of contamination	i.e. if observed effects result from a recent contamination or from contamination during previous years
Can compare levels of pesticide contamination between different regions in the UK	—
Can be an early warning system	e.g. by indicating effects of pesticide contamination on the genetic structure of populations
Is robust and objective	i.e. scientific background and validation
Is transparent	i.e. principles and calculations are easy to understand for non-specialists
Can give a good summary	—
Results are easy to interpret	—
Is quick	i.e. can be applied already in the field to give some indication of potential pesticide contamination
Is cheap	e.g. low costs for lab material or no sampling necessary in addition to GQA data

Table 4.3: Reviewed approaches in terms of current Environment Agency procedures

Assessment in terms of current Environment Agency procedures	Explanation
Is currently used	i.e. implemented in current procedures
Is ready to use for diagnosis	i.e. is ready to be implemented
Missing information	i.e. information that is a prerequisite for implementing the approach
Could be easily added to current procedures	i.e. could be added with minor changes in practice
Could only be adopted with major revision of current practice	—

Table 4.4: Reviewed approaches that can/could be used as a diagnostic tool to identify pesticide contamination

		Biochemical biomarkers	DNA biomarkers/ microarrays	Pesticide Index	LIMPACT	SPEAR concept/ SPEAR-based indicator	RPDS/RPBBN
Features	Biological level	Molecular techniques	Molecular techniques	Community, taxonomy-based approach	Community, taxonomy-based approach	Community, trait-based approach	Community, taxonomy-based approach
	Methodology	Detecting enzyme activity responding to toxin-induced stress	Detecting changes in gene expression responding to toxin-induced stress	Scoring approach based on empirical list of pesticide sensitive species	Expert system, heuristic, rule-based	Concept: identifying pesticide-sensitive species. Indicator: linking exposure and response	Expert systems, pattern recognition (RPDS: clustering; RPBBN: reasoning, also prognostic)
	Designed for pesticides?	Can be designed to detect pesticides	Can be designed to detect pesticides	Yes	Yes	Yes	No
	Clear link between response and exposure?	AChE: Yes, for organophosphates and carbamates	Potentially, but link still needs to be established	No	Yes	Yes	N/A
	Currently used in EA procedures?	No	No	No	No	No	Partially (not for pesticides)
Requirements	Diagnostic tool for pesticide contamination?	(1) AChE: Yes, to detect effects and identify the toxicant	(0.5) Potentially, to detect effects and identify the toxicant	(1) Yes, to identify levels of contamination	(1) Yes, to identify levels of contamination	(1) Indicator: Yes, to identify levels of contamination	(0.5) Potentially if empirical pesticide data were available
	Indicate trends over time	(0) No, there are no long-term effects	(0.5) Difficult, could indicate changes in expression patterns	(1) Yes	(0) No	(1) Indicator: Yes	(0.5) Potentially if empirical pesticide data were available
	Sensitive to subtle changes due to pesticides?	(1) AChE: Yes	(0.5) Potentially	(0) Unlikely	(1) Yes, since rules are based on subtle changes	(1) Indicator: Yes	(0.5) Potentially if empirical pesticide data were available
	Can identify magnitude of contamination?	(1) Qualitative	(0.5) Potentially, qualitative and quantitative	(1) Qualitative	(1) Quantitative	(1) Indicator: Quantitative	(0.5) Potentially if empirical pesticide data were available
	Can identify type of contaminant?	(1) AChE: Yes, can identify organophosphates and carbamates	(0.5) Potentially	(0) Unlikely	(0) No	(0.5) Indicator: Potentially if the trait ”physiological sensitivity” could be disaggregated	(0.5) Potentially if empirical pesticide data were available
	Compare levels of pesticide contamination between different regions in the UK	(0.5) Potentially.	(0.5) Potentially	(0) No (dependent on selected taxa)	(0) No (dependent on selected taxa)	(1) Indicator: Yes (trait-based)	(0.5) Potentially if empirical pesticide data were available
	Robust and objective?	(1) Yes	(0.5) Potentially	(0.5) Potentially	(0.5) Potentially	(1) Indicator: Yes	(0.5) Potentially
	Transparent and easy to understand for non-	(1) Yes, colour changes in biochemical assays	(0.5) Not at present but could be designed to	(1) Yes, scoring approach yields single	(0.5) Concept; Yes. It is complex to follow the	(1) Yes. Concept: logical combination of traits	(0.5) Concept: Yes. Underlying mathematical

		Biochemical biomarkers	DNA biomarkers/ microarrays	Pesticide Index	LIMPACT	SPEAR concept/ SPEAR-based indicator	RPDS/RPBBN
	specialists?	indicate altered enzyme activity, easy to interpret	easily identify toxicant by pattern of response.	value	decision-making process	Indicator: exposure-response curve	operations are reasonably complex
	Good summary of diagnosis, easy to interpret?	(1) Yes	(1) Yes	(1) Yes, scoring yields single value	(1) Yes, one contamination class is assigned	(1) Yes, indicator yields single value	(0.5) Potentially.
	Quick – applicable in the field?	(0) No (lab-based analysis)	(0) No (lab-based analysis)	(1) Yes (inventory monitoring can indicate absence of pesticide sensitive species)	(0) No (sorting of samples and software required)	(1) Yes (inventory monitoring can indicate absence of pesticide sensitive species)	(0) No (sorting of samples and software required)
	Cheap?	(0) AChE assay: No. Additional sampling, equipment, consumables costly to set up	(0) Not at present	(1) Yes	(0) No (additional bio-monitoring required)	(1) Indicator: Yes	(1) Yes
Assessment	Suitable diagnostic tool?	Yes (AChE assay to identify/exclude organophosphates/ carbamates)	Yes, to identify type of contaminant, if exposure and effects were linked	Yes, qualitative, if pesticide-sensitive species were identified	Yes, quantitative, if indicator taxa were identified for the UK	Indicator: Yes, quantitative and quick	Potentially, if empirical pesticide data were available
	Should be combined with other approaches?	To be used after contamination has been identified	To be used after contamination has been identified	Yes, biomarkers to identify type of contaminant	Yes, biomarkers to identify type of contaminant	Indicator: Yes, biomarkers to identify type of contaminant	Yes, biomarkers to identify type of contaminant
	Ready to use for diagnosing pesticide contamination in the UK?	Close to being used – assays need to be set up	Not at present	No (approach needs refinement)	No (approach needs additional data for re-calibration)	Yes, because is trait-based not taxonomy-based	No (approach needs additional data for re-calibration)
	Missing information?	Sufficient tests in the field with freshwater macroinvertebrates and known contaminants	Early stages of development, needs validation with different pesticides	Absence of potentially pesticide-sensitive families in different UK regions	Chemical monitoring data, in depth taxonomic identification (genus or species)	UK species and information about traits for UK endemic species	Chemical monitoring data for calibration
	Could be easily added to current EA procedures?	No, additional sampling and methods are required	N/A (exposure-response link missing)	Yes, after method refinement	No (additional bio-monitoring required)	Yes, although genus or species identification required	N/A (approach needs refinement)
	Could only be adopted with major revision of current practice?	Yes, need to either set up or collaborate with lab to undertake assays	N/A	N/A	Yes	N/A	N/A
Sum of scores (based on requirements)		7.5 (-1) Link between lab and field	5.0 (-1) Link between lab and field	7.5 (-0.5) Taxonomy-based, training data set required	5.0 (-0.5) Taxonomy-based, training data set required	10.5	5.5 (-0.5) Taxonomy-based, training data set required
Total		**6.5**	**4.0**	**7.0**	**4.5**	**10.5**	**5.0***

* (+1) Provided pesticide data were available, these are the only taxonomy-based approaches included in the review that could account for community variability due to other factors e.g. physical variables.

4.2 Diagnostic abilities of the reviewed approaches

4.2.1 Diagnostic and non-diagnostic approaches

The critical assessment of diagnostic abilities split the approaches into two groups. The first group included the Pesticide Index, LIMPACT and the SPEAR concept/indicator, all of which operate at the community level and can diagnose pesticide contamination (Figure 4.1). Diagnostic approaches that could be adapted to be pesticide-specific were also included, with the AChE assay representing the most promising biochemical biomarker, and molecular biomarkers as well as the software systems RPDS and RPBBN operating at the community level. The results of the critical assessment are presented in the following sections (Section 4.2.2 to 4.2.7).

The second group of non-diagnostic approaches included PERPEST, RIVPACS, PSYM and the relative sensitivity concept. PERPEST predicts the probability of effects of pesticides on structural and functional endpoints in freshwater communities from micro(mesocosm) studies. By definition the tool is prognostic, although the underlying case-based reasoning method could in principle be adapted to diagnose pesticide contamination. Case-based reasoning relies on an ample number of cases that relate a combination of (meaningful) biological endpoints to levels of pesticide contamination, linking patterns in community composition to exposure. For complex communities, it is difficult to identify patterns and establish cases. This means that other techniques that do not involve subjective interpretations of the data, such as clustering in the RPDS software or probabilistic reasoning in RPBBN, may be more suitable for the task.

RIVPACS and PSYM predict community composition in freshwater habitats. Although these are prognostic tools, they have the potential to support a diagnostic system where the expected community composition could be compared to the observed composition to assess the level of biological impairment. This is illustrated by the RIVPACS system being used to calculate the Pesticide Index. The relative sensitivity concept was developed to predict the physiological sensitivity of aquatic macroinvertebrates to organic compounds, including pesticides. Prognostic by definition, the concept estimates species' sensitivity and could support a diagnostic tool by identifying species or higher taxa that are potentially sensitive to pesticides.

In summary, RIVPACS, PSYM and the relative sensitivity concept are non-diagnostic approaches that could support diagnostic tools. The case-based reasoning methodology underlying PERPEST could be used for diagnostic systems in principle, but given the problem of establishing diagnoses from complex patterns of symptoms, other methods such as clustering or probabilistic reasoning are probably more suitable.

4.2.2 Biochemical biomarkers

Biochemical biomarkers could potentially identify the environmental stresses affecting an organism and diagnose the impact. Biochemical biomarkers have been shown to respond to pesticide exposure in some instances and could be designed to detect pesticide effects. The benefits of these approaches are:

- AChE biomarkers could potentially identify or exclude types of contaminant. AChE biomarkers have been shown to detect very low levels of carbamates and organophosphates and be sensitive to subtle changes. They have shown good responses in field studies, but more field tests with freshwater macroinvertebrates and known contaminants are needed.

- AChE biomarkers can measure the magnitude of recent contamination only since animals that do not die from exposure recover their enzyme activity.
- Colour changes in the biochemical assay indicate responses and are easy to interpret (in terms of contamination, not impact).
- Biomarkers are lab-based techniques: this means that it is not possible to evaluate responses quickly in the field. Tests based on AChE would require additional biomonitoring.
- Relatively speaking it is cheap to employ AChE biomarkers, but set-up costs for assays may be expensive and additional sampling, equipment and consumables are needed (for a costed plan, see Appendix III).
- Due to its specificity to organophosphates and carbamates, biomarkers based on AChE can be considered suitable (though costly) for use after pesticide contamination has been diagnosed, to identify the type of contaminant that caused the biological impairment.
- Prior to applying AChE methods, decisions would have to be made on which organism to use. Standard operating procedures with quality control checks are available for chironomids, *Daphnia* and *Gammarus* and only small changes would be needed to adapt the assay to other organisms.

Biochemical biomarkers are not used in current Environment Agency procedures, but could be used in an integrated assessment system. Such a system could employ a general stress biomarker first, such as GST or CYP450. If it showed a response, both AChE and metallothionein biomarkers could identify organophosphates, carbamates and metals. However if none of these responded, it would remain relatively difficult to identify the source of pollution using biochemical biomarkers, unless there was already a candidate pesticide. Most biomarkers are not time- or dose-responsive and lack specificity when confronted with confounding field variables or complex mixtures of chemicals (Forbes *et al*., 2006). Mayer *et al.* (1992) listed the following criteria for selecting and validating biomarkers: easy to use and cost-effective; enhanced sensitivity over acute toxicity; minimal or well-understood variability in response; dose- and time-responsive; and linked to physiological processes with biological significance. In practice, meeting all these criteria is impossible, but many can be attained with well-designed and replicated experiments which measure temporal and dose-dependent changes in biomarker (Handy *et al*., 2003).

A further factor is deciding what organisms to use for measurements. If animals from natural populations are used, genetic variation and local adaptations may introduce an added level of variation when comparing responses. This will be less of an issue if the animals used are upstream and downstream of a pollution source in a single water body. The alternative is to apply laboratory organisms *in situ* (Crane *et al*., 2000), which removes the variation in genetic background, but could stress the organisms and is more time-consuming. The approach to adopt should be considered on a case-by-case basis.

Biochemical biomarkers could play a role in diagnosing pesticide contamination, but still need to be validated under field conditions, with biomarker responses linked to pesticide effects on higher levels of biological organisation (populations and communities).

4.2.3 DNA biomarkers – Gene expression biomarkers

Molecular biomarkers of DNA damage are potentially valuable tools to assess effects of acute and chronic exposure of aquatic organisms to genotoxic substances. Gene expression biomarkers can be specific in their responses, while genotoxic biomarkers that indicate strand breakage lack specificity. Specifically:

- Gene expression biomarkers offer the potential of a toxicant fingerprint, where patterns of response are linked to pesticide class and even type.
- If clear links between response and exposure are established, DNA biomarkers will both qualitatively and quantitatively indicate contamination levels and will be sensitive to subtle changes.
- Measurement of gene expression can offer a large toolbox of responses that can lead to toxicant fingerprints, which could be used potentially to identify stressors in a simplistic and easy-to-understand format (a picture).
- However, biomarker analyses are lab-based, which means that it is not possible to evaluate responses quickly in the field.
- DNA biomarkers are costly in terms of developing and running microarrays.
- DNA biomarkers/microarrays may offer a solution in the future to identifying the type of pesticide that causes observed biological impairment.
- At present, however, DNA biomarkers are not ready for use, because work has do be done to link exposure to specific changes in gene expression and to establish fingerprints of gene expression for single substances. In addition, DNA biomarker responses need to be linked to pesticide effects at population and community level.

4.2.4 Pesticide Index

The Pesticide Index belongs to the group of taxonomy-based systems that are designed as diagnostic tools. The index is based on scoring invertebrate families in terms of their sensitivity to pesticides, but identification of sensitive families is based on correlation (families absent in areas lacking organic stresses and potentially exposed to pesticides) rather than observing cause-effect of pesticides. In particular:

- The index is designed to give qualitative information on contamination. Its list of potentially pesticide-sensitive taxa does not distinguish between types of compounds; therefore the index is unlikely to identify the type of pesticide contamination. Further, the Pesticide Index is based on the presence of expected taxa, which makes it unlikely to respond to subtle changes in abundance.
- The scoring approach is transparent and scores a site based on expected but absent taxa, indicating the relative magnitude of contamination.
- The Pesticide Index can run on current biomonitoring data. Inventory monitoring can show absence of species and thus could give a first indication of impacts attributable to pesticides. For confirmation, sorting in the lab is necessary to obtain a sample, and specialist software is needed.
- The index may not work well in regions where the taxa are absent as a result of their natural distribution patterns. Therefore, the empirical list of

pesticide-sensitive taxa should be extended on the basis of presence/absence data from other regions in England and Wales.

- The index is not used in current Environment Agency practices. However, it could be employed as an indicator of pesticide contamination if empirical data were used to relate the absence of taxa to pesticide exposure.
- The index could be combined with molecular methods, with the Pesticide Index used to diagnose the magnitude of contamination and biomarkers applied subsequently to identify the type of contaminant.

4.2.5 LIMPACT

The LIMPACT system is currently the only diagnostic expert system for pesticide effects in aquatic ecosystems. The system is rule-based and uses information on abundance patterns of indicator species to diagnose the contamination of test sites. The rules are developed from observed abundance patterns at sites of different contamination levels, and identification of indicator taxa is based on abundance dynamics at sites of known levels of pesticide exposure. Specifically:

- LIMPACT is designed to give quantitative information on contamination (not detected, low, moderate and high), but does not identify the contaminant.
- LIMPACT is sensitive to subtle changes, because the rules are based on such changes. The rule-based approach is easy to understand and LIMPACT yields a single value. However, the number of rules makes the decision process complex to follow.
- The LIMPACT system cannot be used quickly in the field, since sorting of samples and specialist software is required. In principle the approach could be used to diagnose contamination based on GQA data, but requires a temporal resolution of samples (spring, summer, autumn) that is currently not covered by GQA procedures.
- LIMPACT is taxonomy-based and therefore may not to work well in areas where indicator taxa are absent due to their natural distribution patterns. Unless biological and pesticide monitoring data were available to revise the system, LIMPACT could not be applied in England and Wales, because results might be flawed due to differences in the presence and abundance of indicator taxa compared to German lowland conditions.
- If LIMPACT was restructured for the UK, the system could only be adopted with major revision of Environment Agency practices, because additional biomonitoring in summer would be necessary. It is unclear whether monitoring data from spring and autumn only would be sufficient for an assessment.
- LIMPACT could be combined with molecular methods, with LIMPACT used to diagnose pesticide contamination and biomarkers subsequently applied to identify the type of contaminant.

4.2.6 SPEAR

The SPEAR concept is currently the only trait-based approach to identify pesticide-sensitive species (taxa). The approach uses mechanistic understanding of the factors affecting the sensitivity of species to pesticides. Field studies in Finland, France and

Germany have shown a firm link between insecticide and fungicide exposure and abundance of SPEAR. Further, these studies revealed a linear exposure-response relationship that holds for different geographical regions. This relationship can be used to diagnose the magnitude of pesticide contamination from biological data (SPEAR-based indicator). It is assumed that this relationship holds for the UK, but field investigations are suggested to test this. In particular:

- Currently, the relationship cannot be used to indicate different types of pesticide (such as insecticides/fungicides), but this may be possible if the SPEAR classification is refined with respect to the physiological sensitivity of taxa to substance groups with particular modes of action. However, there is currently no indication of this possibility given the lack of sufficient toxicity tests of taxonomic groups for substance groups with particular modes.
- The SPEAR-based indicator is sensitive to subtle changes, since a long-term decrease in percentage SPEAR abundance was found to occur at lower levels of modelled runoff than long-term decreases in other community measures such as diversity or EPT numbers.
- Both the SPEAR concept and the SPEAR-based indicator are transparent for non-specialists because of the logical combination of traits and the use of a unique exposure-response relationship for diagnosis.
- The SPEAR-based indicator can run on current biomonitoring data and yields a single value of the magnitude of pesticide contamination (in TU). Inventory monitoring in the field can show absence of pesticide-sensitive species and give a first indication of pesticide contamination. For confirmation, sorting in the lab is necessary to obtain a sample. No particular software is required, because data management and calculation of SPEAR values can be done with standard database software such as Microsoft Excel or Microsoft Access.
- The SPEAR-based indicator is not used in current Environment Agency procedures, but is a transparent and simple indicator of pesticide contamination. It could be combined with molecular methods, with the SPEAR indicator used to diagnose the magnitude of contamination and biomarkers applied subsequently to identify the type of contaminant.
- The trait-based indicator is ready for use in the UK, while the SPEAR classification list may need updating on UK species and their traits considered in the classification concept. The SPEAR indicator could be easily added to current Environment Agency procedures with minor changes in practice (no additional biomonitoring), but identification of taxa to a lower taxonomical level than the family level would be beneficial.

4.2.7 RPDS and RPBBN

Both RPDS and RPBBN are diagnostic systems being developed for Environment Agency assessment of biological quality, but currently they are not able to diagnose pesticide pollution. RPDS is based on clustering using the MI-max algorithm and RPBBN is based on probabilistic reasoning. The strength of both approaches is that they do not require a priori knowledge, but are based on the self-organisation of data.

- Both systems could be used to diagnose pesticide effects and potentially to identify the magnitude of contamination and type of contaminant. However, the empirical pesticide data needed to calibrate the systems are not currently available.

- The concept is reasonably transparent, but the underlying algorithms for clustering and reasoning are complex.
- Using both systems could be cheap, because they could run on current GQA biomonitoring data, but both require special software and rely on sorted samples. This makes it impossible to apply them directly in the field.
- Both RPDS and RPBBN could be suitable tools to indicate pesticide contamination in freshwaters, if empirical pesticide data were available for the database and to calibrate the clustering algorithms and probability networks respectively.
- Both methods could be combined with molecular methods to identify the type of contaminant.

4.3 Comparative assessment of diagnostic approaches

Approaches that can or could be used as diagnostic tools for pesticide contamination were compared, to identify those that could meet most of the end users' requirements (Table 4.4).

For sub-organism approaches, the AChE assay represented the most promising biochemical biomarker, scoring higher than the group of molecular biomarkers. An extensive body of research shows that AChE assays may be sensitive to subtle pesticide-induced changes, may identify magnitude and type of contamination, and may be robust, objective, transparent and cheap, while much of this is still unknown for molecular biomarkers.

Scores for sub-organism approaches were lower than the highest scores from the group of community-based approaches. It is complicated to establish a link between biomarker responses and higher level responses in the field, because of compensatory mechanisms that regulate population dynamics in natural systems. Although sub-organism approaches score less or similar to community-based ones, the strength of AChE assays in particular is the ability to identify or exclude types of contaminants that may be responsible for the observed biological impairment.

Within the group of community-based approaches, the SPEAR concept/indicator and Pesticide Index scored more than RPDS/RPBBN and LIMPACT; unlike the latter, the SPEAR concept and Pesticide Index were found to be transparent, quick and cheap. Both LIMPACT and RPDS/RPBBN scored similarly. However, the total score for the latter could be increased by one, provided pesticide data were available for the model, because RPDS/RPBBN would be the only taxonomy-based approach to take into account natural distribution patterns, making it usable in different regions of England and Wales.

5 A suitable indicator – recommendations

5.1 Conclusions from critical assessment of approaches

Currently, no single approach could meet all the expectations of potential end users for such a biological indicator. However, three types of approaches were found to be promising tools which could meet some end user requirements:

- Firstly, the SPEAR concept/indicator and, after validation, the Pesticide Index, because they are transparent, quick and cheap.
- Secondly, the AChE assay, because this technique has the potential to identify or exclude organophosphate or carbamate insecticides as contaminants responsible for observed biological impairment.
- Thirdly, RPDS and RPBBN, because these software tools (that are not yet pesticide-specific) do not rely on a priori knowledge but on self-organisation of data. In addition, they would be the only taxonomy-based approaches to account for variability due to other factors (such as physical variables).

The major gap in community-based approaches is that they cannot be tested or used in streams in England and Wales, because combined datasets of biological and pesticide monitoring data are not available. Such a dataset would be needed for testing (SPEAR indicator, Pesticide Index) and calibrating (RPDS/RPBBN) the methods.

Major gaps in biomarker methods are the lack of sufficient tests in the field, and the link between biomarker responses and higher level responses, which is difficult to establish due to compensatory mechanisms that regulate population dynamics in the field.

Since the reviewed methods have different strengths, a 'tiered' approach might be the most promising to assess pesticide contamination. This tiered approach would enable the strengths of higher and molecular-level approaches to be combined, screening for pesticide contamination using the higher level approaches and identifying the type of contaminant using molecular ones.

5.2 A tiered approach to diagnosing pesticide contamination based on macroinvertebrate data

A tiered approach to screening for pesticide contamination at larger scales should use community-based methods for the first tier (large-scale screening, quick, transparent, cheap). When the results of the screening suggest intermediate or high pesticide contamination at a site, a more complex analysis should be triggered that uses biochemical/molecular biomarkers to identify/exclude types of contaminants. The development of a tiered approach is suggested and described in Figure 5.1.

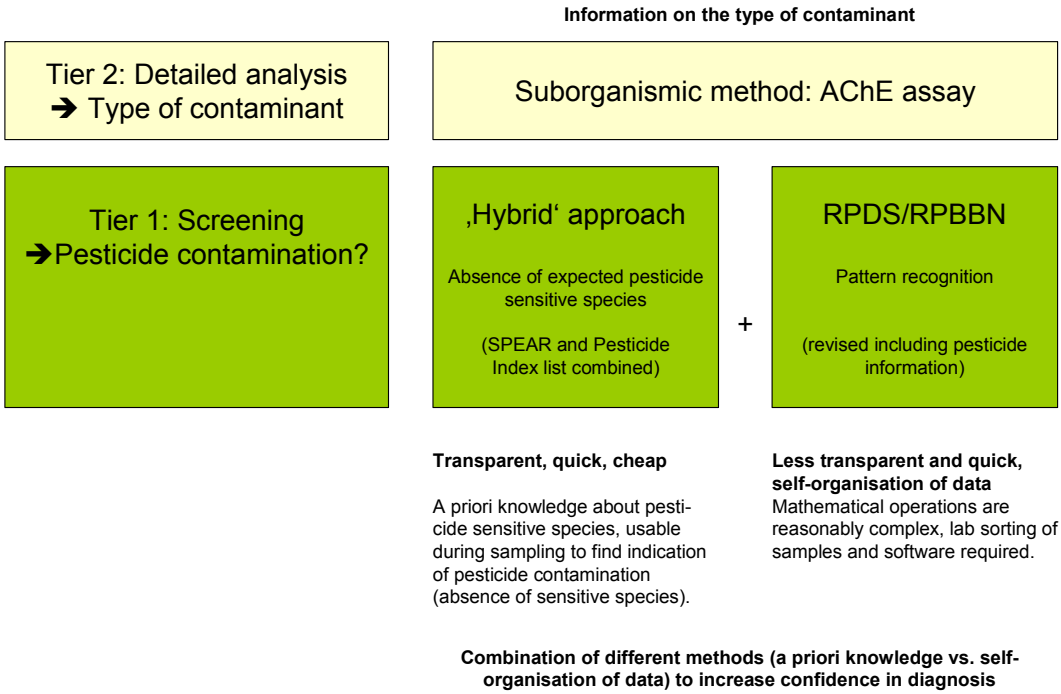

Figure 5.1: A tiered approach to diagnosing pesticide contamination using macroinvertebrate data and combining the strengths of different indicator types

5.2.1 First tier: Hybrid approach and RPDS/RPBBN

At the first tier, a 'hybrid' approach combining the SPEAR indicator and Pesticide Index and the RPDS/RPBBN software would be used to give a diagnosis. If the diagnosis of each approach indicated (intermediate or high) pesticide contamination, the second tier would be triggered for a refined diagnosis on the potential type of contaminant.

Hybrid approach: The hybrid approach would compare predicted (RIVPACS) and observed community composition and indicate the potential level of pesticide contamination based on the number and abundance of expected pesticide-sensitive species absent from a site. Pesticide-sensitive species would be identified by means of a species list that would combine both the mechanistic knowledge from the SPEAR concept with the empirical findings on pesticide-sensitive species incorporated in the Pesticide Index. A prerequisite for this would be to further develop the Pesticide Index by linking presence/absence of species to pesticide contamination and thus to update the list of pesticide-sensitive species.

RPDS/RPBBN: This diagnostic software could be restructured to diagnose pesticide contamination. A prerequisite for this would be a comprehensive dataset to restructure/update the system to indicate pesticide contamination in England and Wales. A suitable training set for RPDS/RPBBN would consist of several hundred sites and would include faunal and chemical data from streams investigated before and during the main period of agrochemical application.

Pros and cons: The first tier as described above would be advantageous, since it combines ecological a priori knowledge about pesticide-sensitive species from the

laboratory and the field (hybrid approach) with information gained from data clustering (RPDS/RPBBN). The hybrid approach could be applied in the field during inventory monitoring for a quick assessment of whether pesticide-sensitive species were absent (some users consider use of the indicator in the field to be very beneficial). However, for confirmation and precise calculation, lab sorting and computing would be required. The method is transparent and easy to understand for non-specialists (another requirement of end users) whereas for RPDS/RPBBN, the underlying algorithms make the approach less easy to understand for non-specialists. Combining results from the hybrid approach and the RPDS/RPBBN would provide a diagnosis based on two different methodologies (a priori knowledge versus self-organisation of data) and thus would more be more reliable than a diagnosis derived from only one type of method.

5.2.2 Second tier: AChE assay

The second tier would be triggered if both first tier approaches indicated intermediate or high pesticide contamination. The aim of the second tier would be to identify or exclude the possible type of contaminant.

AChE assay: The AChE assay is relatively easy to use, is extremely sensitive to organophosphates and carbamates, and is clearly considered a good potential biomarker. However, a prerequisite for using the assay for second tier assessment would be to link biomarker response to exposure conditions in the field.

Pros and cons: The second tier would be advantageous because it offers the possibility of additional information on the type of contaminant, along with the magnitude of contamination assessed in the first tier. Potential end users are eager to diagnose the type of contaminant from biological samples, but at the same time appear reluctant to use molecular methods because of low signal-to-noise ratios.

5.2.3 Research requirements

The proposed approach would require a field dataset of pesticide and biological monitoring data to establish exposure-response relationships for a number of agricultural water bodies in England and Wales. With this dataset, it would be possible to address knowledge gaps for methods included in the tiered approach, and to validate existing approaches for UK conditions. The value of such field data for each method, however, would depend on the extent of the dataset.

For the first tier hybrid approach, the value of a field dataset including measures of pesticide exposure and observed effects at community level would be threefold. Firstly, the dataset could be used to evaluate the empirical list of potentially pesticide-sensitive taxa in the Pesticide Index. Linking the presence or absence of taxa to pesticide contamination would be a prerequisite to including information from the empirical taxa list in the hybrid approach. Secondly, the data could be used to update the empirical list with key taxa outside of the Kent region where the Pesticide Index was developed. Thirdly, the hybrid approach could be tested for England and Wales and it would be possible to establish nationwide reference values in terms of the presence of pesticide-sensitive taxa at sites of different contamination levels.

A dataset to suit these purposes should include a minimum of 20 sampling sites along a pesticide gradient. With this set, it would be possible to test the hybrid approach with the list of pesticide-sensitive taxa taken from the trait-based SPEAR indicator. To test and update the empirical list taken from the taxonomy-based Pesticide Index, more than 20 sites per region would be necessary to account for the presence or absence of key taxa as a result of their natural distribution patterns. The described datasets could

be of some value for updating the RPDS/RPBBN software, but judging from the large number of sampling sites used to set up the software, a larger set of several hundred sites would be necessary to adapt the systems to diagnose pesticide contamination.

For the second tier AChE assay, a field dataset would be very valuable to investigate biomarker responses along a pesticide gradient. A suitable dataset (corresponding to the above described set of 20 sites, for example) would include chemical water samples and possibly biological samples, if samples from the field community were to be used in the assay. In this instance, the measurement of AChE in individuals from a wild population would be the best approach.

These datasets could help address knowledge gaps in the tiered approach and aid development of the approach as an indicator of pesticide contamination in freshwaters.

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Glossary

Acetylcholine receptor (AChR)	Ion channel that opens in response to acetylcholine binding, thereby converting a chemical signal into an electrical one.
Alanine	Crystalline amino acid ($C_3H_7NO_2$) that is a constituent of many proteins.
Biomarker	Biological response to an environmental chemical, which gives a measure of exposure and sometimes also a toxic effect.
Biomarker of effect	Biological response to an environmental stress that indicates what systems have been affected and may indicate mode of action.
Biomarker of exposure	Biological response to an environmental stress, which indicates that an organism has been exposed.
Biotransformation	Chemical conversion of substances by living organisms or enzyme preparations.
Carboxylesterase	An enzyme of wide specificity that catalyzes the hydrolytic cleavage of the ester bond in a carboxylic ester to form an alcohol and a carboxylic acid.
Catalase (CAT)	Enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen.
Cholinergic nerve axon	A nerve activated by acetylcholine.
Conjugation	Binding to.
Cytochrome P450 (CYP450)	A generic term for a large number of evolutionary related oxidative enzymes.
Denatured DNA	A structural change in macromolecules caused by extreme conditions.
DNA microarray	A glass slide to which a collection of DNA fragments has been attached.
Downregulated	Expression of the gene is decreased or halted altogether.
Electrophilic	Attracted to electrons in a chemical reaction that accepts electrons.
Endocrine	Any of the glands of the endocrine system that secrete hormones directly into the bloodstream.
Endocrine-disruption	Chemicals that either mimic a natural hormone, fooling the body into over-responding to the stimulus or responding at inappropriate times, or that block the effects of a hormone from certain receptors by blocking the receptor site on a cell.
Glutathione S-transferase (GST)	The glutathione S-transferase (GST) family of enzymes comprises a long list of cytosolic, mitochondrial and microsomal proteins that are capable of multiple reactions with a multitude of substrates, both endogenous and xenobiotic.
Heat shock proteins (HSP)	Heat shock proteins (HSP) are a group of proteins whose expression is increased when the cells are exposed to elevated temperatures. This increase in expression is transcriptionally regulated. This dramatic upregulation of HSP induced mostly

	by heat shock factor (HSF) is a key part of the heat shock response. Production of high levels of HSP can also be triggered by exposure to different kinds of environmental stress conditions, such as infection, inflammation, exposure of the cell to toxins (ethanol, arsenic, trace metals and ultraviolet light, among many others), starvation, hypoxia (oxygen deprivation), nitrogen deficiency (in plants), or water deprivation. Consequently, the heat shock proteins are also referred to as stress proteins and their upregulation is sometimes described more generally as part of the stress response.
Hexachlorobenzene	Hexachlorobenzene, or perchlorobenzene, is a chlorinated hydrocarbon with the molecular formula C_6Cl_6. It is a fungicide formerly used as a seed treatment, especially on wheat.
Immunological methods	Use of biochemical properties of the immune system to detect proteins.
Induction	A process in which a molecule (such as a drug) induces (initiates or enhances) the expression of an enzyme.
Inhibition	Inhibition of the expression of an enzyme by another molecule.
Insecticide resistance	Naturally occurring, inheritable ability of individuals in a population to survive treatment with an insecticide that would normally give effective control.
Lactate	L-lactate is constantly produced from pyruvate by fermentation.
Metabolite	Intermediates and products of metabolism.
Metabolomics	Metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind" - specifically, the study of their small-molecule metabolite profiles
Mixed function oxidases (MFO)	Monooxygenases, or mixed function oxidase, transfer one oxygen atom to the substrate, and reduce the other oxygen atom to water.
Non-polar	Without charge separation; not soluble in water.
Nucleic acid	Any of a group of complex compounds found in all living cells and viruses, composed of purines, pyrimidines, carbohydrates, and phosphoric acid. Nucleic acids in the form of DNA and RNA control cellular function and heredity.
Organophosphate oxons	A chemical oxon is an organic compound derived from another chemical in which a phosphorus-sulfur bond in the parent chemical has been replaced by a phosphorus-oxygen bond in the derivative. Important examples of oxons can be found in the family of pesticides known as organophosphates. Some of these chemicals, such as chlorpyrifos, diazinon and parathion, do not manifest their main toxicity in their original form. Rather, an animal's liver replaces a phosphorus-sulfur bond with a phosphorus-oxygen bond, turning these chemicals into oxons. The oxons then inhibit an enzyme that breaks down acetylcholine, an important neurotransmitter.
Osmoregulation	Maintenance of an optimal, constant osmotic pressure in the body of a living organism.
Oxidative stress	A condition of increased oxidant production in animal cells characterized by the release of free radicals and resulting in

	cellular degeneration.
Oxyradical	An oxygen molecule with an unpaired electron. Because they have a free electron, such molecules are highly reactive.
Polyaromatic hydrocarbon (PAH)	Any of a class of carcinogenic organic molecules that consist of three or more benzene rings and are commonly produced by fossil fuel combustion. Also called polynuclear aromatic hydrocarbon.
Polymerase chain reaction (PCR)	A technique for amplifying DNA sequences in vitro by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase. It can amplify a specific sequence of DNA by as many as one billion times.
Primer	A primer is a nucleic acid strand or related molecule that serves as a starting point for DNA replication. A primer is required because most DNA polymerases (enzymes that catalyze the replication of DNA) cannot begin synthesizing a new DNA strand from scratch, but can only add to an existing strand of nucleotides.
Proteomics	Proteomics is a term in the study of genetics that refers to all the proteins expressed by a genome. Proteomics involves the identification of proteins in the body and the determination of their role in physiological and pathophysiological functions.
Pyruvate	A salt or an ester of pyruvic acid, a colourless organic liquid, $CH_3COCOOH$, formed as an intermediate in carbohydrate metabolism and fermentation and as an end product in glycolysis.
QTPCR	Quantitative PCR – a variation of PCR where the amount of product is quantified allowing an accurate measurement of gene expression.
Reverse transcriptase (RT)	Polymerase that catalyzes the formation of DNA on an RNA template, found in oncogenic viruses containing RNA, especially the retroviruses.
RNA	A polymeric constituent of all living cells and many viruses, consisting of a long, usually single-stranded chain of alternating phosphate and ribose units with the bases adenine, guanine, cytosine and uracil bonded to the ribose. The structure and base sequence of RNA are determinants of protein synthesis and the transmission of genetic information. Also called ribonucleic acid.
Thin-layer chromatography	Widely-used chromatography technique to separate chemical compounds.
Transcriptomics	The transcriptome is the set of all messenger RNA (mRNA) molecules, or "transcripts", produced in one or a population of cells. The term can be applied to the total set of transcripts in a given organism, or to the specific subset of transcripts present in a particular cell type. Unlike the genome, which is roughly fixed for a given cell line (excluding mutations), the transcriptome can vary with external environmental conditions.
Upregulated	Expression of the gene is increased.

Appendices

I. Questionnaire

on expectations from a suitable freshwater biological indicator of pesticide contamination

Questionnaire

1 Name *Please insert text*

2 Job title *Please insert text*

Current approach

The following questions are intended to help us understand the approach that you are currently using to identify biological impairment of surface waters, including impairment due to pesticides.

3 Do you use biological data collected as part of the Environmental Agency's General Quality Assessment scheme (GQA) to identify biological impairment?

Please indicate with "x":
No
Yes

Do you collect any additional data or use further data sources? E.g. do you carry out additional investigative monitoring or do you identify taxa to a higher taxonomic resolution?

Please indicate with "x":
No, I do not use any additional data.
Yes, I do use additional data.

If yes, please specify what additional data you use and why.

Please insert text

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Current approach

4 Which measures (e.g. of taxa richness) or techniques (e.g. ordination techniques) are you currently using to indicate biological impairment in freshwater habitats including impairment due to pesticides ? Why do you use these particular measures/techniques?

Measures/Techniques: *Please insert text*

5 What in your opinion are the advantages of these measures/techniques?

Advantages (please describe briefly): *Please insert text*

6 What in your opinion are the disadvantages of these measures/techniques?

Disadvantages (please describe briefly): *Please insert text*

Requirements

The following questions are intended to help us understand your requirements for a biological indicator of pesticide contamination.

7 Would a biological indicator of pesticide contamination be useful to you?

Please indicate with "x":
No. ☐
Yes. ☐

If yes, please explain briefly why and how you would use this indicator.

Please insert text

8 Which endpoints are you interested in (e.g., genetic structure of populations, population structure, community composition)?

Please indicate briefly:

Please insert text

Requirements

9 Please rank the following six attributes of a potential indicator with respect to their relevance for your work. (The attribute with the highest relevance should be given a rank value of "1", the least relevant attribute should be given a rank value of "6".)

a ... the ability to distinguish the impact of pesticides from other stressors*
**Other stressors: e.g. nutrient enrichment or temporary droughts*

b ... the ability to inform about the relative order of magnitude* of pesticide contamination
** Relative order of magnitude: contamination at site x is higher than contamination at site y*

c ... the ability to give an early warning*
** Early warning: e.g. by indicating effects of contamination on the genetic structure of populations*

d ... the ability to identify the time period of contamination*
** Time period of contamination: i.e. if observed effects result from a recent contamination or from contamination during previous years*

e ... the ability to allow trends over time* to be assessed
** Trends over time: shifts in contamination levels over a period of several years e.g. due to changes in agricultural practice or management measures*

f ... the ability to compare levels of pesticide contamination between different regions* in the UK
** Different regions: to allow for a comparison between different habitats and therefore different communities*

Optimal indicator

10 If we had the ability to design a new biological indicator of pesticide contamination to your specifications, what would they be?

Please insert text

Thank you very much!

If you have any difficulties in answering the questionnaire, please do not hesitate to contact:

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e-mail: carola.schriever@ufz.de **phone: +49 (0)341-235-2442**

II. Tabular summary of reviewed approaches

Table II.1 Tabular summary of pesticide-specific approaches at the molecular level

		Acetylcholinesterase	Gene expression	DNA microarrays
General	Category	Biochemical biomarker	Molecular/DNA biomarker	Molecular/DNA biomarker
	Type	Existing method well developed in many organisms.	Existing method, which can be readily applied to any gene.	Existing method, which is being developed currently for use in testing freshwater pesticides and other environmental stressors.
	Aim	To use the target site of specific pesticides to measure the presence of that pesticide in the field.	To measure expression of any gene.	Simultaneous measurement of thousands of genes to detect differences in expression between a treatment and control or between two treatments.
	Concept	Qualitative, enzyme inhibition.	Quantitative/qualitative, can detect amounts and also which genes are responding.	Quantitative/qualitative, shows which genes are responding and by how much.
	Endpoint 1	Freshwater macroinvertebrate but is also used in fish and marine organisms.	Freshwater macroinvertebrate and fish.	Freshwater macroinvertebrates and fish.
	Endpoint 2	Suborganismal, enzyme inhibition.	Suborganismal, gene expression which may or may not lead to changes in proteins.	Suborganismal, gene expression which may or may not lead to changes in proteins.
	Methodology	Biochemical method, comparison between animals in polluted site vs. control site.	Molecular biological method using quantitative PCR and stats.	Molecular biological method using DNA microchips to measure hybridisation of expressed genes to known sequences.
	Underlying data	N/A	N/A	N/A
	(Required) Input data	N/A	N/A	N/A
	Specificity to different modes of actions	Specific to inhibition of AChE.	Specificity can be extremely high, or in some cases low, depending on the gene.	Specificity can be extremely high, or in some cases low, depending on the gene. Pattern of expression can be a fingerprint of a particular pollutant.
	Software existing	There is software to convert microtiterplate assays in mean scores.	Yes	Yes
Successful field evaluation	Geographical region	Various	Various	Various
	Tested water body type	Lagoon, rivers, estuaries, marine.	Lagoon, rivers, estuaries.	Lagoon, rivers, estuaries
	Number of test sites	Unknown	Unknown	Unknown
	Sources of pesticide pollution	Unknown	Unknown	Unknown
	Type of pesticide pollution	Organophosphates, carbamates.	Any	Any

Table II.1 Tabular summary of pesticide-specific approaches at the molecular level

		Acetylcholinesterase	Gene expression	DNA microarrays
	Reliability	High reliability, some instances where responses found with other types of pollutant, but generally very specific and is linked to higher effects.	Reliability has potential to be high, but not enough data currently.	Reliability has potential to be high, but not enough data currently.
	Robustness	Good responses in field studies.	Is robust if trained scientists undertake the work.	Is robust if trained scientists undertake the work.
	References			
General comment				
Requirements	Diagnostic tool to identify pesticide contamination	Yes, inhibition will indicate pesticide contamination and there have been a number of studies that show this links to effects.	Yes, can identify pesticide if prior work has been done, but need to do the work to make links to effects.	Yes, can identify pesticide if prior work has been done, but need to do the work to make links to effects.
	Distinguish pesticides from other stressors	Yes	If the gene has been previously identified as responding specifically to a particular type of stress, then yes.	If the gene has been previously identified as responding specifically to a particular type of stress, then yes.
	Sensitive to subtle changes	Yes	Yes, very.	Yes, very.
	Indicate trends over time	No, there are no long-term effects.	Difficult, but could indicate changes in expression patterns.	Difficult, but could indicate changes in expression patterns.
	Indicate the relative order of magnitude of pesticide contamination	Yes, dose-dependent.	Yes, depending on the gene, could be dose-dependent.	Yes, depending on the gene, could be dose-dependent.
	Indicate the time period of contamination	Possibly, it will tell you it happened recently since animals that don't die recover their activity.	Unsure, need more data.	Unsure, need more data.
	Compare levels of pesticide contamination between different regions in the UK	Yes, as long as you know the baseline activity.	Yes	Yes
	Be an early warning system	Yes, could get effects before levels are high enough to kill the organism.	Yes	Yes
	Objective (robust)	Yes	Yes	Yes
	Transparent	Yes, if are doing a comparison between control and polluted sites, easy to see difference using stats.	Should be easy to understand that gene expression has changed.	Interpretation is currently complex but in the future we should have easy pattern recognition.

Table II.1 Tabular summary of pesticide-specific approaches at the molecular level

		Acetylcholinesterase	Gene expression	DNA microarrays
	Giving a good summary	Yes	Yes	Yes
	Results are easy to interpret	Yes	Yes	Yes
	Quick	Not in the field but assays can be done very quickly in the lab (few hours).	No, has to be done in the lab.	No, has to be done in the lab.
	Cheap	Relatively speaking it is cheap, but additional sampling, equipment and consumables needed.	No, development expensive, although final assay may be relatively cheap.	No, development expensive, although final assay may be relatively cheap.
Assessment in the context of current Environment Agency procedures	Currently used	No	No	No
	Ready to use for diagnosis	Yes, has already been tested in the field with a number of organisms.	No	No
	Missing information	Sufficient tests in the field with freshwater macroinvertebrates and known contaminants.	Early stages of development, needs validation with different pesticides.	Early stages of development, needs validation with different pesticides.
	Could be easily added to current techniques or current monitoring.	No	No	No
	Could only be adopted with major revision of current practice	Yes	Yes	Yes

Table II.2 Tabular summary of pesticide-specific approaches at community level

		Species at risk (SPEAR) concept Liess and Von der Ohe (2005)	**SPEAR-based indicator**	**Relative sensitivity concept** Von der Ohe and Liess (2004), Wogram and Liess (2001)	**Pesticide Index** Humpheryes (pers. communication)	**LIMPACT** Neumann *et al*. (2003)	**PERPEST** Van den Brink *et al*. (2002)
General	Category	Community level, trait-based	Community level, trait-based	Community level, taxonomy-based	Community level, taxonomy-based	Community level, taxonomy-based	Community level, taxonomy-based
	Type	Classification/Indicator system	Indicator	Classification system	Empirical approach	Indicator system (diagnostic model)	Prognostic model
	Aim	Identifying species (taxa) at risk from pesticides according to traits.	Prognosis/Diagnosis	Identifying taxa that are sensitive to organic pollutants including pesticides.	Designed to identify sites exposed to pesticides.	Classifying sites as unimpacted, medium impacted or highly impacted from pesticides in an investigated year.	Predicting probability of effects.
	Concept	Qualitative: species (taxon) at risk, yes or no.	Quantitative	Qualitative: taxon is more sensitive than other (ordinal)	Qualitative	Quantitative: Not detected, low (sum of TU per year and site < -4), medium (sum TU per year and site < -2) and high (sum TU per year and site >= -2)	Qualitative
	Endpoint 1	Freshwater macroinvertebrates	Freshwater macroinvertebrates	Freshwater macroinvertebrates	Freshwater macroinvertebrates	Freshwater macroinvertebrates	Macroinvertebrates, fish and community metabolism in freshwater systems
	Endpoint 2	Genus, species, family	Community composition	Genus, species, family	Community composition	Species level to family level	Macroinvertebrates, fish, community metabolism
	Methodology	Mechanistic, trait information is transformed into binary values (taxon is at risk due to trait, yes or no), binary values are combined by Boolean AND, taxon is at risk if each of the considered traits indicates risk.	Relationship between measured TU and observed percentage SPEAR abundance (Liess and Von der Ohe, 2005).	Tolerance of test species to a substance in relation to tolerance of *daphnia magna* to same yields relative sensitivity of test species; the arithmetic mean of the relative sensitivity gives the relative tolerance of the next higher taxonomic level.	Scoring approach: scores taxon said to be sensitive to pesticides and calculates overall scores per site from the taxa that are expected (based on RIVPACS) but missing.	Heuristic rule-based expert system, empirical rules considering abundance dynamics of 39 indicator taxa during spring, summer and autumn.	Case-based expert system, (dis)similarity of known cases, based on effects of 22 herbicides and 24 insecticides (49 herbicide experiments and 55 insecticide experiments); 421 cases in total

Table II.2 Tabular summary of pesticide-specific approaches at community level

		Species at risk (SPEAR) concept Liess and Von der Ohe (2005)	**SPEAR-based indicator**	**Relative sensitivity concept** Von der Ohe and Liess (2004), Wogram and Liess (2001)	**Pesticide Index** Humpheryes (pers. communication)	**LIMPACT** Neumann *et al*. (2003)	**PERPEST** Van den Brink *et al*. (2002)
	Underlying data	Standard acute toxicity tests, life-history information from literature.	Measured concentrations of insecticides and fungicides, observed community composition.	Standard acute toxicity tests.	RIVPACS predictions, empirically derived list of species that may be sensitive to pesticides.	Database of streams with different pesticide exposure.	Mesocosm studies
	(Required) Input data	Information on the following traits: physiological sensitivity to organic pollutants including pesticides, generation time, migration ability, time of emergence.	Biological samples before and after exposure.	N/A	Family invertebrate data	Abundance data from four samplings (spring, summer1, summer2, autumn) and nine water quality parameters.	Concentration
	Specificity to different modes of actions	Unknown	Unknown	N/A	Not specific	Unknown	Limited to insecticide and herbicide moa, but no specificity.
	Software existing	No	No	N/A	Yes	Yes	Yes
Successful field evaluation		Firm link between measured pesticide levels and SPEAR, tested in different geographical regions across Europe.	Adequate prognosis of observed effects of short-term parathion-ethyl exposure.	N/A	Limited evaluation.	No independent dataset, testing with training set.	No
	Geographical region	Germany, North-German lowlands, Brittany, France and Helsinki region, Finland.	Germany, North-German lowlands.	N/A	Southern England	Germany, North-German lowlands.	N/A
	Tested water body type	Streams	Stream	N/A	Rivers and streams	Small lowland streams	N/A
	Number of test sites	49 (Germany: 20; France: 16; Finland: 13).	1	N/A	Unclear	104	N/A
	Sources of pesticide pollution	Agriculture (field crops).	Agriculture (field crops).	N/A	Sheep dipping; cereal crops; fruit crops.	Agriculture (field crops).	N/A

Table II.2 Tabular summary of pesticide-specific approaches at community level

		Species at risk (SPEAR) concept Liess and Von der Ohe (2005)	**SPEAR-based indicator**	**Relative sensitivity concept** Von der Ohe and Liess (2004), Wogram and Liess (2001)	**Pesticide Index** Humpheryes (pers. communication)	**LIMPACT** Neumann *et al*. (2003)	**PERPEST** Van den Brink *et al*. (2002)
	Type of pesticide pollution	Organophosphates, organochlorines, strobilurines, triazoles, organic phosphorous acid at concentrations >= 1:1,000 LC50 *daphnia magna*, several others at lower concentrations.	Organophosphate (parathion-ethyl)	N/A	Drift, drainflow, surface runoff, accidental spillage.	Organophosphates, carbamates, organochlorines, strobilurines, triazoles, >= 1:1,000 LC50 *daphnia magna*, several others at lower concentrations	N/A
	Reliability	Firm link between decrease of SPEAR abundance in relation to overall abundance (%SPEAR abundance) and measured pesticide levels (temporal coincidence, no significant influence of other environmental parameters), in each of the studied regions significant decrease in %SPEAR abundance at concentrations >= 1:100 *daphnia magna*	N/A	N/A	Unclear	No independent dataset, testing with training set.	N/A
	Robustness	Robust to geographical variation.	N/A	N/A	Unclear	No independent dataset, testing with training set.	N/A
	References	Liess and Von der Ohe, 2005; Schäfer *et al*, 2007.	Schriever and Liess, 2006.	N/A		Neumann *et al*., 2003.	N/A
General comment		A long-term field study from Germany linked governmental inventory monitoring data on stream invertebrate	Predictions matched observed loss of SPEAR after short-term exposure to parathion-ethyl .		It has not yet been possible to see data showing that taxon sensitivity is clearly due to pesticides rather than	Positive and negative indicator taxa.	N/A

Table II.2 Tabular summary of pesticide-specific approaches at community level

		Species at risk (SPEAR) concept Liess and Von der Ohe (2005)	**SPEAR-based indicator**	**Relative sensitivity concept** Von der Ohe and Liess (2004), Wogram and Liess (2001)	**Pesticide Index** Humpheryes (pers. communication)	**LIMPACT** Neumann *et al*. (2003)	**PERPEST** Van den Brink *et al*. (2002)
		communities to modelled pesticide runoff; long-term decrease in %SPEAR abundance at sites of medium to very high runoff inputs; long-term decrease in other community measures such as diversity or EPT numbers only at high to very high levels of modelled runoff inputs (Schriever *et al*., 2007a)			other diffuse pollution stresses. Identification of sensitive families is based on correlation (families absent in areas lacking organic stresses and potentially exposed to pesticides) rather than observing cause-effect of pesticides.		
Requirements	Diagnostic tool to identify pesticide contamination	Established linear relationship between TU and %SPEAR abundance can be used to diagnose pesticide contamination based on %SPEAR abundance at test sites.	Prognostic use of the relationship between TU and %SPEAR abundance, but diagnostic use is also possible.	N/A	Designed as diagnostic tool.	Yes	No
	Distinguish pesticides from other stressors	Yes, tested for streams with agricultural catchment.	N/A	N/A	No evidence currently available.	Yes	N/A
	Sensitive to subtle changes	Yes, field study using modelled runoff levels showed that long-term decrease in %SPEAR abundance at lower runoff levels than long-term decrease in other community measures such as diversity or EPT	N/A	N/A	Unlikely	Yes, since rules are based upon such subtle changes.	N/A

Table II.2 Tabular summary of pesticide-specific approaches at community level

	Species at risk (SPEAR) concept Liess and Von der Ohe (2005)	**SPEAR-based indicator**	**Relative sensitivity concept** Von der Ohe and Liess (2004), Wogram and Liess (2001)	**Pesticide Index** Humpheryes (pers. communication)	**LIMPACT** Neumann *et al*. (2003)	**PERPEST** Van den Brink *et al*. (2002)
Indicate trends over time	N/A	Yes	N/A	No evidence currently available.	N/A	N/A
Indicate the relative order of magnitude of pesticide contamination	Yes (making use of established relationship between TU and %SPEAR abundance).	Yes	N/A	No evidence currently available.	Yes	N/A
Indicate the time period of contamination	Yes (field studies showed significant decrease from pre to main application period of agrochemicals in the study regions).	N/A	N/A	No evidence currently available.	No	N/A
Compare levels of pesticide contamination between different regions in the UK	Possible (field studies demonstrated robustness).	N/A	N/A	N/A	No	N/A
Be an early warning system	Possible (sensitive to subtle changes, see below).	N/A	N/A	Unlikely	No	N/A
Objective (robust)	Yes	N/A	N/A	Lacks clear evidence of cause-effect relationship.	Yes	N/A
Transparent	Few rules, easily understandable for non-specialists.	Exposure-response relationship, easily understandable for non-specialists.	N/A	Yes	Concept: Yes, following the decision-making process is reasonably complex.	N/A
Giving a good summary	Yes (single value that summarises community composition).	Yes	N/A	Yes, scoring yields single value.	Yes, one contamination class is assigned.	N/A
Results are easy to interpret	Yes (%SPEAR abundance related to orders of magnitude of pesticide contamination)	Yes	N/A	Yes (but lacks clear evidence of cause-effect relationship).	Classifying sites as unimpacted, medium impacted or highly impacted by pesticides.	N/A

Table II.2 Tabular summary of pesticide-specific approaches at community level

		Species at risk (SPEAR) concept Liess and Von der Ohe (2005)	**SPEAR-based indicator**	**Relative sensitivity concept** Von der Ohe and Liess (2004), Wogram and Liess (2001)	**Pesticide Index** Humpheryes (pers. communication)	**LIMPACT** Neumann *et al*. (2003)	**PERPEST** Van den Brink *et al*. (2002)
	Quick	Yes (data from quick inventory monitoring can indicate contamination if pesticide-sensitive species are absent, but for confirmation sorting in the lab and standard software is necessary).	N/A	N/A	Yes (data from quick inventory monitoring can indicate contamination if pesticide-sensitive species are absent, but for confirmation sorting in the lab and specific software is necessary).	No	N/A
	Cheap	Yes (can run on GQA data, additional sampling during main period of agrochemical application may be necessary).	N/A	N/A	Yes	Additional sampling necessary.	N/A
Assessment in the context of current Environment Agency procedures	Currently used	No	No	N/A	No	No	N/A
	Ready to use for diagnosis	Yes	Yes, trait-based and therefore easier to transfer to different regions than taxonomy-based approaches.	N/A	No (approach needs refinement).	No (approach needs additional data for re-calibration).	N/A
	Missing information	Endemic species in England and Wales (gaps can be filled by literature review and expert questioning).	UK species and information about traits for UK endemic species.	N/A	Empirical data relating loss of taxa to pesticide exposure in different regions of the UK.	Chemical monitoring data for calibration in depth taxonomical identification (genus or species).	N/A
	Could be easily added to current techniques	No changes in practice, just additional way of looking at species.	Yes, though in depth taxonomic identification required (genus/species)	N/A	Yes	No (additional biomonitoring required) .	N/A
	Could only be adopted with major revision of practices	N/A	N/A	N/A	N/A	Yes	N/A

Table II.3 Tabular summary of approaches at molecular level that are non-specific to pesticides

		Heat shock proteins (HSP)	Glutathione S-transferase (GST)	Cytochrome P450 monooxygenases (CYP450)	Catalase (CAT)	Steroid metabolism
General	Category	Biochemical biomarker	Biochemical biomarker	Biochemical biomarker	Biochemical biomarker	Biochemical biomarker
	Type	Existing method, measurement of heat shock protein induction.	Existing method, but only developed as a biomarker in a handful of freshwater invertebrates.	Existing method but with a number of different methodologies, quite difficult to measure in invertebrates.	Existing method that has been developed in a few organisms.	Existing method to measure the metabolism of testosterone.
	Aim	To indicate cellular stress.	To look for quantitative changes in GST activity to indicate increased conjugation of toxins.	To measure increased expression of detoxification enzyme to indicate exposure to various toxins.	To detect oxidative stress.	Detect endocrine disruption.
	Concept	Quantitative	Quantitative, amount of activity.	Quantitative, amount of activity.	Quantitative/qualitative.	Quantitative/qualitative.
	Endpoint 1	Freshwater macroinvertebrate/fish.	Freshwater macroinvertebrate but is also used in fish and marine organisms.	Freshwater macroinvertebrate but is also used in fish and marine organisms.	Freshwater macroinvertebrate/fish.	Freshwater macroinvertebrate/fish.
	Endpoint 2	Suborganismal, induction of protein.	Suborganismal, enzyme induction.	Suborganismal, enzyme induction.	Suborganismal, enzyme inhibition or induction.	Suborganismal, metabolism of testosterone.
	Methodology	Biochemical method, comparison between animals in polluted site vs. control site.	Biochemical method, comparison between animals in polluted site vs. control site.	Biochemical method, comparison between animals in polluted site vs. control site.	Biochemical method, comparison between animals in polluted site vs. control site.	Biochemical method, comparison between animals in polluted site vs. control site.
	Underlying data	N/A	N/A	N/A	N/A	N/A
	(Required) Input data	N/A	N/A	N/A	N/A	N/A
	Specificity to different modes of actions	Responds also to heat and seasonal changes, metals.	OK for pesticides, but doesn't differentiate between classes, also responds to temperature stress.	Shows general stress and is quite good for detecting PAHs.	Specific for oxidative stress, some pesticides can cause this.	Very little data on effect of pesticides.
	Software existing	No	Software converts microtitre plate assays in mean scores	Software converts microtitre plate assays in mean scores.	Software converts microtitre plate assays in mean scores	No
Successful field evaluation	Geographical region	Various	Various	Various	Various	Various
	Tested water body type	Lagoon, rivers, estuaries.	Lagoon, rivers, estuaries.	Lagoon, rivers, estuaries.	Lagoon, rivers, estuaries	Lagoon, rivers, estuaries
	Number of test sites	Unknown	Unknown	Unknown	Unknown	Unknown

Table II.3 Tabular summary of approaches at molecular level that are non-specific to pesticides

		Heat shock proteins (HSP)	Glutathione S-transferase (GST)	Cytochrome P450 monooxygenases (CYP450)	Catalase (CAT)	Steroid metabolism
	Sources of pesticide pollution	Unknown	Unknown	Unknown	Unknown	Unknown
	Type of pesticide pollution	Organophosphates, herbicides, not specific.	Has worked with organophosphates, OC, herbicides.	Organochlorines, organophosphates, herbicide, pyrethroids.	Pyrethroids.	Fungicide, organophosphates.
	Reliability	Poor	Can demonstrate stress but is confounded by responses linked to seasonal changes and temperature.	Good if monitoring PAHs, but low response in macroinvertebrates.	Not enough info.	Not enough info.
	Robustness	Low	A number of instances where sensitivity is low.	Low sensitivity.	Not enough info.	Not enough info.
	References					
General comments						
Requirements	Diagnostic tool to identify pesticide contamination	Shows that sufficient stress to cause denaturation of proteins, but not specific to pesticides.	If responding to pesticides, it does show that the organism is exposed, but insufficient data to link with effects.	If responding to pesticides, it does show that the organism is exposed, but insufficient data to link with effects.	Oxidative stress can be very harmful, so if this is caused by a pesticide, it will reveal effects.	If pesticide is responsible, then it shows it is severely disrupting reproduction, but insufficient data so far in freshwater invertebrates.
	Distinguish pesticides from other stressors	No	No	No	No	Not enough info.
	Sensitive to subtle changes	Possibly	Sometimes	Not in macroinvertebrates.	Not enough info.	Not enough info.
	Indicate trends over time	No	No	No	No	Not enough info.
	Indicate the relative order of magnitude of pesticide contamination	Possibly if get a response, it could be dose-dependent.	If you do get a response, it could be dose-dependent.	If you do get a response, it could be dose-dependent.	If you do get a response, it could be dose-dependent.	Not enough info.
	Indicate the time period of contamination	Induction responses can be fast.	Unclear, but induction responses can be fast.	Unclear, but induction responses can be fast.	Unknown	Not enough info.
	Compare levels of contamination	Yes	Possibly	Possibly	Possibly	Yes

Table II.3 Tabular summary of approaches at molecular level that are non-specific to pesticides

		Heat shock proteins (HSP)	Glutathione S-transferase (GST)	Cytochrome P450 monooxygenases (CYP450)	Catalase (CAT)	Steroid metabolism
	between different regions in the UK					
	Be an early warning system	Shows animals are stressed.	Of stress, yes.	Of oxidative stress, yes.	Of stress, yes.	Shows presence of endocrine disruption.
	Objective (robust)	Yes	Yes	Yes	Yes	Yes
	Transparent	Yes, if doing a comparison between control and polluted sites, easy to see difference.	Yes, if doing a comparison between control and polluted sites, easy to see difference.	Yes, if doing a comparison between control and polluted sites, easy to see difference.	Yes, if doing a comparison between control and polluted sites, easy to see difference.	No
	Giving a good summary	Yes	Yes	Yes	Yes	No
	Results are easy to interpret	Yes	Yes	Yes	Yes	No
	Quick	No	Not in the field but assays can be done very quickly in the lab (few hours).	Methods we have used are very time-consuming.	Unknown	No, either measured using CYP450 techniques or using TLC methods.
	Cheap	No	Relatively speaking it is cheap, but additional sampling, equipment and consumables needed.	Depends on the method used.	Depends on the method used.	No
Assessment in the context of current Environment Agency procedures	Currently used	No	No	No	No	No
	Ready to use for diagnosis	No	No	No	No	No
	Missing information	Yes	Yes	Yes	Yes	Yes
	Could be easily added to current techniques or current monitoring.	No	No	No	No	No
	Could only be adopted with major revision of practices	Yes	Yes	Yes	Yes	Yes

Table II.4 Tabular summary of approaches at the community level that are non-specific to pesticides

		PSYM Biggs *et al*. (2000)	**RIVPACS** Wright *et al*. (1998)	**RPDS** Walley and O'Connor (2002)	**RPBBN** Walley and O'Connor (2002)
General	Category	Community level/taxonomy-based	Community level/taxonomy-based	Community level/taxonomy-based	Community level/taxonomy-based
	Type	Empirical system	Empirical system	Expert system based on clustering	Expert system based on probabilistic reasoning
	Aim	Designed to assess ecological integrity of small lakes, ponds and canals.	Designed to assess ecological integrity of rivers and streams.	Designed to diagnose biological impairment in rivers and streams through pattern recognition.	Designed to diagnose impacts of water quality stresses using a Bayesian Belief Network.
	Concept	Quantitative, based on presence/absence data	Quantitative, based on presence/absence data	Qualitative	Qualitative
	Endpoint 1	Macroinvertebrates, wetland plants	Macroinvertebrates	Macroinvertebrates	Macroinvertebrates
	Endpoint 2	Family	Family	Family	Family
	Methodology	Statistical	Statistical	To simulate and systematise pattern recognition process used by experts in interpreting complex data sets: clustering using MI-max algorithm.	Allows prediction of 'what if' scenarios when physical and/or chemical conditions are modified.
	Underlying data	Database of minimally impaired sites (pesticide free).	Database of minimally impaired sites (some sites may be occasionally exposed to pesticides).	Environment Agency GQA invertebrate and water chemistry data.	Environment Agency GQA invertebrate and water chemistry data.
	(Required) Input data	Family invertebrate and plant data; 13 field and map-based environmental variables.	Family invertebrate data; 12 field and map-based environmental variables.	Family level invertebrate data, 1995 UK General Quality Assessment programme.	Invertebrate data, 1995 UK General Quality Assessment programme.
	Specificity to different modes of actions	Not specific	Not specific	N/A	N/A
	Software existing	Yes	Yes	Yes	Yes
	Successful field evaluation	Evaluation in progress.	Very extensively evaluated.	Limited evaluation in England and Wales.	Limited evaluation in England and Wales.
	Geographical region	United Kingdom.	United Kingdom.	England and Wales.	England and Wales.
	Tested water body type	Ponds and lakes.	Rivers and streams.	Rivers and streams.	Rivers and streams.
	Number of test sites	None specifically for pesticides.	None specifically for pesticides.	None specifically for pesticides.	None specifically for pesticides.
	Sources of pesticide pollution	N/A	N/A	N/A	N/A
	Type of pesticide pollution	N/A	N/A	N/A	N/A

Table II.4 Tabular summary of approaches at the community level that are non-specific to pesticides

		PSYM Biggs *et al*. (2000)	**RIVPACS** Wright *et al*. (1998)	**RPDS** Walley and O'Connor (2002)	**RPBBN** Walley and O'Connor (2002)
	Reliability	N/A	N/A	N/A	N/A
	Robustness	N/A	N/A	N/A	N/A
	References				
General comments		Could be used to provide an expected list of taxa sensitive to pesticides. Requires that such taxa are identified in the UK.	Could be used to provide an expected list of taxa sensitive to pesticides. Requires that such taxa are identified in the UK.	Could be used to match patterns of invertebrate assemblages to pesticide stress if empirical pesticide data were available.	Could be used to predict effects if empirical relationships between invertebrate occurrence and pesticide concentrations were available.
Requirements	Diagnostic tool to identify pesticide contamination	Not a diagnostic system.	Not a diagnostic system.	Does not currently have the capability to diagnose pesticide pollution.	Does not currently have the capability to diagnose pesticide pollution.
	Distinguish pesticides from other stressors	Potentially if pesticide-sensitive taxa could be identified.	Potentially if pesticide-sensitive taxa could be identified.	Potentially if empirical pesticide data were available.	Potentially if empirical pesticide data were available.
	Sensitive to subtle changes	Unknown	Unknown	Unknown	Unknown
	Indicate trends over time	Potentially if pesticide-sensitive taxa could be identified.	Potentially if pesticide-sensitive taxa could be identified.	Potentially if empirical pesticide data were available.	Potentially if empirical pesticide data were available.
	Indicate the relative order of magnitude of pesticide contamination	Potentially if pesticide-sensitive taxa could be identified.	Potentially if pesticide-sensitive taxa could be identified.	Potentially if empirical relationships between pesticide exposure and invertebrate assemblages data were identified.	Potentially if empirical relationships between pesticide exposure and invertebrate assemblages data were identified.
	Indicate the time period of contamination	Unlikely	Unlikely	Unlikely	Unlikely
	Compare levels of pesticide contamination between different regions in the UK	Potentially if pesticide-sensitive taxa could be identified.	Potentially if pesticide-sensitive taxa could be identified.	Potentially possible if sufficient empirical data were available.	Potentially possible if sufficient empirical data were available.
	Be an early warning system	Unlikely	Unlikely	Unlikely	Unlikely
	Objective (robust)	Potentially if pesticide-sensitive taxa could be identified.	Potentially if pesticide sensitive taxa could be identified.	Potentially	Potentially
	Transparent	Yes	Yes	Concept: Yes, underlying mathematical operations are	Concept: Yes, underlying mathematical operations are

Table II.4 Tabular summary of approaches at the community level that are non-specific to pesticides

		PSYM Biggs *et al*. (2000)	**RIVPACS** Wright *et al*. (1998)	**RPDS** Walley and O'Connor (2002)	**RPBBN** Walley and O'Connor (2002)
				reasonably complex.	reasonably complex.
	Giving a good summary	Potentially if pesticide-sensitive taxa could be identified.	Potentially if pesticide-sensitive taxa could be identified.	Potentially	Potentially
	Results are easy to interpret	Potentially if pesticide-sensitive taxa could be identified.	Potentially if pesticide-sensitive taxa could be identified.	Reasonably	Reasonably
	Quick	No (requires sorting of sample to obtain data).	No (requires sorting of sample to obtain data).	No	No
	Cheap	Yes	Yes	Yes	Yes
Assessment in the context of current Environment Agency procedures	Currently used	Yes (but not for pesticide risk assessment).	Yes (but not for pesticide risk assessment).	Yes (but not for pesticide risk assessment).	Yes (but not for pesticide risk assessment).
	Ready to use for diagnosis	No	No	No	No
	Missing information	Pesticide-sensitive taxa have not been identified in the UK.	Pesticide-sensitive taxa have not been identified in the UK.	Pesticide-sensitive taxa have not been identified in the UK; RPDS does not have empirical pesticide data.	Pesticide-sensitive taxa have not definitively been identified in the UK; RPBBN does not have empirical pesticide data.
	Could be easily added to current techniques or current monitoring.	N/A	N/A	N/A	N/A
	Could only be adopted with major revision of current practice	N/A	N/A	N/A	N/A

III. Costed plan of AChE assay

Method

The AChE assay procedure was based on the generic assay reported by Ellman *et al*. (1961) adapted for use in microtiter plates (Fisher *et al*., 2000). Pooled frozen organisms, originated from the mass exposure experiment, were homogenized in 1.5 ml Eppendorf tubes with ice-cold 0.02 M sodium hydrogen phosphate buffer (PB), pH 8.0, containing one per cent Triton-X-100 (Sigma, Poole, UK). The homogenization was manual, with a microcentrifuge tube pestle (50 cycles, 10 seconds). Ice-cold PB (without Triton-X-100), in a 10:1 ratio, was added to the initial homogenate. The final homogenates were mixed and centrifuged at 14,000 *g* and 2 to 4°C for four minutes. Supernatants were transferred to a clean precooled tube, mixed with a whirlimixer (Fisher Scientific, Loughborough, UK), and assayed immediately. Additions to the microtiter plate were made in the following order: 100 µl of 8 mM 5,5′-dithio-bis(2-nitrobenzoate) (D-8130, Sigma) in PB supplemented with sodium hydrogen carbonate at 0.75 mg/ml; 50 µl of assay blank (PB containing 0.1 per cent Triton-X-100), supernatant, or quality-control enzyme (eel cholinesterase, C-3389 Sigma); and 50 µl of 16 mM acetylthiocholine iodide in PB (A-5751 Sigma). The microtiter plate was inserted into the integral incubator of a Dynex MRXII plate reader and incubated at 30°C for five minutes. This was followed by measuring the change in optical density per minute at 405 mm and 30°C over 10 minutes with intermittent shaking. Enzyme activity was expressed in moles/L/min/g protein. Protein concentration in homogenate supernatants was determined by using a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The protein standard curve was prepared with a series of bovine serum albumin (BSA) standards diluted in blank buffer.

Costs

Initial investment of equipment

- Ultracentrifuge for Eppendorf tubes, with cooling. **approx. £2,000**
- Microtitre plate reader (Dynex MRXII, Dynex Technology). **approx. £4,000**
- Multi-channel pipettes (50-250 µl). **£400 each x 2**
- Waterbath at 30^{o}C (only if using frozen larvae).
- Ice bucket and water ice.
- 50 ml glass beaker with deionised water for washing homogenisation pestle.
- Whirlimixer

AChE assay cost

Plasticware

- Small weighing boats for use as reagent troughs for filling multi-channel pipettes –10p each (need three per plate) **£0.30**
- Gilson p1000, p200 and tips – 1p each **£0.10**
- Multi-channel pipette tips – 50p each **£1.50**
- 1.5 ml Eppendorf tubes – 11p each **£1.10**
- Teflon Eppendorf pestle for homogenisation in Eppendorf – 5p each (reusable) **£0.20**
- Clean, flat bottom 96 well microtitre plates, 70p each. Recently used plates may be recycled by rinsing thoroughly in warm tap water followed by deionised water. **Do not use detergents**. **£0.70**
- **Total** **= £3.90/plate**
  (most of these costs were from Sigma – significant discounts on plasticware are available if bought in bulk).

Chemical reagents

- 100 µl x 8 mM 5,5′-dithio-bis(2-nitrobenzoate) (D-8130, Sigma) = 10ml approx/plate **(60p)**
- 50 µl x 16 mM acetylthiocholine iodide (A-5751 Sigma) = 5 ml per plate **= 10p/plate**
- Triton X-100 £25.80 100 ml **pennies**
- Eel cholinesterase 500 units = £18.20 (0.5 units/ml) 2 ml/assay (QC) = one unit = **4p**
- Phosphate buffer **pennies**
- **Total** **approximately £1.00/plate to compare two sites**

AChE assays should be accompanied by a protein reading. The protein assay kits are from BCA Pierce and cost £3.20 per plate in reagents (plus £2.00 in plasticware) = **£5.50 total.**

Summary

AChE assay cost = **£4.90**

Protein assay cost = **£5.50**

Total cost (not including equipment and staff) = **£10.40/plate to compare two sites.**

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