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Identifying freshwater invertebrate taxa susceptible to AChE-acting pesticides☆

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ABSTRACT

Globally pesticide use has been associated with negative impacts on riverine invertebrate communities, but chronic exposure effects to most specific groups of pesticides are not well understood. In this paper, we sought to identify invertebrate species most vulnerable to effects of AChE-acting pesticides in UK rivers for potential application in environmental monitoring. We did this using a combination of the conservation of molecular target for AChE-acting pesticides (identified using the SeqAPass tool), laboratory-based toxicity data, and both biological traits and life history information. We then applied this information to assess for evidence of impacts on these riverine invertebrate communities in the Anglian region of England where there is high pesticide use. Sensitive genera to AChE-acting pesticides included *Aedes, Anopheles, Cloeon, Ischnura*, and the tolerant genera were *Culex, Daphnia, Lymnaea*. Using the UK Environment Agency's biota monitoring data spanning over period of 28 years (1984–2011) we evidence absence of the taxa most sensitive to AChE-acting pesticides (*Ischnura, Aedes and Anopheles*) but presence of AChE pesticide tolerant taxa (*Lymnaea*) at riverine sites in the Anglian region with high AChE pesticide use.

1. Introduction

Chemical pollution has been shown to impact adversely on freshwater invertebrates globally (Clements, 1994; Beasley & Kneale, 2002; Smith et al., 2008; Dijk, Staalduinen & Sluijs, 2013; Beketov et al., 2013) and for pesticides, accidental spills and some specific surface water outflow events have been shown to cause declines in aquatic invertebrate populations, and in some cases localised extinctions (Raven & George, 1989; Beketov et al., 2013; Reiber et al., 2021). Associations are reported between declining riverine invertebrate populations and chronic (year on year) pesticide exposures (Dijk et al., 2013), however, these associations are compounded by a wide range of other, often highly variable water physicochemical factors including organic enrichment/eutrophication, pH, temperature, flow, and salinity (Heugens et al., 2008; Stampfli et al., 2013; Macaulay et al., 2021; Bray et al., 2021a). Trait bioindicators for pesticide effects on aquatic invertebrates, such as the SPEAR*pesticides* (Liess & Von Der Ohe, 2005), are influenced too by water physicochemical properties (e.g., nutrients, salinity) (Malherbe, Van Vuren and Wepener, 2018; Jones et al., 2023) complicating the task of identifying the impact of pesticides as a contributing factor in the status of aquatic invertebrate populations. SPEAR*pesticides* has been shown to be poorly correlated with pesticide exposure in mesocosm experiments using malathion (Bray et al., 2021b).

Determining sensitivity of riverine invertebrates to pesticides is complex as this is affected by the absorption, distribution, metabolism, and excretion of the pesticide within the organism's body (toxicokinetics) and the way in which the pesticide affects the organism's physiology that leads to its toxic effects (toxicodynamics). Understanding the potential impacts of pesticides on riverine invertebrate populations and ecosystems clearly requires understanding both the toxicokinetics and toxicodynamics of these compounds. Thus, in addition to the presence (conservancy) of the pesticide target site, at the

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organism level, vulnerability to pesticide effects in the natural environment will be affected by the organism's biological traits and life histories (Rico & Van den Brink, 2015). These factors include where the organism lives in the riverine environment (sediment dwelling organisms tend to have higher exposures), and both structural (e.g. epidermal/cuticle thickness) and physiological features (e.g., ability to metabolise and excrete the chemical) of the organism. An organism's sensitivity to chemicals furthermore is dependent on the ability to recover from an exposure. The toxicity of pesticides to different organisms can be assessed through laboratory-based exposure experiments, but these data are lacking for the majority of taxa and pesticides. Extrapolating for pesticide sensitivity across taxa is challenging not least because many of the detoxifying metabolic enzymes (e.g. Cytochromes P450s (CYP), Glutathione S-transferases (GST)) have diverged within the invertebrate phyla (Spurgeon et al., 2020). It is the case, however, that those taxa more closely related to terrestrial species against which pesticides are targeted (i.e. those targeted for plant protection) will have a higher level of conservancy of the molecular target site and thus likely a higher sensitivity to a given pesticide (Brasseur et al., 2023).

The UK's pesticide application (kg/ha) is amongst the highest in Europe and globally (7th highest application out of an assembled listing for 30 European and African countries assessed between 2000 and 2012) and harmful effects on riverine invertebrates for acute exposures from pesticide spills are well established (Raven & George, 1989; Sharma et al., 2019). Mortalities associated with pesticide spills include for acetylcholinesterase -AChE-inhibiting chemicals (e.g. organophosphates and carbamates) (Raven & George, 1989; Dowson et al., 1996) which are among the most commonly used pesticides for agriculture and indoor purposes (Karami-Mohajeri & Abdollahi, 2011). AChE inhibiting pesticides act by inhibiting an enzyme (acetylcholinesterase) that degrades the neurotransmitter acetylcholine which is essential in the functioning of the central nervous system (CNS) of humans, other mammals and in invertebrates (Jones, 2005). The build-up of acetylcholine can result in rapid twitching of voluntary muscles and eventual paralysis (Fulton & Key, 2001).

Globally pesticide concentrations in rivers for AChE inhibiting pesticides vary widely. The highest exposure concentrations recorded for key selected pesticides include, up to 0.65 μg/l for diazinon, 10.08 ng/l for dimethoate, 3.24 ng/l for malathion, 0.0676 μg/l for chlorpyrifos, 3.03 ng/l for primiphos-methyl, 2.19 ng/l for fenitrothion, 0.83 ng/l for methidathion, and, 2.83 ng/l for parathion (Wee et al., 2016; Behrooz et al., 2021; Montuori et al., 2022). In the UK, riverine concentrations of pesticides belonging to the AChE group also show wide variation, with levels recorded historically up to 0.94 μg/l for dimethoate, 0.86 μg/l for diazinon, 0.22 μg/l for fenitrothion, 0.11 μg/l for malathion, 0.05 μg/l for parathion, 0.12 μg/l for carbaryl, and 2500 μg/l for chlorpyrifos (from spill event; Raven and George, 1989; Croll, 1991; Long et al., 1998). More recently measured concentrations for AChE-acting pesticides have been measured up to 0.14 μg/l for fenitrothion, 0.109 μg/l for malathion, 0.0028 μg/l for pirimicarb, 0.006 μg/l for diazinon, and 0.02 μg/l for dichlorvos (Comber, Mistry & Sturdy, 2012; Proctor et al., 2019). However, most individual pesticides in English rivers are reported to be below quantification limits (<0.005 μg/l) for extensive periods of monitoring, as determined using liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry – see WIMS Environment Agency database (https://environment.data.gov.uk/water-quality/view/landing). Maximum average concentrations of total AChE pesticides (sustained over 36-days) have been recorded up to 0.72 μg/l (Poyntz-Wright et al., 2024). Despite the wide geographical use of AChE-acting pesticides (Kadiru, Patil & Souza, 2022) the extent to which sustained exposures to environmentally relevant concentrations are impacting UK aquatic invertebrate populations is still relatively poorly understood.

In the UK's comprehensive national monitoring program, there is a lack of geographically concurrent biota and chemical sampling therefore in seeking to identify invertebrate species most vulnerable to pesticides acting through AChE for their potential application in environmental monitoring and to assess for the influence of chronic exposure to these pesticides on riverine invertebrate populations, we adopted an alternative weight of evidence. In this approach, we first identified those invertebrate taxa likely to be the most sensitive to these pesticides considering the presence of molecular target site for AChE pesticides. We then looked to assess their innate sensitivity to AChE pesticides from the available laboratory testing data, in combination with information on the species biological traits and life history information (to assess exposure susceptibility). This information was then applied to assess for the prevalence of AChE sensitive taxa versus AChE tolerant taxa in the Anglian region of the UK, a region dominated by arable farming with a high associated use of pesticides (Poyntz-Wright et al., 2023), using the UK Environment Agency's biota monitoring data collected over a 28 year period (1984–2011).

2. Method

2.1. Data collection

Laboratory toxicity test information for pesticide effects on invertebrate families was derived from the ECOTOX database (https://cfpub.epa.gov/ecotox/search.cfm). Invertebrate sequence data, for molecular target site comparisons, was collected from the NCBI database using the SeqAPASS tool. Macroinvertebrate monitoring data for riverine sites in the Anglian region was sourced from ChemPop, CEH BIOSYS database (https://environment.data.gov.uk/ecology/explorer/), and chemical and physical site data were sourced from the WIMS database (https://environment.data.gov.uk/water-quality/view/landing).

2.2. *Identification of AChE sensitive* ***terrestrial invertebrates (individual level)***

We first determined the most sensitive **terrestrial** invertebrate species to AChE-acting pesticides from laboratory toxicity studies based on acute exposures. Using the ECOTOX database, we identified terrestrial species' sensitivity to the 34 AChE pesticides monitored by the UK Environment Agency. We selected LC50 or LD50 as the endpoint for each pesticide based on the fact this parameter had the largest dataset, offering the broadest range of species for comparison. The dose units for pesticides in laboratory experiments varied and included mg/kg of body weight and mg/l. To assess the overall sensitivity of each species to the AChE-acting pesticides as a whole, we calculated the mean toxicity concentration for each dose unit (mg/kg body weight and mg/L water concentration) separately. This approach enabled us to identify the most sensitive taxa for each dose unit, without making any assumptions regarding their equivalence. Although we recognise that the route of application (exposure) can affect the toxicity of a chemical we averaged the dose units for the LC50/LD50s collectively in our analysis for all the chemical exposures thus maximising the number of species assessed. Whilst this has its limitations, it nevertheless allowed us to identify taxa that were some of the most sensitive to the effects of these groups of pesticides, which was the primary purpose of this part of the study analysis. We incorporated data only from studies that satisfied the following criteria: experiments were conducted with biological replicates and exposure durations were between 1 and 7 days. This exposure period was adopted to maximise the acute data available for the analysis whilst also ensuring data were largely comparable across studies (see Fig. S8). For some chemicals no laboratory data were available fitting these criteria and the final list of chemicals used in our analyses for AChE-acting pesticides included, azinphos-ethyl, azinphos-methyl, carbaryl, carbofuran, carbophenothion, chlorpyrifos-ethyl, coumaphos, Demeton-s-methyl, diazinon, dichlorvos, dimethoate, ethion, fenitrothion, fonofos, malathion, methomyl, oxamyl, parathion-ethyl, parathion-methyl, phorate, pirimicarb, pirimiphos-methyl, triazophos.

2.3. *Identification of* **aquatic invertebrates** *potentially sensitive to AChE-acting pesticides based on conservancy of molecular target sites* ***(individual level)***

AChE-acting pesticides are designed to target terrestrial invertebrates. Here we assessed the potential susceptibility of **aquatic invertebrates** to AChE-acting pesticides by first comparing the conservation of their molecular target sites with terrestrial invertebrates with established sensitivities to AChE pesticides. We identified the most sensitive terrestrial species with available genomic information of the molecular target site AChE to use them as the comparators with aquatic invertebrates. For each dose unit—mg/kg of body weight and mg/l—a single most sensitive terrestrial taxon was designated the comparator. We employed SeqAPass to assess the conservancy of the target site of AChE pesticides in aquatic invertebrates with the terrestrial taxa. SeqAPass (accessible at https://seqapass.epa.gov/seqapass/) is a tool designed for predicting potential organisms' susceptibility to chemicals by evaluating the conservancy of molecular targets. This process entailed examination of the conservancy at the primary amino acid sequence level (Level 1) and a comparison of protein functional domain sequences (Level 2). The Level 1 comparison involves querying the AChE target site, within the NCBI database to generate the corresponding query sequences. The chosen sequences encompassed the entire molecular target sites and were free of any association with pesticide resistance. The complete molecular target sequences of all aquatic invertebrates in the NCBI database were then compared to the terrestrial query sequences, generating a BLAST output. The BLAST outputs provide the percentage sequence similarity of each aquatic invertebrate to the queried terrestrial invertebrates. Based on this similarity, we determined whether aquatic invertebrate taxa were likely or not to be sensitive to AChE pesticides. Sensitivity is determined by comparing the percentage sequence similarity to a predefined 'cut-off.' We set a 'cut-off' by analysing the distribution of sequence similarities (as percentages) calculated from each invertebrate's target protein. This analysis identified the lowest sequence similarity to the reference taxa in the data and was called the local minimum. We then compared the local minimum with ortholog candidate data. The 'cut-off' is determined as the point where an ortholog candidate shows a level of similarity equal to, or higher, than the local minimum observed in the data. If the sequence similarity is equal to or greater than this 'cut-off,' the aquatic invertebrate is considered sensitive. For the Level 2 analysis, assessing the conservation of functional domains at the molecular target sites (AChE), we compared the sequences of functional domains in aquatic invertebrates to those of terrestrial invertebrates, again generating BLAST outputs. The resulting BLAST output provided information on the percentage similarity for each functional domain sequence in aquatic invertebrates in relation to the corresponding sequences in the queried terrestrial invertebrates. We then assessed the percentage sequence similarity in relation to the sensitivity 'cut-off' (cut-off as defined above). Aquatic invertebrates with functional sequences exhibiting a similarity greater than the 'cut-off' were considered sensitive to AChE-acting pesticides. Aquatic invertebrate taxa were included in these analyses where there was a clearly identified AChE binding site (i.e. a relatively conserved primary amino acid sequence compared with their sensitive terrestrial counterparts) and coding for at least one conserved functional domain in the AChE receptor and thus likely to have the potential to be sensitive to AChE-acting pesticides. Taxa without an apparent molecular target site were excluded assuming they would not be responsive/sensitive to AChE-acting pesticides. Fig. 3 provides a detailed representation of these analyses and further details on the methods are provided in LaLone et al. (2013).

2.4. *Identification of aquatic invertebrates predicted to be most sensitive to AChE pesticides (population level)*

Aquatic invertebrates potentially sensitive (i.e. demonstrating significant conservancy of molecular target sites for pesticides) or tolerant to AChE-acting pesticides were then assessed based on laboratory toxicity data. For each of these aquatic invertebrate genera and every AChE pesticide found in English rivers, we gathered data on the LC50 and established the overall acute toxicity level for each genus to AChE pesticides by calculating the mean toxicity concentration across all pesticides (see Fig. 1).

We then set out to estimate the sensitivity of aquatic taxa for chronic exposures (i.e. those exposures commonly occurring in riverine environments albeit these exposures may occur in a pulsatile manner). Chronic exposure data for most pesticides were lacking so we established an acute/chronic effect ratio for AChE-acting pesticides for the genera where these data were available (see Table S2 and Fig. S8). From this, for each genus and pesticide, we identified the (fold) difference between the average acute sensitivity and average chronic sensitivity (see Table S3). The mean effect ratios for the AChE-acting pesticides for acute and chronic exposures were then determined for all genera/pesticide combinations (Table S3). Subsequently, the overall mean of acute/chronic effect ratios was then used to extrapolate the potential chronic toxicity threshold for the genera based on available acute toxicity information (Figs. 2 and 3), as illustrated below:

$$\text{Chronic AChE sensitivity of genus} = \frac{\textit{Mean acute (AChE) concentration per genus}}{\textit{Chronic adjustement factor}}$$

The chronic adjustment factor was based on exposure studies which covered a range of life stages, environmental conditions (temperature, pH), pesticides and genera. For AChE pesticides we categorized genera as either sensitive or tolerant by comparing their calculated chronic AChE sensitivity to the maximum average concentrations for AChE-acting pesticides recorded over 36-day periods ($n = 44$) in an English river (Poyntz-Wright et al., 2024). This field maximum average concentration is the highest sustained AChE pesticide concentration identified in an English river and taxa with chronic sensitivity level greater than 0.72 μg/l are unlikely to be negatively affected by sustained pesticide levels in English rivers. Aquatic invertebrate genera where a concentration >0.72 μg/l was needed to induce a chronic lethal effect were deemed tolerant, and those for which a concentration of ≤ 0.72 μg/l was chronically lethal were deemed sensitive. These categories simply separated genera into those likely to be more tolerant (present) or sensitive (absent) to high chronic AChE pesticide pollution in riverine sites. Some chemicals had no laboratory data available fitting the criteria previously outlined in section 2.1.

2.5. *Identification aquatic invertebrate taxa predicted to be most sensitive or tolerant to AChE pesticides based on trait and life history information (population level)*

We used biological traits and life history information, to determine the potential susceptibility of aquatic invertebrates to pesticides in the natural environment. In this analysis, we considered only aquatic taxa identified in the previous sections with conserved molecular target sites

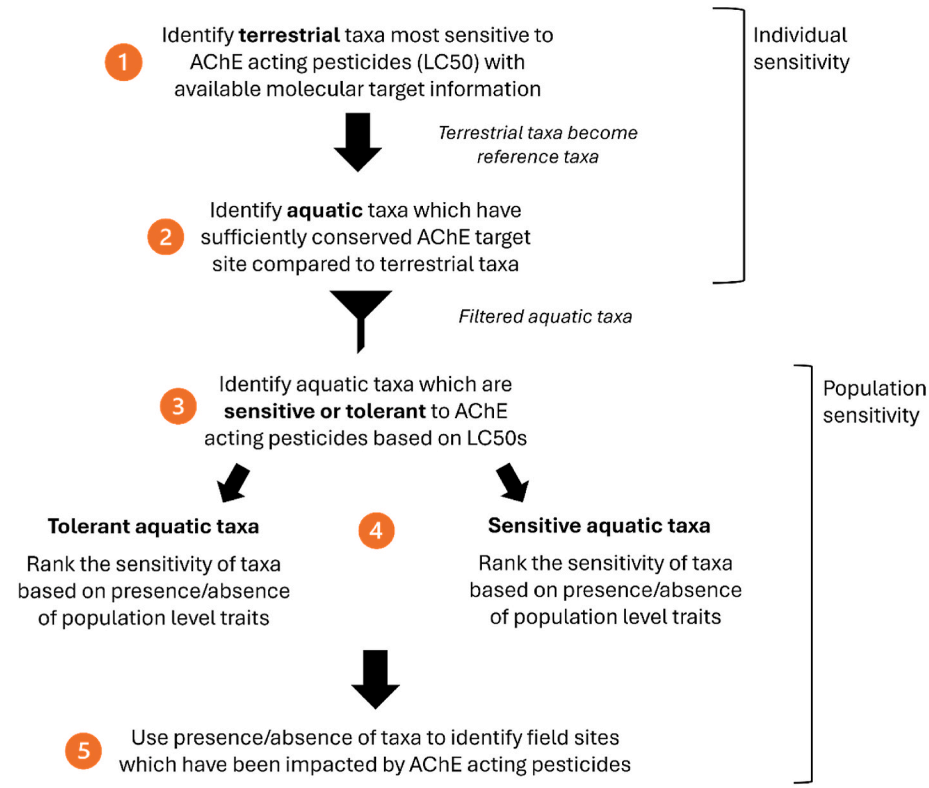

Fig. 1. A graphical representation of the methodological steps involved in our analysis.

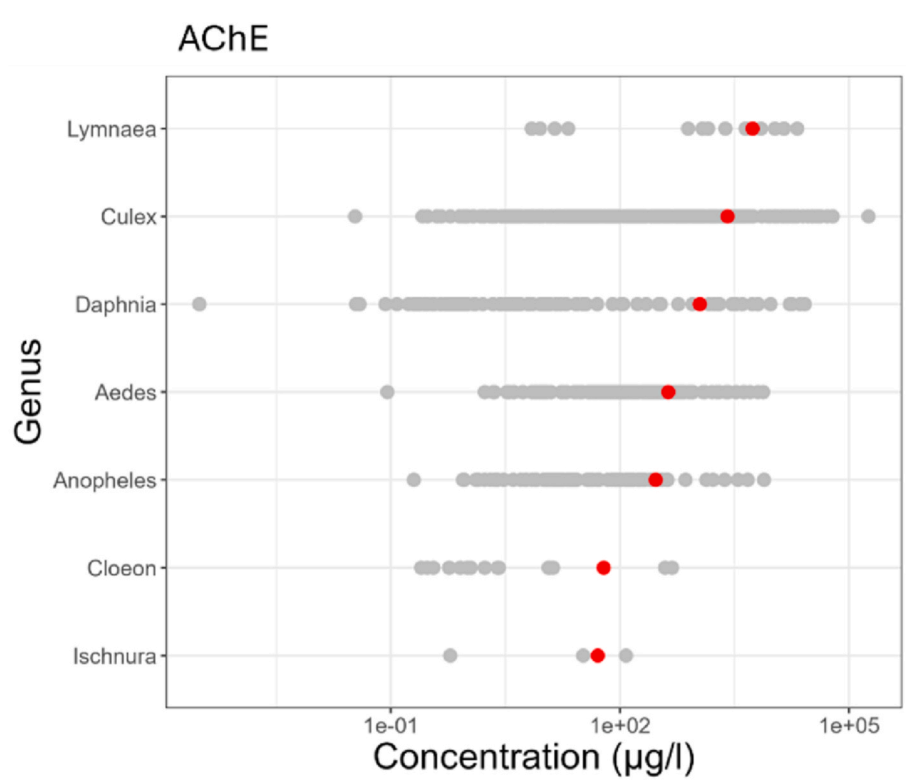

Fig. 2. Sensitivity of aquatic invertebrates to AChE-acting pesticides (mortality) for acute exposures. Grey dots are the LC50/EC50 from studies for that genus. Red dots indicate the mean LC50/EC50 from across all the studies. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

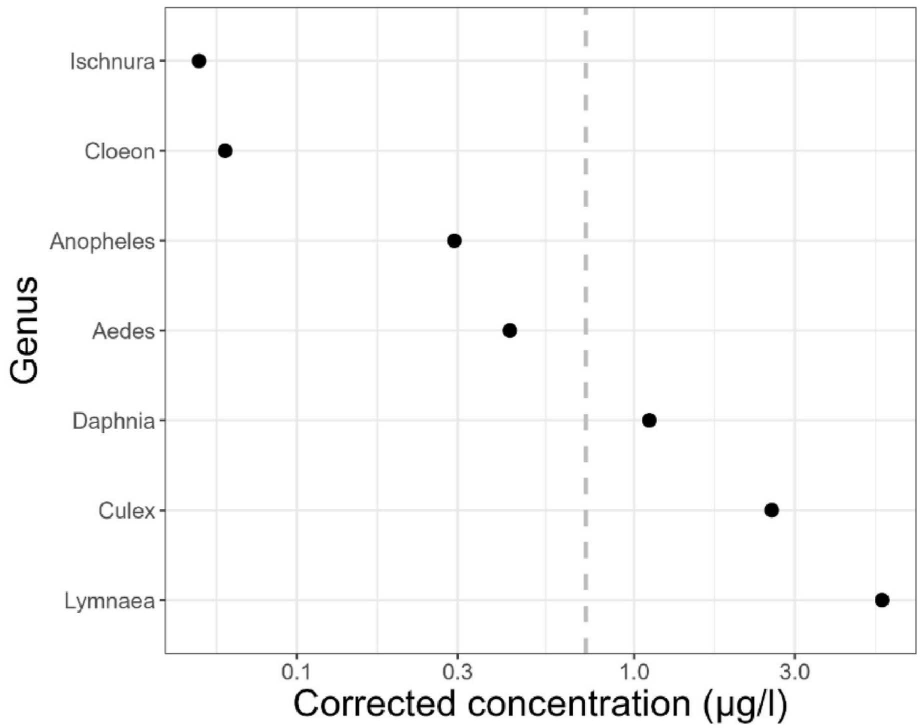

Fig. 3. Chronic sensitivity of aquatic invertebrates to AChE-acting pesticides (LC50) derived from applying the chronic adjustment factor to the acute effect concentrations (average difference between acute and chronic sensitivities of genera to AChE-acting pesticides). The chronic adjustment was a factor of 1000 (see Tables S1–3 and Fig. S8). Grey dashed line represents maximum recorded sustained concentration observed in an English river (0.72 μg/l; Poyntz-Wright et al., 2024).

(acetylcholinesterase) and for which laboratory toxicity information to AChE pesticides was available. The traits considered important for the susceptibility of aquatic taxa to pesticides, based on available literature, included; 1) **poor drift** (rare: catastrophic only) from upstream refuges, resulting in a lack of replacement of taxa downstream after a pollution event and consequently a smaller population (slower recovery); 2) **long life cycle duration** (>1 year) results in greater exposure to pesticides over taxa's life-time; 3) **few generations** per year (semi/uni-voltine) means potentially slower population recovery from pesticide impact; and 4) **predatory lifestyle** which can result in biomagnification of pesticides from prey resulting in greater exposure (Baird & Van den Brink, 2007; Ippolito, Todeschini & Vighi, 2012; Mondy & Usseglio-Polatera, 2013; Rico & Van den Brink, 2015). Drift and generations per year are also considered in the well-known bioindicator for pesticides SPEAR*pesticides*, whereas life cycle duration and predatory lifestyle are not. We accessed information from the following databases to compile the traits of aquatic invertebrates (genera): Sarremejane et al. (2020), Invertebres D'eau Douce. Systematique, Biologie, Ecologie | Request PDF (researchgate.net) and Aquatic invertebrate traits database | NIWA. The traits in the databases were weighted based on the proportion of species in the genera with the trait (fuzzy coded see Chevene, Doleadec & Chessel (1994) for explanation). The decision to weight dominant traits within genera was motivated by the recognition that the different species within a genus can exhibit considerable variation in their biological traits. By identifying the most dominant (or common) traits among species within a genus, we aimed to capture the overarching sensitivity profile of that genus to pesticides. This approach facilitated a direct comparison of genera in terms of their sensitivity to pesticides We identified the most dominant trait as that which was most common (present in the greatest proportion of species) within a given genera. Consequently, for each trait either taxa were defined as susceptible ('1' – trait present) or not susceptible ('0' – trait absent). The scores across all traits were then summed to provide an overall ranking for AChE sensitivity. Taxa with a higher rank (total score) had a greater number of pesticide susceptible traits were deemed more likely to be sensitive to the effects of pesticides. We have assumed that traits equally contribute to determining the sensitivity of taxa to AChE acting pesticides as there is not a robust method for quantifying how the different traits compare in affecting pesticide exposure sensitivity.

2.6. Identifying pesticide impacted sites based on indicator taxa in the Anglian region

We identified sites in the Anglian region where riverine invertebrate communities were more likely to have been exposed to pesticide pollution using the BIOSYS database synthesized by ChemPop at the UK Centre for Ecology and Hydrology (UKCEH) (Qu et al., 2023). The dataset was comprised of observations from 213 riverine sites in the Anglian region, collected over a span of 28 years. We assessed for the presence and absence of tolerant and pesticide sensitive pesticide taxa, identified as such in the preceding sections. Sensitive genera to AChE-acting pesticides included *Aedes*, *Anopheles*, *Cloeon*, *Ischnura*, while tolerant genera were comprised of *Culex*, *Daphnia*, *Lymnaea*. We identified which sites had AChE genera with only sensitive, only tolerant or both sensitive and tolerant genera. Sites with only sensitive, and both sensitive and tolerant genera potentially experienced low pesticide pressure, while sites with only tolerant genera potentially experienced high pesticide pressure.

We then identified a subset of field sites where pesticides likely had the least and the most impact on riverine invertebrate communities over the 28 years. This was done by hypothesising that sites experiencing the lowest pesticide pressure were likely to contain the most sensitive taxa (genus) and sites potentially experiencing the highest pesticide pressure had the most tolerant taxa (genus) present only. For AChE-acting pesticides, the most sensitive genera were *Ischnura, Aedes and Anopheles* and the most tolerant genus was *Lymnaea*.

2.7. Data analysis

All data was processed and graphs curated using R programme R Core Team (2021) version 4.2.1 and QGIS (2022) was used for mapping. Code is avaliable at: https://github.com/ImogenPW/Identifying-AChE-and-GABA-acting-pesticide-bioindicator-taxa. We employed the use of T-tests to compare the difference in means of land-use between sites with only tolerant taxa (*Lymnaea*; n = 26) and sites with both tolerant and sensitive taxa (*Lymnaea*, *Ischnura, Aedes and Anopheles*; n = 131). We corrected for multiple testing using the Bonferroni adjustment method.

3. Results

3.1. Identifying **terrestrial invertebrates** sensitive to AChE-acting pesticides

Terrestrial invertebrate sensitivity to AChE pesticides differed across species, as illustrated in Figs. S1–4. For those species which had target gene sequence information available in the NCBI database, *Myzus persicae* (mg/l) and *Stomoxys calcitrans* (mg/kg) were amongst the taxa exhibiting highest sensitivity to AChE-acting pesticides. *Dermestes ater* (mg/kg) and *Hemisarcoptes caccophagus* (mg/l) were the most tolerant taxa to these pesticides.

3.2. Identifying potential sensitive **aquatic freshwater invertebrates** based on conservancy of molecular target sites

Aquatic invertebrate taxa with both a primary amino acid sequence and at least one functional domain conserved to a level greater than the susceptibility 'cut-offs' (defined in section 2.2), and thus potentially sensitive to AChE pesticides, include *Aedes, Anopheles, Aphelenchoides, Cloeon, Culex, Daphnia, Dreissena, Ecdyonurus, Ephemera, Helobdella, Hirudo, Ischnura, Lymnaea, Orthetrum, Polypedilum, Pomphorhynchus, Sympetrum* (see Figs. S3–4).

3.3. Identifying freshwater invertebrates sensitive to AChE pesticides based on available laboratory toxicity data

Aquatic invertebrates are presented for which there was both a conserved molecular target site and available laboratory-based effect concentrations. For the remaining genera listed there was no laboratory effects data available and therefore we could include them in our sensitivity analyses. For AChE-acting pesticides, *Ischnura* was the most sensitive genus, whereas *Lymnaea* was the least sensitive genus (Fig. 2). However, after applying a chronic adjustment factor to determine the more likely sensitive taxa in the field to sustained pesticide pollution, and considering the maximum average AChE-acting pesticide concentration in rivers, the most likely genera to be affected by AChE pesticides included *Ischnura, Cloeon, Anopheles* and *Aedes* (LC50 < 0.72 μg/l; Fig. 3). Genera *Daphnia, Culex* and *Lymnaea* were deemed least likely to be affected by AChE-acting pesticides as chronically adjusted LC50s were greater than the maximum average AChE-acting pesticide concentration recorded in the field; see Fig. 3).

3.4. Identifying invertebrate taxa most sensitive to pesticide AChE-acting pesticides

Susceptibility of aquatic genera to AChE-acting pesticides differed based on population relevant traits, within sensitive and tolerant taxa groupings. The most sensitive (susceptible) taxa to AChE-acting pesticides were *Ischnura*, *Aedes* and *Anopheles* followed by *Cloeon* (see Table 1), whereas the most tolerant taxa to AChE-acting pesticide were *Lymnaea* and (followed by) *Culex* (see Table 1).

3.5. Anglian region locations with AChE pesticide -sensitive and -tolerant taxa

We identified sites in the Anglian region of England which have potentially been impacted by pesticides (AChE-acting pesticides) based

Table 1
Ranking of aquatic taxa sensitivity to AChE pesticides based on chronic sensitivity to pesticides and population relevant traits.

	Genus	Poor drift	Long lifecycle duration	Few generations per year	Predatory lifestyle	Total score	Rank
Sensitive	*Ischnura*	1	0	1	1	3	1
	Aedes	1	0	1	1	3	1
	Anopheles	1	0	1	1	3	1
	Cloeon	1	0	0	0	1	2
Tolerant	*Culex*	1	0	1	1	3	1
	Lymnaea	1	0	1	0	2	2

*Blue shaded rows indicate non-sensitive taxa and rank. Grey shaded rows indicate sensitive taxa and rank. A higher **total score** means the taxa are more susceptible to AChE-acting pesticides. Poor drift (drift is rare - yes (1)/no (0)), long life cycle duration (life >1 year – yes (1)/no (0)), few generations (semi/uni-voltine – yes (1)/no (0)), predatory lifestyle (feeding habit is predatory - yes (1)/no (0)).

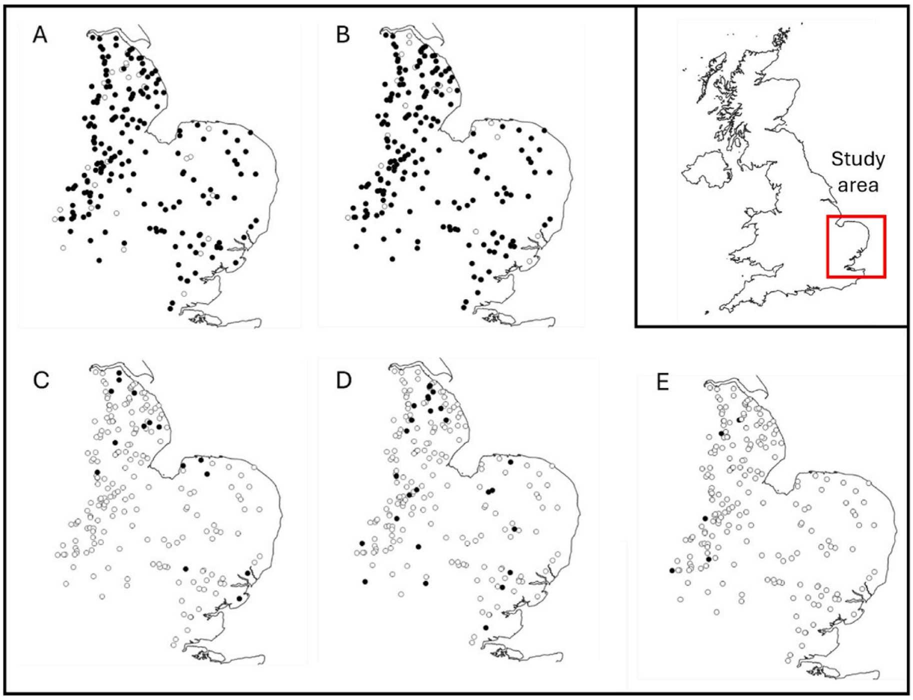

Fig. 4. Maps show the presence geographically of freshwater invertebrate taxa across sites sampled in the Anglian region of England between 1984 and 2011. A) presence of sensitive taxa (*Ischnura, Cloeon, Aedes* and *Anopheles*), B) presence of tolerant taxa *(Lymnaea* and *Culex*), C) presence of only sensitive taxa and D) presence of only tolerant taxa. Closed circles indicate presence of taxa, open circles indicate taxa absence. E) sites with neither tolerant nor sensitive taxa (closed circles indicate where there is neither taxa, open circles indicate where taxa have been recorded). See Table S5 for the number and percentage of sites. No sites contained *Aedes*.

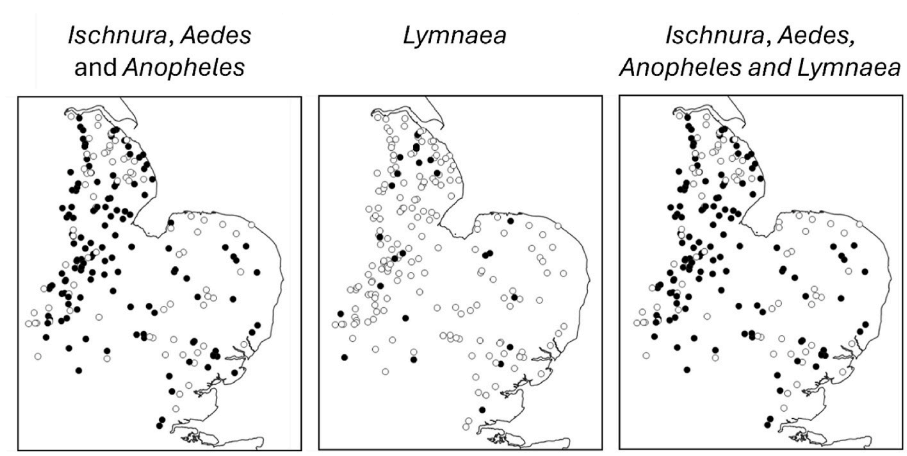

Fig. 5. Geographical locations for the presence (closed circles) and absence (open circles) of the most sensitive taxa – *Ischnura, Aedes and Anopheles* - and tolerant taxa - *Lymnaea* - to AChE pesticide at riverine sites in the Anglian region from 1984 to 2011. Plot (a) presence/absence of *Ischnura, Aedes and Anophelese (most sensitive taxa)*, (b) sites where **only** *Lymnaea* (most tolerant taxa) was recorded present/absent, and (c) *Ischnura, Aedes and Anopheles* and *Lymnaea* taxa were recorded present/absent. All sites with *Anopheles* contained *Ischnura.* No sites contained Aedes.

on the presence and absence of pesticide tolerant and sensitive invertebrate taxa over a 28-year period (between 1984 and 2011; Fig. 4 and S9). For taxa with available trait/life-history information we identified a total of 26 sites (12% of those assessed) which had only tolerant AChE taxa (*Lymnaea*), and 17 sites (8% of those assessed) with only sensitive AChE taxa recorded (*Ischnura* and *Cloeon*), see Fig. 4 and S8. *Culex* and *Anopheles* were recorded present at sites with both tolerant and sensitive taxa. There were 167 sites which had both tolerant and sensitive taxa (*Lymnaea, Culex, Anopheles, Cloeon and Ischnura*) detected over the monitoring period indicating low pesticide impact (see Fig. S8 'total taxa' map and Fig. S7).

We identified sites likely most and least impacted by AChE-acting pesticide pollution based on the occurrence of the most- and least-sensitive taxa to this class of pesticides. The most sensitive AChE taxa (*Ischurna, Aedes and Anopheles*) were recorded at 134 sites and these sites therefore have likely experienced low AChE pesticide pollution (*Lymnaea* was also present at 132 of these sites; see Fig. 5). Twenty-six sites had only the most tolerant taxon *Lymnaea* recorded present over the 28 years (Fig. 5) and may thus likely have experienced high AChE pesticide pollution.

4. Discussion

Using molecular, laboratory toxicity, biological trait and life history information, we hypothesised that six invertebrate taxa are either tolerant or sensitive to AChE-acting pesticides. Based on this, we identified 26 sites where AChE-acting pesticides likely had adverse impacts on riverine invertebrate communities in the Anglian region of England between 1984 and 2011.

4.1. Identification of taxa with conserved pesticide molecular target site/s

Many aquatic invertebrate taxa were identified with high conservancy in the molecular target site for AChE-acting pesticides compared with terrestrial invertebrate taxa sensitive to these pesticides, rendering them potentially susceptible to their toxic effects. Based on the molecular target site conservancy, seventeen aquatic taxa were identified as potentially sensitive to compounds targeting acetylcholinesterase (Figs. S3–4). It is important to recognise that other aquatic invertebrates may also have high conservancy of the target sites for these pesticides (making them potentially susceptible to their effects) but the limited available genomic information prevented a comprehensive analyses for aquatic phyla as a whole (Hotaling, Kelley & Frandsen, 2020). Notably, for those phyla deemed sensitive to the effects of the AChE-acting pesticides, the level of conservation of target sites in the primary amino acid and functional domain sequences differed, in some cases considerably, and even between aquatic invertebrates within the same order (Figs. S3–4). For AChE sensitive taxa, variation in the conservation of sequences differed between species within a given taxon by up to 50%. These difference are likely a result of natural selection and microevolutionary or macroevolutionary changes (Klerks, Xie & Levinton, 2011; Spurgeon et al., 2020; Brasseur et al., 2023). Taxa with a more conserved target site for the chemical are generally more sensitive to that chemical, and thus have a greater likelihood for an adverse exposure effect (i.e. lower LC50s; LaLone et al., 2013). This is well illustrated for the conservancy of voltage sodium channel sequences shown to predict taxa sensitivity to permethrin (LaLone et al., 2013). However, the presence or absence of conserved molecular target sites is one of many factors that influences a species overall sensitivity.

4.2. Sensitivity of aquatic invertebrates to AChE-acting pesticides based on laboratory toxicity data

Based on the laboratory toxicity data (mortality) we found marked differences in the relative toxicity thresholds to AChE-acting pesticides for invertebrates with conservation of molecular target sites, with acute toxicity (LC/LD50s) concentrations ranging between 0.07 and 5 mg/l (Fig. 2), supporting that factors other than conservancy in the molecular target site for these pesticides can have a considerable bearing on susceptibility to their effects. Within some genera there were taxa sensitive to AChE-acting pesticides and others that were tolerant, as occurred for example in the *Diptera* (Fig. 3). Some of these differences likely relate to variations other aspects of the organisms enzymes systems involved in pesticide metabolism (i.e. cytochrome P450s (CYPs), glutathione S-transferases (GSTs)), chemical transporters, and off-target receptor binding sites that have been shown to impact sensitivity to pesticides in invertebrates (Spurgeon et al., 2020).

After adjusting acute toxicity levels for chronic exposures (by applying a chronic adjustment factor), we identified four taxa, all belonging to the Insecta, which would likely be affected by AChE-acting pesticides for the sustained exposure concentrations observed in England's rivers ($\leq$0.72 μg/l; Fig. 3). Species with chronic toxicity thresholds to AChE pesticides above 0.72 μg/l (Fig. 3) and unlikely to be affected in English rivers through sustained exposure to these pesticide included *Daphnia*, *Culex* and *Lymnaea*. Lack of available data, however, for both chronic and acute exposures, was a major limitation for analyses for many genera, identifying a major knowledge gap for assessing the risks of these pesticides to freshwater invertebrates.

4.3. Susceptibility of taxa based on population traits/life history information

Although laboratory experiments provide information on the innate sensitivity of organisms to pesticides, biological traits will also affect an organism's susceptibility to pesticides in the natural environment (Vignati, Ferrari & Dominik, 2007; Reiber et al., 2022). The population traits considered in the current analysis are those previously determined to affect the susceptibility of taxa to pesticides and encompassed those which affect the ability of population to recover (Kreutzweiser & Sibley, 1991; Galic et al., 2012; Katagi & Tanaka, 2016; Baudrot et al., 2020). Considering the innate sensitivity of taxa together with population traits, the most sensitive taxa to AChE-acting pesticides were from the orders Odonata and Diptera (Insecta), and the most tolerant taxa was from the order Achatinoidea (Gastropoda), see Table 1. This finding supports the identification of Mollusca as the most dominant taxa at pesticide impacted sites (Becker et al., 2020). Elsewhere, high abundance of Gastropoda taxa have been noted in drainage ditches (in Argentina) with high concentrations of AChE-acting pesticides (azinphos-methyl and chlorpyrifos), and at concentrations greater than those observed in England (Macchi et al., 2018; Poyntz-Wright et al., 2024). In Egypt *Lymnaea* (Gastropoda) has been found surviving at high concentrations of AChE-acting pesticides in water courses, and again at concentrations exceeding those observed in the England's rivers (Sayed et al., 2021; Poyntz-Wright et al., 2024), further illustrating *Lymnaea's* high tolerance to AChE-acting pesticides. Supporting our findings for *Ischnura* studies on pond and riverine systems in mainland Europe and in Pakistan have provided evidence to show that natural populations of *Ischnura* are sensitive to AChE-acting pesticides (Van Praet et al., 2014; Arambourou & Stoks, 2015; Ilahi et al., 2020). Further a study on a Western Cape river in South Africa has shown exposure to AChE acting pesticides caused a major reduction in natural populations of Diptera (Bollmohr & Schulz, 2009). Overall, the invertebrates identified within our study show differences in their sensitivities to those used in regulatory studies (e.g. Daphnia and Gammarus) illustrating that regulatory species do not fully represent the sensitivity range for AChE acting pesticides in the natural environment. Incorporating species like *Ischnura, Aedes, Anopheles* and *Lymnaea* into regulatory testing protocols would help ensure greater protective regulatory standards for the diversity of invertebrate populations occurring in UK rivers.

Individual traits relating to, for example, respiratory type and level of sclerotization can also affect sensitivity to pesticides (Ippolito et al., 2012; Rico & Van den Brink, 2015) but these factors were not taken into account in our analyses as their contribution to population-level sensitivity is currently unclear.

4.4. Identification of riverine sites with invertebrate populations potentially impacted by AChE-acting pesticides

For numerous sites across the Anglian region the most sensitive taxa to AChE-acting pesticides (*Ischnura, Aedes and Anopheles*) were found to be absent (with only tolerant taxa (*Lymnaea*) present) over a 28-year monitoring period, consistent with an impact of AChE-acting pesticides on riverine invertebrate communities at these sites (Fig. 5). A previous study using the SPEAR*pesticides* bioindicators indicated a more even impact of pesticides across the Anglian region (Poyntz-Wright et al., 2023), however, SPEARpesticide is designed to determine the impact of insecticides as a whole rather than for a specific group, such as here for AChE-acting pesticides.

We attempted to investigate other chemical and physical stressors which might be associated with the present/absence of sensitive and tolerant taxa at the study sites. However robust chemical information was available for two of the sites holding tolerant taxa only thus precluding such analysis (see Figs. S10–S17). These data are much needed as part of long-term field studies to help better define in-field relationships between pesticides and population status for invertebrate taxa over time. Despite the limited data, physical and chemical features of sites did not appear to explain the presence or absence of *Ischnura, Aede and Anopheles*. *Ischnura* prefers muddy substrate with turbid, low velocity waters and are slightly tolerant to organic pollution (Merritt, Moore & Eversham, 1996; Dügel & Kazanci, 2004; Allen, Le Duc & Thompson, 2010). *Aedes* prefer alkaline water with low turbidity and total dissolved solids, and *Anopheles* prefers muddy, gravel and sandy substrates and low turbidity (Azari-Hamidian, 2011; Dalpadado, Amarasinghe & Gunathilaka, 2022). In the current study we found sites with and without *Ischnura, Aedes and Anopheles* that showed similar flow rates, pH, turbidity (NTU), percentages of sand, silt, clay, pebbles and boulders and organic pollution (nitrate, nitrite, orthophosphate, ammonia, ammoniacal nitrogen and biological oxygen demand concentrations; Figs. S10–S17).

At some sites only sensitive taxa were present (Fig. 4). The reason for the absence of pesticide tolerant taxa at these sites is unclear. Sampling time is unlikely the reason for the absence of *Culex* as this is commonly found during spring and autumn and *Lymnaea* is found all year round (ECDC, 2020; Fodor et al., 2020) and all sites were sampled during spring and autumn (see supplementary material – S19). High concentrations of nutrients, for example, has been shown to be associated with absence of *Lymnaea* (Buglife, 2013; Benali, Bouderbala & Chevre, 2022), whereas this is not the case for *Culex* (Muturi et al., 2007). Site specific information was limited for rivers in the current study, and clearly metadata on these sites for wider features of the water physicochemistry, flow, substrate, nutrient levels etc., would be valuable for information to help answer this question.

In these analyses defining the relative risk of riverine invertebrates to AChE-acting pesticides was limited by the lack of both chronic laboratory toxicity information and data for sustained recorded riverine AChE-acting pesticide concentrations. The average acute to chronic sensitivity ratio (ACR; LC50s) we applied of 1000 (our defined chronic adjustment factor) for the riverine invertebrates exposed to AChE-acting pesticides, however, aligns with other studies assessing effects of other pesticides on aquatic invertebrates where differences of several orders of magnitude have been calculated (Raimondo, Montague & Barron, 2007; May et al., 2016; see Table S4 for ACRs). Also, the 'mean maximum' sustained river chronic AChE-acting pesticide concentration of 0.72 μg/l was derived from a Midlands river in England (the only location for which well-defined riverine concentrations for AChE are available in the literature; Poyntz-Wright et al., 2024) and, given the Anglian region has higher pesticide use generally than other regions in England (Poyntz-Wright et al., 2023), the sustained concentrations of AChE-acting pesticide in Anglian could exceed the current 0.72 μg/l limit used in this study. Nevertheless, the defined cut-off level of 0.72 μg/l still appropriate for the purpose of separating riverine invertebrates into groups which are likely to be more – or less-at risk for exposure to AChE-acting pesticides in Anglian rivers. It should be recognised that the current analysis takes place over 28-years, and the level of pesticide contamination at sites may have changed over this time. However, based on the presence of pesticide bioindicator taxa for sites in the Anglian region this would indicate a fairly consistent impact of pesticides over the time period (Poyntz-Wright et al., 2023). Differences in land-use (arable, woodland, urban, and semi-natural habitat) did not appear to explain differences in the between potentially AChE-acting pesticides impacted versus non-impacted sites (see S18).

5. Conclusion

We have identified four candidate invertebrate taxa, namely, *Ischnura, Aedes, Anopheles* and *Lymnaea*, as potentially useful for detecting AChE-acting pesticide pollution in English rivers, being highly sensitive and tolerant, respectively, to this class of pesticides. Furthermore, our findings indicate riverine invertebrate populations at many sites across the Anglian region of England have been impacted by AChE-acting pesticide pollution with no other obvious physical or chemical features of these rivers determined explaining the absence of taxa sensitive to AChE-acting pesticide, accepting the limitations of the data outlined above.

In the final analysis, although there are key data gaps that need filing to better understand the interactive effects of pesticides including with multiple abiotic factors (e.g., temperature, pH, and water flow) and biological interactions (e.g., species competition and predation) that can significantly influence the susceptibility of invertebrates to pesticide exposure in complex natural environments, adopting to sample for the invertebrate species we have identified as sensitive to AChE acting pesticides in the long-term monitoring programmes operated by the UK Environment Agency would improve our understanding on the impact of this widely used group of pesticides on British riverine invertebrate populations regionally and temporally, and support the conservation of the more susceptible species.

CRediT authorship contribution statement

Imogen P. Poyntz-Wright: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Xavier A. Harrison:** Writing – review & editing, Methodology. **Andrew Johnson:** Writing – review & editing. **Susan Zappala:** Writing – review & editing. **Charles R. Tyler:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125217.

Data availability

Data will be made available on request.

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