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Gene-to-ecosystem impacts of a catastrophic pesticide spill: testing a multilevel bioassessment approach in a large river ecosystem

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Title: Gene-to-ecosystem impacts of a catastrophic pesticide spill: testing a multilevel bioassessment approach in a large river ecosystem

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Summary

1. Pesticides can have strong deleterious impacts in freshwaters, but understanding how these effects cascade through natural ecosystems, from microbes to apex predators, is limited because research that spans multiple levels of biological organisation is rare.

2. We report how an accidental insecticide spill altered the structure and functioning of a river across levels ranging from genes to ecosystems. We quantified the impacts on assemblages of microbes, diatoms, macroinvertebrates and fish and measured leaf-litter decomposition rates and microbial functional potential at upstream control and downstream impacted sites two months after the spill.

3. Both direct and indirect impacts were evident across multiple levels of organisation and taxa, from the base of the food web to higher trophic levels. At the molecular level, differences in functional gene abundance within the impacted sites reflected a combination of direct and indirect effects of the pesticide, via elevated microbial populations capable of utilising chlorpyrifos as a resource (i.e. direct effect) and oxidising ammonia released by decaying macroinvertebrate carcasses (i.e. indirect effect).

4. At the base of the food chains, diatom taxa found only in the impacted sites were an order-of-magnitude larger in cell-size than the largest comparable taxa in control communities, following the near-extirpation of their consumers. Population biomass of the key detritivore *Gammarus pulex* was markedly lower, as was the rate of litter decomposition in the impacted sites. This was partially compensated for, however, by elevated microbial breakdown, suggesting another indirect food-web effect of the toxic spill.

5. Although many species exhibited population crashes or local extirpation, total macroinvertebrate biomass and abundance were largely unaffected due to a compensatory elevation in small tolerant taxa such as oligochaetes, and/or taxa which were in their adult aerial life-stage at the time of the spill (e.g. chironomids) meaning they avoided contact with

the polluted waters and were therefore able to repopulate quickly. Mass-abundance scaling of trophic links between consumers and resources revealed extensive restructuring within the food web.

6. This case study shows that pesticides can affect food-web structure and ecosystem functioning, both directly and indirectly across levels of biological organisation. It also demonstrates how an integrated assessment approach, as adopted here, can elucidate links between micro-biota, macroinvertebrates and fish, for instance, thus improving our understanding of the range of biological consequences of chemical contamination in natural ecosystems.

Introduction

Freshwaters are exposed to multiple pesticides and other toxic chemicals at local to global scales (Schinegger *et al.* 2011; Beketov *et al.* 2013; Stehle & Schulz 2015). Ecotoxicological experiments in the laboratory have revealed with great accuracy and precision how these can affect the survival of target species (e.g. *G. pulex*; Xuereb *et al.* 2007), and community- and ecosystem-level responses have been demonstrated in micro- and mesocosm experiments (e.g. Van den Brink *et al.* 1995; Van Wijngaarden *et al.* 1996; Traas *et al.* 2004; Halstead *et al.* 2014) and field surveys (Chung, Wallace & Grubaugh 1993; Triebskorn *et al.* 2003; Malaj *et al.* 2014). In the last decade, new indices of community response have been proposed specifically to detect pesticide pollution (e.g. Liess & Ohe 2005; Schäfer *et al.* 2007; Liess, Schäfer & Schriever 2008) and to link community change to toxicants in field data (e.g. Kefford *et al.* 2010).

Despite these advances, a mechanistic understanding of both the toxic effects of pesticides (i.e. direct) and those mediated via the food web (i.e. indirect) across multiple levels of

biological organisation (i.e. from genes to ecosystems) is still limited in natural settings (Kohler & Triebskorn 2013). This is likely because there are relatively few opportunities to understand how pesticides affect whole rivers or lakes, due to the logistical, ethical, and legal difficulties in conducting such a study in a controlled manner. Here, we address this research gap by quantifying the gene-to-ecosystem consequences of a major pesticide spill that caused widespread kills of macroinvertebrates over 15 km in a large lowland river by combining citizen science biomonitoring data with a suite of non-traditional measures of ecosystem impact.

Invertebrate data were collected by citizen scientists prior to, during and after the spill enabling before-after-control-impact (BACI) assessment. These data enabled the UK Environment Agency to identify chlorpyrifos as the cause of the catastrophic mortality following the spill. Chlorpyrifos is a widely used organophosphate pesticide (insecticide and acaricide) which attacks insect (and arachnid) nervous systems. Since insects are core intermediate species in almost all stream food webs, perturbations to their populations have potential to ripple through the entire food web, as bottom-up effects on the fish assemblage and top-down effects on the microbial communities that drive a range of biogeochemical processes. Specifically, chlorpyrifos can affect microbial, macroinvertebrate and fish populations, both directly and indirectly (see reviews by Barron & Woodburn 1995; Brock, Lahr & Van den Brink 2000; Giddings *et al.* 2014), food-web structure (Traas *et al.* 2004) and can suppress macroinvertebrate-mediated litter breakdown (Maltby & Hills 2008). Placing the potentially subtle effects of pesticides within a coherent multilevel framework requires a combination of structural and functional measures from the microbial community at the base of the food web to apex predators. This has been partially achieved in some studies using mesocosms (e.g. Van den Brink *et al.* 1995; Van Wijngaarden *et al.* 1996; Kersting & Van den Brink 1997; Halstead *et al.* 2014), but rarely in natural settings (Kohler & Triebskorn 2013), and never in a manner that simultaneously captures molecular-level

responses through to the full complexity of the food web in the same system.

Here we present data that reveal how chlorpyrifos affected the structure and functioning of the river food web, based on several complementary approaches including the abundance of targeted functional genes, those responsible for the degradation of chlorpyrifos(Kwak *et al.* 2012), for example, measures of microbial and macroinvertebrate resource use and "trivariate analysis" (*sensu* Cohen *et al.* 2009). This collection of measures across multiple levels of organisation provides a vital bridge between field and laboratory-based findings and highlights the advantages of using a holistic approach to understand chemical stressor impacts in natural ecosystems.

We test the following hypotheses:

1. The structure (assessed using the abundance of functional gene loci) and functional capacity of the microbial assemblage will change due to direct effects (i.e. the pesticide provides an additional substrate) and indirect effects (i.e. increased organic substrates are derived from decaying macroinvertebrates) of the pesticide.

2. Compensatory mechanisms will be evident in the food web in the aftermath of the spill, with less pesticide-sensitive, small, opportunistic, vagile, and fast-growing taxa (e.g. chironomids) higher in abundance and/or biomass in the absence of larger, slow-growing taxa (e.g. *Gammarus pulex*), relative to control communities.

3. Leaf litter breakdown will be impaired by the loss of key detritivores, with microbial activity hence accounting for a greater proportion of total litter breakdown.

4. The food web will undergo extensive restructuring, particularly in terms of altered mass-abundance scaling relationships of the links between nodes. Local extirpations of intermediate species (e.g. herbivorous insects) will release basal species under top-down control (e.g. benthic algae) while suppressing bottom-up fluxes to higher

trophic levels (e.g. fish).

Methods

Study site

The River Kennet is a lowland chalk tributary (catchment area 1200 km^2) of the River Thames in southern England, designated as a UK Site of Special Scientific Interest (SSSI). The river is groundwater-dominated, has hard water and is nutrient-rich (Fig. 1; Table 1). Its diverse fauna is dominated by Gammaridae, Baetidae, Ephemerellidae, Simuliidae and Chironomidae, which support an economically important salmonid game fishery (Wright *et al.* 2002; 2004).

On 1 July 2013, following their routine biomonitoring, a citizen-science group (Action for the River Kennet, ARK) reported a large-scale macroinvertebrate kill along a 15-km stretch of the river. On 2 July 2013, an Environment Agency pollution incident team collected the first samples for, and detected, the organophosphate chlorpyrifos. This insecticide attacks the nervous system of insects by inhibiting acetylcholinesterase, and can be toxic to fish and meiofauna (Carr, Ho & Chambers 1997; DeLorenzo, Scott & Ross 1999). Concentrations of of 0.52-0.82µg L^{-1} were recorded coming from the main tertiary sewage treatment works in Marlborough, Wiltshire, on 2 and 5 July, respectively (Fig. 1), probably resulting from a "down-the-drain" incident. The peak concentration was most likely missed by the sampling team, but even the measured concentration is sufficient to be acutely toxic to arthropods (Giddings *et al.* 2014), particularly over extended periods (i.e. >24 hours; Rubach, Crum & Van den Brink 2011). Chlorpyrifos was also detected at concentrations between 0.06-0.07 µg L^{-1} across the impacted study site on 5 July. By 9 July 2013 the pesticide was undetectable, indicating that a single pulse was received and remained in the water column for a few days.

Contribution of citizen scientists

Citizen scientists from ARK were trained by the Riverfly Partnership to collect and identify aquatic macroinvertebrates and had collected data for multiple sites for several years prior to and following the spill (Fig. S1). During the current study, they collected one monthly kick sample (3-minutes duration) from an upstream control and downstream impacted site (Fig. 1). A standard hand net (1-mm mesh) was used following the Riverfly Monitoring Initiative standard protocol (http://www.riverflies.org). The macroinvertebrates collected were identified live on the bank, without magnification, and abundance ranked per sample as: 0 = 0 individuals; 1-9 = 1; 10-99 = 2; 100-1000 = 3; >1000 = 4, for eight key groups: 1. cased Trichoptera; 2. caseless Trichoptera; 3. Ephemeridae; 4. Ephemerellidae; 5. Heptageniidae; 6. Baetidae; 7. Plectoptera; 8. Gammaridae, which were summed to give a total score based on the number and diversity of the target taxa. These data provide a critical BACI element to the study, enabling us to track the impact of the spill through both space and time.

Mean annual water chemistry data were obtained for Environment Agency monitoring stations located 2.3 km upstream and 2.7 km downstream from the spill and were similar across the study site (Table 1). These water chemistry data, combined with the ARK monitoring data of macroinvertebrates, showed no evidence of organic pollution from the sewage treatment works, indicating that sewage was an unlikely cause of the macroinvertebrate mortality event (Fig. S1).

Sampling protocol

Comprehensive biological sampling began in September 2013, as soon as possible after the chlorpyrifos spill had been identified as the causal agent, using an experimental design comprising three upstream control and three downstream impacted reaches, each 50 m long,

along a *c*. 6 km river stretch (Fig. 1). Sites were c. 1 km apart, with similar channel forms and riparian surroundings. Here we present data from two control and two impacted reaches (Fig. 1) for a suite of structural and functional indicators to test a multilevel bioassessment approach. Three sediment samples, a stone scrape, three Surber samples and depletion electrofishing were used to characterise microbial, diatom, macroinvertebrate and fish structural attributes, respectively. At each site, 10 fine- (0.5mm) and 10 coarse-mesh (10mm) leaf-litter bags were used to determine rates of decomposition driven by microbes alone or by whole communities (Woodward *et al.* 2012). In addition, a sample of river water was collected and incubated with a range of substrates to assess microbial functional capacity.

Microbial functional gene abundance

We used quantitative PCR (qPCR) to examine gene abundance for microbial functional and taxonomic marker genes. 16S rRNA gene abundance was used as a proxy for total bacterial abundance. Direct effects of the chlorpyrifos spill were examined using the organophosphate hydrolase gene (*opd*), which is responsible for the degradation of chlorpyrifos by bacteria; bacterial populations containing this gene have previously been demonstrated to increase in abundance at sites impacted by organophosphate (Kwak *et al.* 2012). Indirect effects were examined by quantifying the abundance of genes coding for enzymes involved in N-cycling: nitrite reductase (*nirS*) and ammonia monoxygenase (*amoA*) from ammonia-oxidising archaea (AOA) and bacteria (AOB) as these are most likely to reflect decomposition of dead arthropods in impacted sites. We hypothesised that decomposition of dead arthropods would result in an increased input of NH_4^+ from ammonification of organic N. We focused on *nirS* and *amoA* genes as both nitrification and denitrification pathways are important in removing N from systems and can be coupled when denitrifiers reduce the $NO3^-$ produced by the nitrifiers that oxidised NH_4^+. By focusing on functions of a range of populations, a change

across all populations combined provides an indicator for community-level effects of chlorpyrifos on river microbes. Full details of DNA isolation, primer details and qPCR cycling conditions are available in the Microbial Functional Gene Abundance section in the Supplementary Material.

Microbial functional potential

Open-water samples were collected from each site and returned to the laboratory in an ice-chilled cooler. Samples were allowed to settle (>10 min), after which a 100-µL aliquot was pipetted into each well of a Biolog EcoPlate, which contained a single carbon substrate, including carbohydrates, polymers, fatty acids and amino acids. Each well also contained the redox dye tetrazolium, which is reduced during microbial respiration, resulting in a measurable colour change. Each EcoPlate contains 31 substrates plus a no-substrate control in triplicate. Plates were incubated in the dark at 22°C for 5 days, after which colour change was quantified by measuring optical density at 600 nm using a Biotek HT absorbance reader (Biotek, Swindon, UK). For each EcoPlate, we calculated the substrate usage by subtracting the mean of the three no-substrate controls from each measurement. Usage was ranked across the substrates in each replicate, and the ranked optical densities were plotted to visualise broad changes across sites.

Population abundance, community structure and food web size-scaling

Quantitative depletion electrofishing was undertaken, with population densities estimated using the R package FSA (Ogle 2012) and iterative Maximum Weighted Likelihood statistics (equation S1 and S2 in Supplementary Material; Carle & Strub 1978). All fishes caught were identified to species and measured by fork length. For each species, individual dry mass was

calculated using length-mass regression equations. Full details of fish dry mass estimation can be found in the Food web characterisation section of the Supplementary Material.

Invertebrates were collected (n = 3 samples per site) using a Surber sampler (0.0625 m^2, 335 µm mesh), preserved in 99.8% ethanol, and later sorted from debris, identified to the highest possible taxonomic resolution (usually species), and counted (Table S1). Dry masses of macroinvertebrates were determined from regressions of linear dimensions using published equations (see Table S2); a subset of 60 individuals were measured per species per site, or every individual where abundance was below 60. We distinguished between arthropods (i.e. insect larvae and Crustacea) and other taxa (i.e. Tricladida, Annelida and Mollusca) based on their sensitivity to chlorpyrifos (Raven & George 1989; Giddings *et al.* 2014).

Diatoms were scraped from 8.64 cm^2 of the upper surface of one cobble at each site using a toothbrush and 3.6 by 2.4 cm photographic slide as a flexible quadrat, preserved using Lugol's iodine, and prepared using standard methods (Battarbee *et al.* 2001). A minimum of 300 diatom valves were identified to species per sample using the keys of Krammer & Bertalot (1986), Krammer *et al*. (1986), Krammer & Lange-Bertalot (1991a b) and abundances per unit area were determined as in Battarbee (1973). Linear dimensions were measured to the nearest 1 µm to estimate diatom biovolume (Table S3; Hillebrand *et al.* 1999). The first 30 specimens of all common (n >30) species were measured and where species were encountered less frequently, all specimens in the count were measured. Carbon content was estimated (Rocha & Duncan 1985) and then converted to dry mass (Sicko-Goad, Schelske & Stoermer 1984).

We used these mass-abundance data from across the different taxa and trophic levels to construct whole-community 'trivariate food webs' - food webs ordinated by overlaying feeding links on the bivariate relationship between species mean body mass and their numerical abundance on a double logarithmic scale - to understand how chlorpyrifos alters

food-web structure. Deviations in *MN* among species pairwise links can be used to identify alterations to biomass fluxes in the food web. For instance, altered consumer-resource feeding "link angles" can reveal rates of change in biomass, population production and population consumption between species-pairs, through to the food web as a whole (*sensu* Cohen *et al.* 2009), and these changes can help us to interpret direct and indirect effects of chlorpyrifos.

Trivariate webs were constructed for all sites. Feeding links were inferred from trophic interactions published in the literature (Table S4). We assumed that if a trophic interaction between two species has been reported in the literature and those same species were present at one of our sites, then that trophic interaction also occurred, as has been validated in other stream food webs (Layer *et al.* 2010; Layer, Hildrew & Woodward 2013). In a few instances, feeding links were assigned on the basis of taxonomic similarity. For example, if a link had been established from the literature for at least one congener it was assumed that different species within the same genus fed upon the same resources and were consumed by the same consumers. It was necessary to extend this assumption to the family level in some instances where information in the primary literature was scarce (Table S5). This minimises bias between nodes where the quantity of directly observed information varies and allows the method to be reproduced exactly (Gray *et al.* 2014).

Ecosystem functioning: leaf-litter decomposition

At each site, the decomposition rate of leaf-litter was determined from leaf-packs containing 3.0 g (±0.3 g SD) black alder (*Alnus glutinosa*) incubated in the river for 9 days. Coarse (150 mm by 100 mm, 10mm mesh) and fine (150 mm by 100 mm, 500 µm mesh) mesh-aperture bags were used to determine the fraction of decomposition contributed by microbes (mass loss from fine mesh bags) and macroinvertebrates (difference in mass loss from coarse and

fine mesh bags). Leaf breakdown rates were expressed as the exponential decay rate coefficient, *k* (see equation S3; Woodward *et al.* 2012).

Data analysis

Trivariate statistics were calculated using the method of Cohen *et al* (2009) in the R package Cheddar (Hudson *et al.* 2012). We used link angles to estimate changes in potential biomass flux between a resource and its consumer. In summary, a link can be viewed as a vector from a resource to its consumer and, considering that macroinvertebrate taxa abundance and/or mass is predicted to decrease at impacted sites, a change in the angle of macroinvertebrate upper- and lower-links would indicate a potential change in biomass flux (Fig. 2).

Linear mixed effect models (LMM) were used to test for differences in mean annual water quality, with treatment and date as fixed and random factors, respectively. Differences in biotic response variables (link angles, species and community abundance and/or biomass, gene abundances and microbial capacity) between control and impacted sites (i.e. condition) were tested using LMM with site and condition as random and fixed factors, respectively. Where necessary a variance structure was used to account for unequal variance between sites in order to meet model assumptions (after Zuur *et al.* 2009). If data were not normally distributed they were Log_{10} transformed to meet the assumptions of the test. All LMM were performed using the nlme package in R (Pinheiro *et al.* 2011) and estimates were made using restricted maximum likelihood or, when testing for differences in group means (e.g. macroinvertebrate communities within and between treatments), using general linear hypotheses tests in the R package multcomp (Hothorn *et al.* 2014).

Results

Macroinvertebrate monitoring by citizen scientists

Within control sites, *G. pulex* had the highest relative abundance (61%), followed by Baetidae (17%), Ephemerellidae (12%), cased Trichoptera (9%) and Plecoptera (1%). The macroinvertebrate assemblage within the impacted site in the three months prior to the spill was similar but following the spill on July 1st 2013, there was a 99.5% reduction in total abundance from the previous month (Fig. 3). By September, total abundance had increased again, but was dominated by Ephemeroptera instead of *G. pulex*, the latter being the slowest taxa to recover, as recorded by the citizen scientists.

Microbial functional gene abundance and functional potential

Analyses of gene abundances revealed that ammonia oxidisers (*amoA*), particularly AOBs, were up to 30-fold higher (t_2 = 4.99; p = 0.03), and populations capable of utilising organophosphate (*oph*) as a resource were up to 7-fold higher in impacted sites compared with control sites (Fig. 4a; t_2 = 6.14; p = 0.02). The elevation in the abundance of these populations suggests both direct (i.e. microbes utilised the insecticide as a resource) and indirect effects (i.e. microbes utilised ammonia released by decaying macroinvertebrates) of chlorpyrifos. However, there was no significant difference in the total abundance of bacteria, nor of the abundance of nitrite reducers or AOAs (Fig. 4a).

The functional microbial assays showed impacted sites had higher overall substrate usage and a shallower rank abundance curve, indicating substantial functional changes in response to the spill. Mean overall carbon usage in the impacted sites was higher than the control sites (Fig. 4b; t_2 = 4.2, p = 0.05). Differences among control and impacted sites suggested elevated rates of substrate usage of simple carbohydrates (e.g. glucose-1-phosphate, t_2 = 4.4, p = 0.05;

α-D-lactose, t_2 = 7.7, p = 0.02) and amino acids in the impacted sites, with little difference in the usage of complex polymers (e.g. Tween 40).

Macroinvertebrate community structure and ecosystem functioning

Total macroinvertebrate biomass and abundance did not significantly differ between the control and impacted sites (t_2 = -1.43; p = 0.29; t_2 = -2.11; p = 0.17). However, arthropod biomass was 92.9% lower in impacted sites than arthropod biomass in control sites and 80.4% lower than biomass of less pesticide-sensitive taxa in impacted sites (Table 2; Fig. 5). In addition, the biomass of macroinvertebrate taxa considered less sensitive to pesticides was 97.2% lower than that of the sensitive arthropods in control sites (Table 2), thus the former were partly compensating for the loss of the latter within impacted sites. *G. pulex* biomass (99.6%) and abundance (99.2%) and *Baetis* biomass (18.7%) and abundance (95.6%) were lower (Fig. 4c; 4d), but chironomid biomass (89.3%) and abundance (92.2%) and oligochaete biomass (85.4%) and abundance (94.5%) was higher in impacted sites compared to control sites (Table 2; Fig. 5). Macroinvertebrate diversity was similar between control and impacted sites (t_2 = -0.39; p = 0.74), as was also true for fish diversity (Table 3), whereas four taxa of large diatoms (*Cymatopleura solea*, *Cymatopleura elliptica, Gyrosigma attenuatum* and *Surirella caproni*) were present only in the impacted sites (Fig. 4d). Microbial decomposition was higher, whereas total decomposition mediated by both microbes and detritivores was lower, in the impacted sites (Table 2; Fig. 4c), probably reflecting the decline of *G. pulex* and partial compensation by increased microbial activity.

Trivariate analysis

Arthropod lower-link angles were less negative (i.e. shallower) than less pesticide-sensitive

taxa in the control communities, but more negative (i.e. steeper) within the impacted communities (Table 2). This indicates altered mass-abundance scaling relationships of the links between nodes and a potential decrease in biomass flux from diatoms to arthropods within the impacted communities (Fig. 2). *G. pulex* and *Baetis* had the highest biomass and numerical abundance within the control macroinvertebrate community, respectively (Figs 4c, 4d), and these species upper-link angles (i.e. to their predators) became shallower at impacted sites (Table 2), thus indicating a potential decrease in biomass flux to fishes from both the detritivore and herbivore food chains. To illustrate the direction of biomass flux through the food web and the connection of a key species to all other taxa via relatively direct and short paths, we constructed an example food chain with *G. pulex* as the focal species (Fig. 6). This highlights the potential for perturbations to ripple rapidly through the network even in this complex food web. More commonly used whole-network metrics, such as the regression slope and intercept, showed no clear differences that could be ascribed to the pesticide spill (Table 3).

Discussion

The documented insecticide spill in the River Kennet affected multiple organisational levels, from individual genes, through to food web structure and an ecosystem process. The location of pesticide-sensitive macroinvertebrate consumers relative to their resources in *MN* space shifted markedly, and the collapse in the population of a previously dominant keystone detritivore, *G. pulex*, was especially notable. This was associated with dramatically impaired rates of detritivore-mediated litter decomposition, with potential repercussions for the higher trophic levels. In this highly interconnected food web (Fig. 6) perturbations could potentially not only easily propagate through species interactions, but could also dissipate effectively. These properties could confer resilience on the system as a whole, as alternative feeding

paths provide relatively direct "short-circuits" in the food web (Fig. 6). Various compensatory mechanisms and hystereses within the food web were evident following the spill, including elevated microbial decomposer activity in the absence of macroinvertebrate detritivores (Fig. 4c) and irruptions and growth of less pesticide-sensitive and *r*-selected taxa capable of exploiting new resources (Fig. 5). The functional potential of the microbial assemblage in particular was higher in the impacted sites, as was the abundance of genes associated with organophosphate use and ammonia oxidation in the aftermath of widespread arthropod deaths (Fig. 4a; 4b). Extended temporal sampling will likely reveal if the sewage treatment work is potentially confounding our interpretation of this result, although there is no suggestion this is the case, as water quality is essentially identical above and below the works (Table 1; Fig. S1).

Microbes account for most of a river's biodiversity, drive key ecosystem processes and biogeochemical cycles (e.g. nitrogen cycle) and interact with higher trophic levels. Our qPCR assays revealed that the abundance of genes associated with the turnover of organophosphate and ammonia was higher in polluted sediment, revealing both direct and indirect effects of the spill on microbial activities.

Strong links between changes in the structure and functioning of the microbial and macroinvertebrate community were evident, as revealed by the changes in decomposition rates associated with these two major biotic drivers (Gessner & Chauvet 2002; Schäfer *et al.* 2007). The microbial community played a key role in maintaining litter decomposition following the macroinvertebrate losses, and microbial functional potential assessed by Ecoplate assays was also elevated at the impacted sites. The large-scale mortality of macroinvertebrates was likely to have released resources readily available for microbial use, promoting the proliferation of fast-growing bacteria able to use a broad range of substrates. Additional data from more extended sampling will eventually help us to better understand the temporal dynamics of the recovery process, by providing deeper insights into the baseline

variability. Even in the current absence of such additional data, our results clearly underline the potential of microbial bioindicators for assessing direct and indirect responses of river ecosystems to environmental impacts.

Employing a highly resolved network-based perspective provided further insights into both direct and indirect effects of the perturbation - from genes to species and from food webs to the ecosystem as a whole - as we were able to connect structural and functional indicators across different levels of biological organisation, as well as improving understanding of the associated responses. For instance, *G. pulex* and *Baetis* represented key nodes in the major detritivore and herbivore food chains, respectively, as is the case in many lowland running waters (Woodward *et al.* 2008; Layer *et al.* 2010), and both populations collapsed in the impacted sites. Our broad multilevel approach revealed how the loss of consumers could result in the release of their resources and potential competitors, and also how major conduits of energy and biomass flux to the species at the top of the food web, including ecologically important and economically valuable fish species, such as trout, could be compromised.

Microcosm and mesocosm experiments have described ecosystem-level responses to, and recovery from, combined pesticide and nutrient additions (Traas *et al.* 2004; Halstead *et al.* 2014), and observational field-based research has demonstrated that recovery of the macroinvertebrate community and leaf-litter decomposition was related to aerial mobility of repopulating taxa (Chung *et al.* 1993). Our study represents a novel approach, integrating a broad range of assessment metrics at multiple levels and this has helped us to better understand the effects of a pesticide spill in a natural setting. The same approach is also more widely applicable to assessments of effects caused by other stressors, such as acidification and eutrophication, where interactions within food webs can shape both the ecosystem impact and the rate and trajectory of recovery (e.g. Ledger & Hildrew 2005; Layer *et al.* 2010; Rawcliffe *et al.* 2010). Thus, such an approach offers a way to move beyond partial taxonomic or trait-based views to one that explicitly incorporates species interactions in food

webs and ecosystem processes in river bioassessment (Gray *et al.* 2014).

Our study also highlights the value of citizen science in biomonitoring and bioassessment, as it enabled us to place the detailed data specifically and intensively collected after the toxic spill in the context of a wide before-and-after-control-and-impact (BACI) -style "natural experiment", which would have otherwise been impossible to employ in the search for causal relationships. Mobile Ephemeroptera (*Baetis* and Ephemerellidae, both active swimmers with an aerial adult life-stage that coincided with the pollution) repopulated the impacted sites more quickly than *G. pulex* (Fig. 3), as did the often opportunistic chironomid species and less sensitive non-arthropod taxa such as oligochaetes (Fig. 5). These responses echo those of small *r*-selected taxa preceding the recovery of larger *K*-selected species in previous studies on pesticide contamination (Chung *et al.* 1993; Liess & Schulz 1999; Beketov *et al.* 2008).

It has been hypothesised that ecological inertia can operate within freshwater food webs, creating 'community closure' or recovery trajectories that are not simple reversals of impacts (e.g. Ledger & Hildrew 2005; Layer *et al.* 2011; 2013). Impacts on key nodes can alter important aspects of food-web structure and associated processes, such that although the latter might operate at similar rates, they may be driven by microbes and *r*-selected taxa instead of *K*-selected taxa, as has been reported in response to pesticide contamination (Chung *et al.* 1993) and other stressors (Hladyz *et al.* 2011). Our initial data demonstrate that, while the R. Kennet's ecological structure and functioning were significantly altered by the toxic spill, there were many alternative nodes and links within the food web that could help confer some level of resilience even in the face of catastrophic population losses.

Future work will require well co-ordinated laboratory and field investigations based on matching methodologies to improve understanding of the links between microbiota and larger organisms before, if ever, one can be used as a proxy for the other (e.g. Triebskorn *et al.* 2003). Nonetheless, our study represents a proof-of-concept as to how vastly different metrics

might be linked and, as more data are generated over time, potential time × treatment interactions can also be more thoroughly explored. Additional metrics based on, for instance, next-generation sequencing (e.g. Rosi-Marshall *et al.* 2013) or measures of whole-ecosystem respiration (e.g. Young, Matthaei & Townsend 2008), could be incorporated to capture the extent of impacts and recovery trajectories more fully.

Although covering only part of the spectrum of responses reported here, other multimetric bioassessments have yielded comparable results, including how pesticides can indirectly release prey species from predation (Papst & Boyer 1980), constrain consumer populations through loss of resources (Brazner & Kline 1990), affect the structure and functioning of aquatic communities in mesocosms (Downing *et al.* 2008; Relyea 2008; Halstead *et al.* 2014) or alter the structure and functioning of natural stream communities (Chung *et al.* 1993; Schäfer *et al.* 2007). Results from correlational studies also suggest that changes at multiple trophic levels may be related to organic chemical contaminants (mostly pesticides) at the continental scale (Malaj *et al.* 2014). Despite this and the worldwide use of, and projected increase in, pesticides, studies of their effects at the ecosystem-level are rare in natural settings (Kohler & Triebskorn 2013). The present study contributes to bridging this gap.

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Table 1. Locations of upstream control and downstream impacted sites as well as of water chemistry monitoring stations of the Environment Agency (EA). Mean and range, in brackets, of annual water chemistry concentrations from Environment Agency monitoring data are shown from sites located between control and impacted reaches. Oxidised nitrogen (oxidised N) is the sum of nitrate (NO3-) and nitrite (NO2-).

Site	Condition	Latitude, Longitude
A	Control	51°4170'N, 1°7536'W
EA Control	Control	51°4163'N, 1°7325'W
C	Control	51°4235'N, 1°7165'W
D	Impacted	51°4227'N, 1°6982'W
EA Impact	Impacted	51°4227'N, 1°6982'W
F	Impacted	51°4269'N, 1°6650'W
Water chemistry	EA Control	EA Impacted
Alkalinity (mg L^{-1})	250 (187-262)	243 (189-254)
Conductivity (µS cm^{-1})	626 (449-738)	609 (492-686)
Oxidised N (mg L^{-1})	6.6 (4.4-7.5)	6.8 (4.4-7.6)
Dissolved oxygen (mg L^{-1})	9.0 (6.9-10.0)	9.6 (6.9-10.9)
Temperature (°C)	11.0 (5.7-14.4)	11.1 (5.7-14.5)
pH	7.6 (7.4-7.8)	7.9 (7.4-8.1)
Ortho-phosphate (mg L^{-1})	0.08 (0.02-0.36)	0.08 (0.02-0.34)

Table 2. General linear model tests of the biomass (mg) and abundance of arthropods and other macroinvertebrates (Tricladida, Annelida and Mollusca, which are considered to be less sensitive to chlorpyrifos than arthropods) per sample; *Baetis*, *G. pulex* (i.e. *K*-selected taxa), chironomid and oligochaete (i.e. *r*-selected taxa) biomass and abundance; arthropod-resource and other-resource trivariate lower-link angles, *Baetis* and *G. pulex* upper-link angles and both total and microbial leaf-litter breakdown rate between control (C) and impacted (I) sites. Significant *p* values (<0.05) are highlighted in bold.

Log_{10} (biomass +1)	Estimate	Std. Error	*z* value	*p*
C:arthropods - C:other	1.62	0.09	17.53	**<0.001**
I:arthropods - I:other	-0.73	0.12	6.00	**<0.001**
C:arthropods - I:arthropods	1.17	0.23	5.19	**<0.001**
C:other - I:other	-1.17	0.25	-4.73	**<0.001**
Log_{10} (abundance +1)				

C:arthropods - C:other	1.28	0.19	6.82	**<0.001**
I:arthropods - I:other	-0.05	0.19	0.25	0.99
C:arthropods - I:arthropods	0.56	0.24	2.37	0.06
C:other - I:other	-0.76	0.24	-3.23	**0.005**
Log_{10} (biomass +1)				
C:*Baetis* - I:*Baetis*	0.62	0.16	4.00	**<0.001**
C:*G. pulex* - I:*G. pulex*	2.30	0.15	15.82	**<0.001**
C:chironomids - I:chironomids	-0.93	0.15	-6.38	**<0.001**
C:oligochaetes - I:oligochaetes	-0.81	0.15	-5.49	**<0.001**
Log_{10} (abundance +1)				
C:*Baetis* - I:*Baetis*	1.21	0.24	4.98	**<0.001**
C:*G. pulex* - I:*G. pulex*	2.31	0.22	10.63	**<0.001**
C:chironomids - I:chironomids	-1.14	0.22	-5.24	**<0.001**
C:oligochaetes - I:oligochaetes	-1.12	0.23	-4.92	**<0.001**
Invertebrate-resource lower-link angles				
C:arthropods - C:other	-0.08	0.02	-3.8	**<0.001**
I:arthropods - I:other	0.2	0.02	10.35	**<0.001**
C:arthropods - I:arthropods	-0.32	0.24	-1.36	0.44
C:other - I:other	-0.04	0.24	-0.18	>0.99
Baetis and *G. pulex* upper-link angles				
C:*Baetis* – I:*Baetis*	-103.71	24.3	-4.27	**<0.001**
C:*G. pulex* – I:*G. pulex*	-62.8	25.73	-2.44	**0.03**
Leaf litter decomposition (*k*)				
I:total - C:total	-0.05	0.01	-6.57	**<0.001**
I:microbial - C:microbial	0.01	0.002	5.75	**<0.001**

Table 3. Properties of the trivariate food webs at control and impacted river sites.

	Site A	Site C	Site D	Site F
Property	Control	Control	Impacted	Impacted
Number of nodes	68	60	64	73
Number of fish species	4	4	5	3
Number of macroinvertebrate taxa	35	23	20	32
Number of diatom taxa	29	33	39	38
Number of links	837	635	739	1060
Linkage density	11.96	10.41	11.37	14.13
Directed connectance	0.17	0.17	0.17	0.19
Trivariate regression slope	-0.98	-0.67	-0.92	-0.95
Trivariate regression intercept	1.29	1.26	1.58	1.35

Figure legends

Fig. 1. River Kennet (UK) with study sites A-C (upward pointing triangles = control) and D-F (downward pointing triangles = impacted). Data for sites A, C, D and F (filled triangles) are presented here. Monitoring data for aquatic macroinvertebrates were collected by citizen scientists upstream (i.e. control site) at Stonebridge Lane and downstream at Elcot Mill (i.e. impacted site) of Marlborough sewage treatment works, where the pesticide entered the river.

Fig. 2. (a) Location of consumers sensitive to pesticides (C^s) and less sensitive to pesticides (C^l) in relation to the consumer resources (R) and predators (P) as viewed on a double-logarithmic scale of body mass versus abundance. (b) Changes within the food web following pesticide exposure can be assessed by using link angles as a proxy for changes in potential biomass flux within the food web: a predicted decrease in C^s *MN* following pesticide exposure and an increase in R *MN* due to the release from top-down consumer control can be assessed using the C^s link angles in relation to C^l and control data; a decrease in C^s lower-link angles would indicate a potential reduction in biomass flux between R-C^s; an increase in C^s upper-link angle would indicate a potential reduction in biomass flux to P and hysteresis within the network whereby P is yet to be impacted by the loss of C^s, or that P has increased reliance on other resources, or a combination of the two.

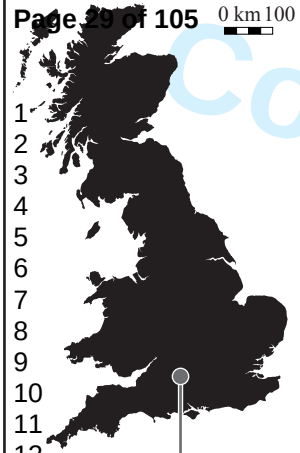
Fig. 3. Top: Aquatic macroinvertebrate monitoring data collected by citizen scientists show macroinvertebrate scores before and after the toxic spill (arrows), based on total abundance of the target taxa. The red line represents an Environment Agency threshold for substantial ecological degradation. Bottom: abundance of key taxa in relation to scores collected from an upstream control at Stonebridge Lane and a downstream impacted site at Elcot Mill (see Fig. 1).

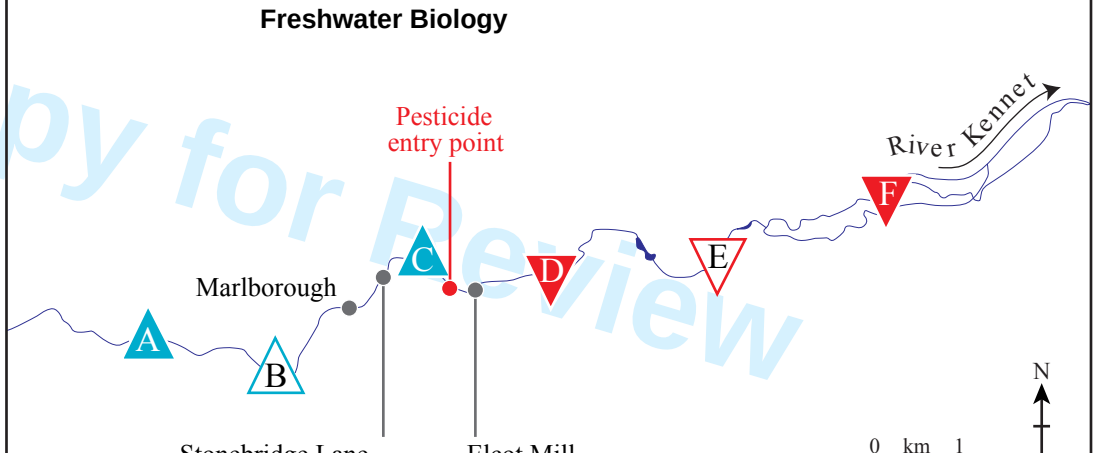
Fig. 4. Vertical arrows indicate notable differences between ecological data from control sites A and C and from impacted sites D and F two months after the toxic spill, error bars represent standard error. (a) Molecular results from microbial qPCR assays targeting the (α) 16S rRNA (microbial abundance), (β) *nirS* (nitrite reductase) (γ) *amoA* (ammonia monooxygenase) AOB (ammonia oxidising bacteria), (δ) *amoA* (ammonia monooxygenase) AOA (ammonia oxidising archaea), (ε) *opd* (organophosphorus hydrolase) genes. (b) Ecoplate microbial functional potential on 31 carbon substrates (x-axis) and their usage (y-axis; measured as optical density at 600 nm after 5 days of incubation at 22 °C as defined in the Methods). (c) Biomass of macroinvertebrates (light shading) and a keystone detritivore, *Gammarus pulex* (dark shading), and leaf-litter breakdown rates by all consumers (light shading) and microbes only (dark shading). (d) Trivariate mass-abundance food webs: green circles = algae (large species found only in the impacted sites highlighted), yellow symbols = arthropods (decreased relative to controls), blue symbols = other macroinvertebrates, black filled diamond = *G. pulex*, black open diamond = *Baetis*, pink symbols = fishes.

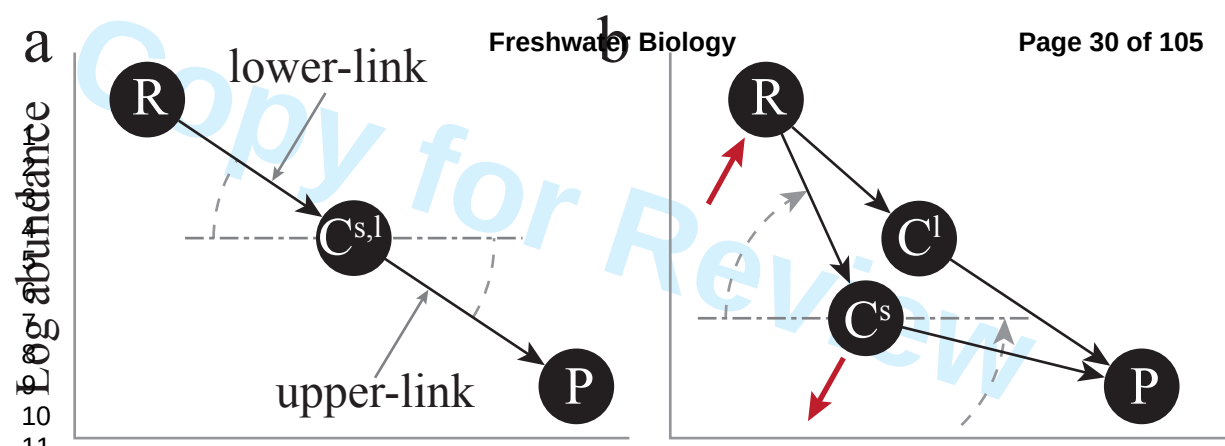
Fig. 5. Macroinvertebrate mean biomass (per sample with standard error) at control and impacted sites in the River Kennet.

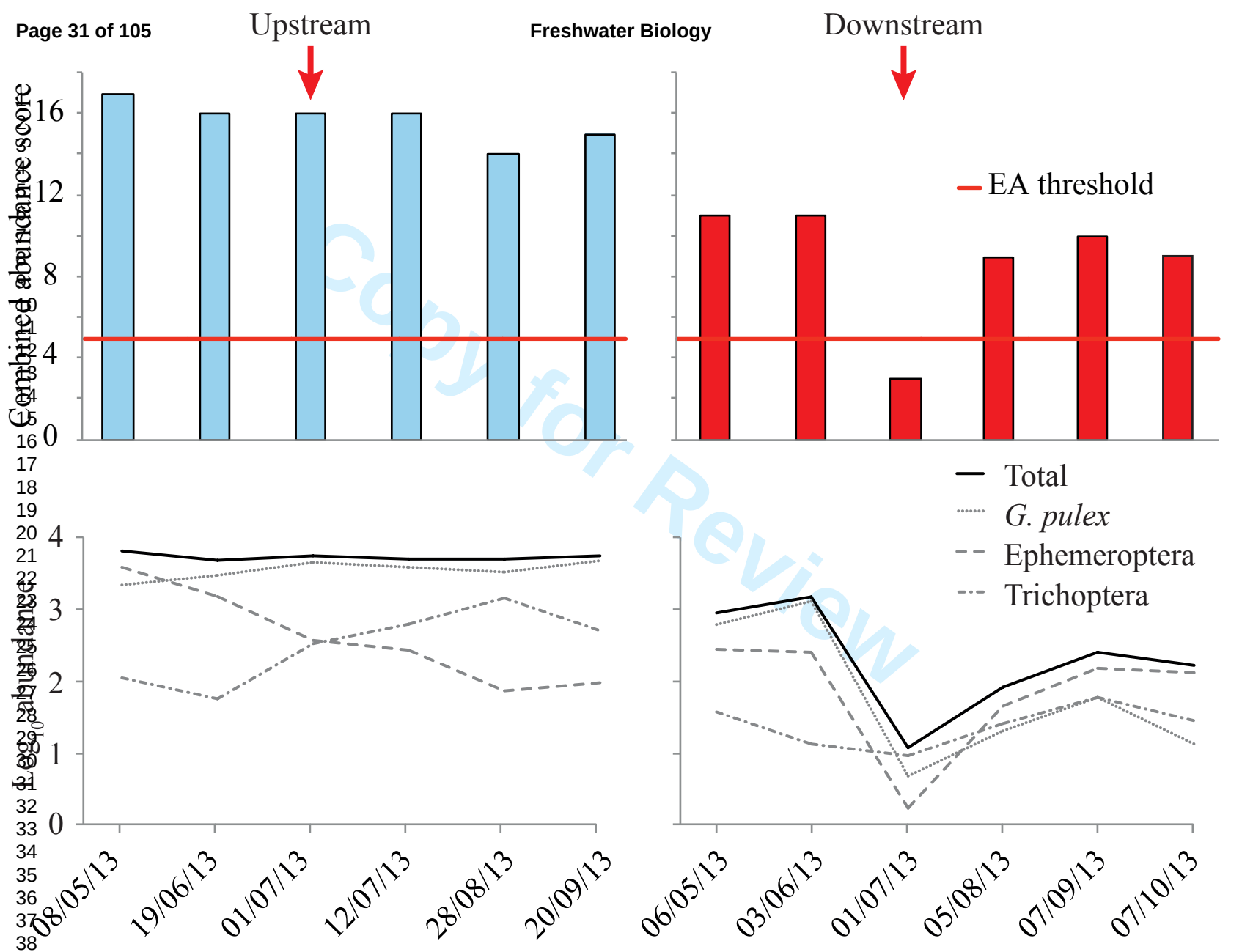
Fig. 6. Aggregated network for the River Kennet food web, highlighting an exemplar food chain from the basal resource to the apex predator; a = coarse particulate organic matter (e.g. leaf litter), b = *Gammarus pulex*, c = brown trout, *Salmo trutta*, d = Eurasian otter, *Lutra lutra*. The two concentric circles of nodes represent the shortest food web distances to or from *G. pulex* – those in the inner circle are a single link removed from *G. pulex*, those in the outer circle are separated by two links in the shortest path. Here, all species are at most 2

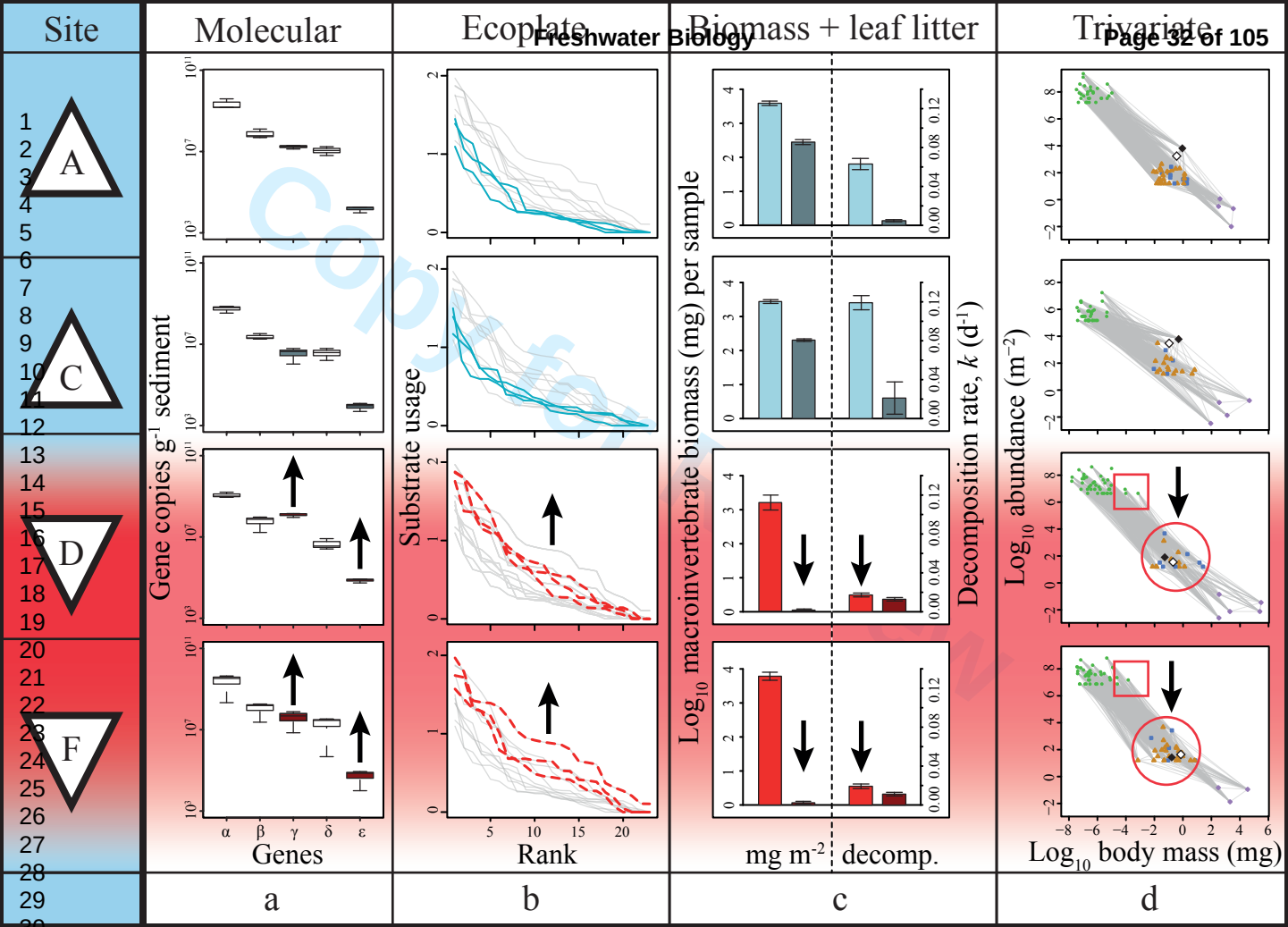
links away from *G. pulex*, although longer food chains are present in the network, as shown by a-b-c-d. Symbols for nodes represent different trophic elements: green circles = producers, blue squares = macroinvertebrates, purple diamonds = vertebrate ectotherms, red triangles = endotherms, black circles = abiotic resources. Light blue and light purple circles = cannibalistic nodes of macroinvertebrates and vertebrate ectotherms, respectively.

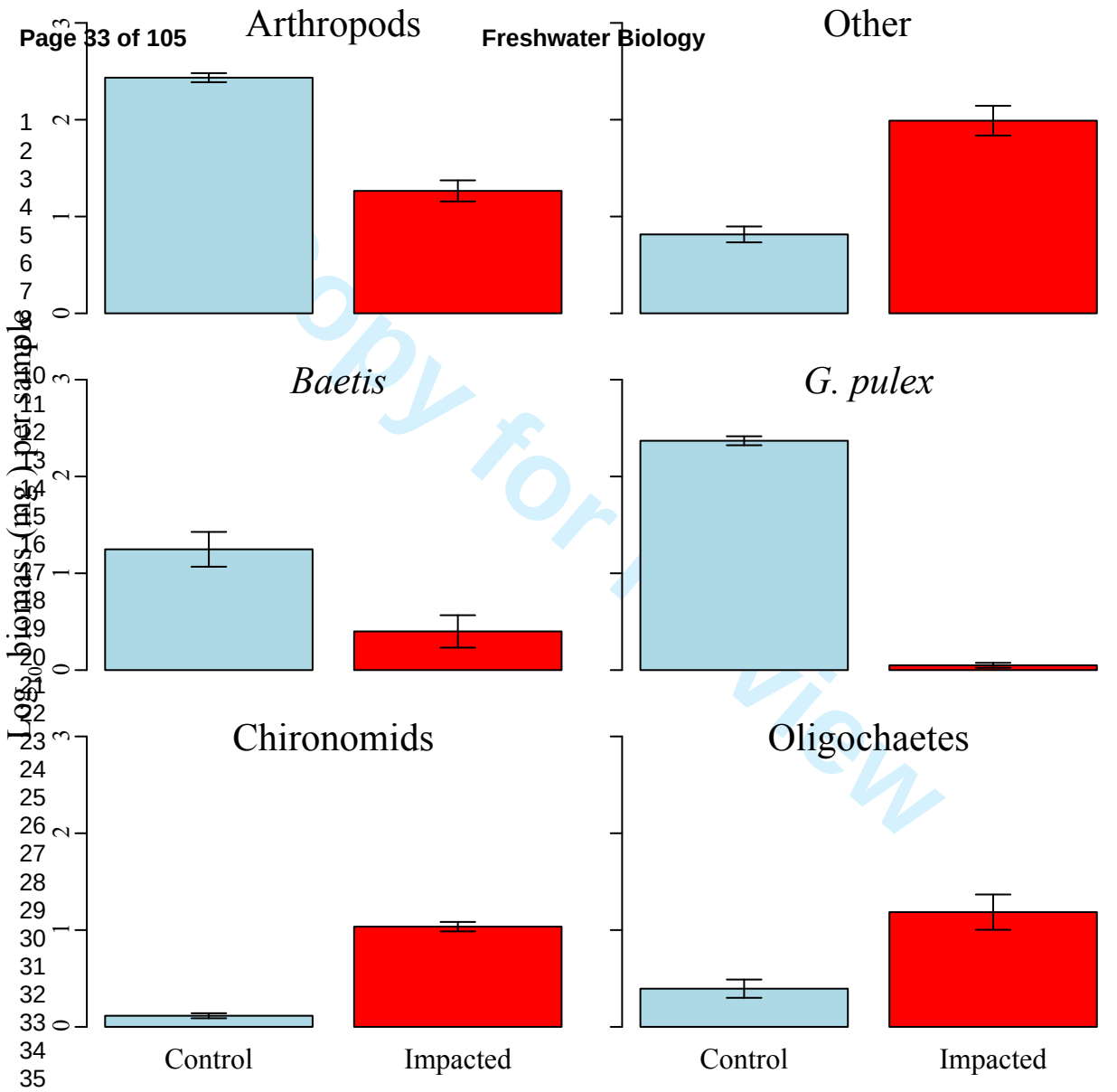


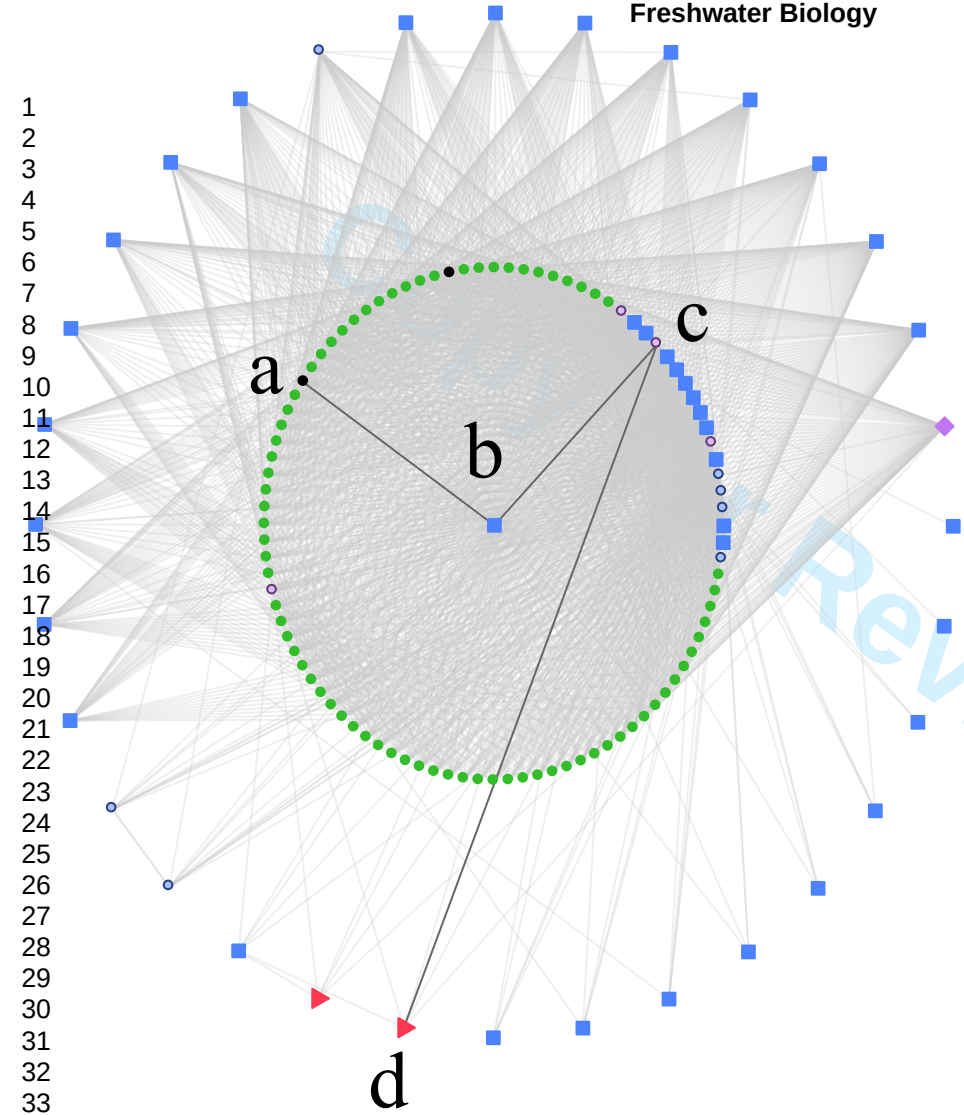


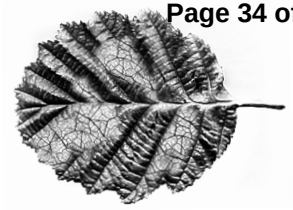


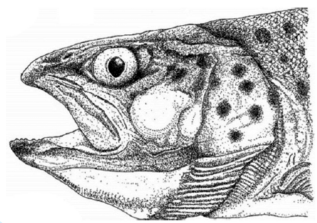



Site
Molecular
Ecoplate
Biomass + leaf litter
Trivariate
A
C
D
F
Gene copies g^{-1} sediment
Genes
α β γ δ ε
Substrate usage
Rank
Log$_{10}$ macroinvertebrate biomass (mg) per sample
Decomposition rate, k (d^{-1})
mg m^{-2}
decomp.
Log$_{10}$ abundance (m^{-2})
Log$_{10}$ body mass (mg)
a
b
c
d









Supplementary Material

ARK macroinvertebrate monitoring data and Environment Agency water chemistry data

Data from ARK monthly aquatic macroinvertebrate and UK Environment Agency water chemistry monitoring data were combined to provide a before-after-control-impact assessment which has not been possible in previous field studies of pesticide spills (Fig. S1). This information was used by the Environment Agency to direct their investigation into the macroinvertebrate loss and monitor for chlorpyrifos. The annual average of dissolved oxygen was significantly higher at the impacted Environment Agency monitoring station (t_{14} = 2.38, p = 0.03) but orthophosphate and oxidised nitrogen were not statistically significantly different between control and impacted monitoring stations (t_5 = 1.83; p = 0.13; t_4 = 0.01; p = 0.99); and there was also no spike in their concentrations at the impacted station the month following the event during macroinvertebrate recovery (0.08 mg^{-1} and 7.57 mg^{-1}, respectively; see also Table 1). These results, combined with ARK macroinvertebrate scores (Fig. S1), indicate that there was no evidence of organic pollution from the sewage treatment works, and that this could therefore not be ascribed as the cause of the macroinvertebrate mortality event.

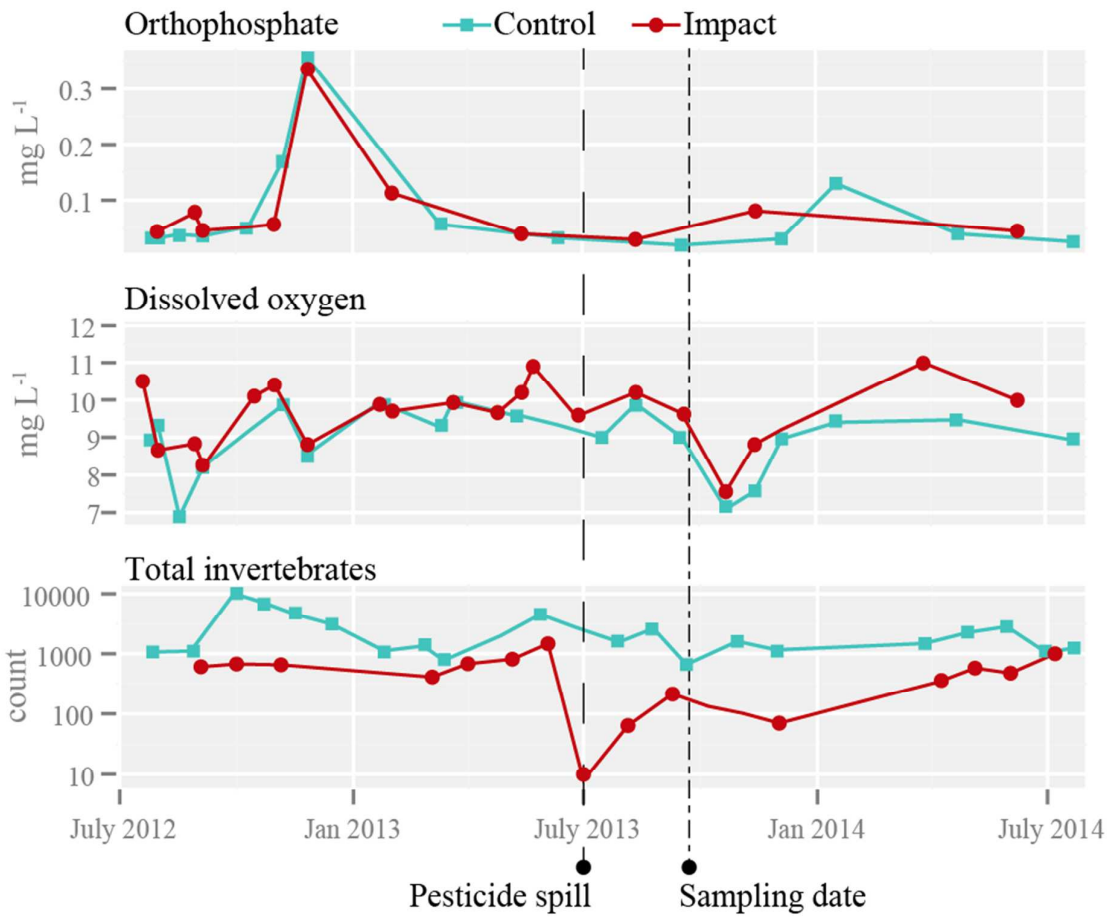


Figure S1. UK Environment Agency water chemistry and ARK aquatic macroinvertebrate data collected between July 2012 and July 2014. Water chemistry samples were collected from an upstream control (blue; adjacent to site B) and a downstream impacted monitoring station (red; adjacent to site E); ARK macroinvertebrate samples were collected from a control at Stonebridge Lane and impacted site at Elcot Mill (see Fig. 1).

Microbial functional gene abundance

DNA isolation: DNA was isolated from 0.25 g sediment samples using a Powersoil DNA Isolation Kit (Mo-Bio Laboratories) in accordance with the manufacturer's instructions. Gene abundances of bacterial 16S rRNA, *nitrite reductase* (*nirS*), ammonia monooxygenase (*amoA*) from ammonia-oxidising archaea (AOA) and bacteria (AOB), and organophosphate hydrolase (*opd*) were quantified by qPCR using.

The following primer pairs:- 16S rRNA: Bakt 341F (CCTACGGGNGGCWGCAG) and Bakt 805R (GAC TAC HVG GGT ATC TAA TCC) (Herlemann *et al.* 2011); *nirS*: *nirSCd3aF* (AAC GYS AAG GAR ACS GG) and *nirSR3cd* (GAS TTC GGR TGS GTC TTS AYG AA) (Throbäck *et al.* 2004); *amoA* (AOA): CrenamoA-23F (ATG GTC TGG CTW AGA CG) and

CrenamoA-616R (GCC ATC CAT CTG TAT GTC CA) (Tourna *et al.* 2008); *amoA* (AOB): *amoA*-1F (GGG GTT TCT ACT GGT GGT) and *amoA*-2R (CCC CTC KGS AAA GCC TTC TTC) (Rotthauwe, Witzel & Liesack 1997); *opd*: OPDF (TCA CAC TGA CTC ACG AGC) and OPDR (CGG CCA ATA AAC TGA CGT).

qPCR cycling conditions: DNA standards were constructed using target template generated by PCR amplification of the target genes from genomic DNA. DNA standards were purified using a GenElute PCR Clean-Up kit (Sigma-Aldrich), prior to quantification on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). The target gene abundance for DNA standards was calculated assuming a molecular mass of 660 Da for double stranded DNA using the following formula: Target abundance = 6.023×10^{23} (copies mol^{-1}) × standard conc. (g μl^{-1}) / MW (g mol^{-1}). Standard curves for each gene were created using ten-fold dilution series ranging from 10^2 to 10^7 gene copies μl^{-1}. For each of the genes the DNA standards, triplicate sediment samples and no-template controls were amplified in triplicate technical replicates on a CFX 96 Real Time System (Bio-Rad) using SensiFAST SYBR No-ROX Kit (Bioline) in 15 µl reactions (7.5 µl of 2 × mastermix, 0.3 µl of forward and reverse primers (10 µM), 5.9 µl PCR grade water (Bioline) and 1 µl of template DNA) using a 2-step cycle programme (initial denaturation/polymerase activation for 3 min at 95°C, followed by 40 cycles of denaturation at 95°C for 5 s and combined annealing and extension at 60°C for 30 s). A dissociation curve was run at the end of each assay to verify that only the expected amplification product was generated in addition to confirming by agarose gel electrophoresis. Gene abundances were quantified against the respective standard curves (all R^2=0.99+) using the CFX Manager software (Bio-Rad) using automatic analysis settings for the Cq values and baseline settings. The limit of detection for all genes was set at 3.3 cycles lower than the Cq value of the no template controls.

Food web taxa population densities

Table S1. Mean numerical abundance per treatment for all nodes in the trivariate food webs (Fig. 4d)

Taxa	Treatment	abundance (m^2)
Achnanthes minutissima	control	1195229384.00
Fragilaria leptostauron	control	654926010.50
Fragilaria capucina	control	296124986.90
Amphora inariensis	control	202495097.30
Cocconeis placentula	control	202417409.60

Melosira varians	control	122089193.40
Nitzschia sublinearis	control	104514701.30
Nitzschia linearis	control	96193580.87
Achnanthes conspicua	control	87095584.38
Synedra ulna ulna	control	87095584.38
Amphora pediculus	control	79085214.48
Achnanthes lanceolata lanceolata	control	70064905.57
Navicula atomus	control	69676467.50
Nitzschia fonticola	control	52335038.26
Gomphonema parvulum	control	43625479.80
Fragilaria capucina gracilis	control	34993608.98
Gomphonema olivaceum	control	34838233.75
Navicula bacillum	control	34838233.75
Nitzschia dissipata	control	34838233.75
Nitzschia sigmoidea	control	34838233.75
Cocconeis pediculus	control	26439425.77
Fragilaria vaucheriae	control	26439425.77
Navicula margalithii	control	26430880.13
Navicula minima	control	17574492.10
Cyclotella radiosa	control	17419116.88
Fragilaria nitzschioides	control	17419116.88
Fragilariforma virescens	control	17419116.88
Meridion circulare	control	17419116.88
Cocconeis pseudothumensis	control	8942621.28
Navicula cryptonella	control	1709127.51
Rhoicosphenia abbreviata	control	932251.37
Achnanthes lanceolata rostrata	control	776876.14
Gomphonema	control	776876.14
Achnanthes clevei	control	310750.46
Fragilaria construens venter	control	310750.46
Gomphonema augur	control	310750.46
Achnanthes helvetica	control	155375.23
Amphora ovalis	control	155375.23
Fragilaria bidens	control	155375.23
Fragilaria capucina rumpens	control	155375.23
Navicula exilis	control	155375.23
Navicula seminulum	control	155375.23
Nitzschia	control	155375.23
Nitzschia amphibia	control	155375.23
Psammodictyon constrictum	control	155375.23
Synedra	control	155375.23
Gammarus pulex	control	6674.00
Baetis	control	1782.67
Agapetus fuscipes	control	1549.33
Polycelis tenuis	control	492.67
Elmis aenea	control	335.33
Oligochaeta	control	218.67
Leuctra inermis	control	208.00

Silo nigricornis	control	201.33
Simulium vernum	control	173.33
Chironomidae	control	156.67
Tanypodinae	control	149.33
Simulium	control	128.00
Paraleptophlebia submarginata	control	120.00
Limnius	control	88.00
Hydracarina	control	84.00
Oreodytes sanmarkii	control	80.00
Ancylus fluviatilis	control	66.67
Bezzia	control	64.00
Athripsodes	control	48.00
Pisidium	control	42.67
Rhyacophila dorsalis	control	34.67
Asellus aquaticus	control	32.00
Glossiphonia complanata	control	32.00
Hemerodromia	control	32.00
Planaria torva	control	32.00
Caenis rivulorum	control	26.67
Dicranota	control	26.67
Serratella ignita	control	24.00
Dendrocoelum lacteum	control	16.00
Drusus annulatus	control	16.00
Dystiscidae	control	16.00
Erpobdella octoculata	control	16.00
Hydropsyche siltalai	control	16.00
Hygrobia hermanni	control	16.00
Limnephilidae	control	16.00
Piscicola geometra	control	16.00
Planorbis	control	16.00
Scirtidae	control	16.00
Cottus gobio	control	0.63
Salmo trutta	control	0.19
Gasterosteus aculeatus	control	0.16
Lampetra planeri	control	0.01
Cocconeis placentula	impact	355252500.30
Melosira varians	impact	314459643.40
Achnanthes minutissima	impact	270097194.50
Synedra ulna ulna	impact	231925123.90
Fragilaria construens venter	impact	196952379.60
Fragilaria leptostauron	impact	103576020.10
Fragilaria capucina rumpens	impact	83934586.33
Amphora pediculus	impact	78860790.32
Amphora inariensis	impact	76471420.07
Fragilaria capucina radians	impact	74608521.18
Fragilaria elliptica	impact	74608521.18
Cyclotella meneghiniana	impact	70292612.62
Nitzschia linearis	impact	63824535.19

Achnanthes lanceolata	impact	62482322.33
Nitzschia fonticola	impact	54672032.04
Navicula margalithii	impact	54556324.04
Nitzschia palea	impact	48377516.61
Gomphonema olivaceum	impact	46861741.75
Navicula minima	impact	46861741.75
Diatoma vulgaris	impact	37419968.60
Gomphonema parvulum	impact	37419968.60
Fragilaria	impact	37304260.59
Fragilaria vaucheriae	impact	34214856.88
Nitzschia sublinearis	impact	32641228.02
Cocconeis pseudothumensis	impact	31241161.17
Fragilaria capucina	impact	31241161.17
Nitzschia dissipata	impact	31241161.17
Encyonema silesiacum	impact	26520274.59
Fragilaria capucina gracilis	impact	24188758.30
Cocconeis pediculus	impact	23430870.87
Cymbella proxima	impact	18652130.30
Nitzschia sigmoidea	impact	16378468.01
Cymatopleura elliptica	impact	15620580.58
Cymbella cistula	impact	15620580.58
Navicula cryptonella	impact	15620580.58
Navicula exilis	impact	14804839.15
Nitzschia recta	impact	13231210.30
Achnanthes lanceolata lanceolata	impact	9326065.15
Meridion circulare	impact	9326065.15
Neidium dubium	impact	9326065.15
Nitzschia capitellata	impact	9326065.15
Cymatopleura solea	impact	8568177.72
Amphora aequalis	impact	7810290.29
Amphora veneta	impact	7810290.29
Cymbella	impact	7810290.29
Navicula	impact	7810290.29
Nitzschia frustulum	impact	7810290.29
Nitzschia heufleriana	impact	7810290.29
Undiff. centric diatom	impact	7810290.29
Achnanthes lanceolata rostrata	impact	4663032.57
Amphora ovalis	impact	4663032.57
Diploneis parma	impact	4663032.57
Gomphonema clavatum	impact	4663032.57
Gyrosigma acuminata	impact	4663032.57
Gyrosigma attenuatum	impact	4663032.57
Hantzschia amphioxys	impact	4663032.57
Navicula lanceolata	impact	4663032.57
Surirella capronii	impact	4663032.57
Oligochaeta	impact	3728.00
Chironomidae	impact	3013.33
Ancylus fluviatilis	impact	736.00

Caenis rivulorum	impact	496.00
Tanypodinae	impact	202.67
Niphargus aquilex	impact	160.00
Elmis aenea	impact	144.00
Silo nigricornis	impact	133.33
Simulium	impact	96.00
Polycelis tenuis	impact	82.67
Pisidium	impact	77.33
Hydracarina	impact	70.67
Gammarus pulex	impact	52.00
Agapetus fuscipes	impact	50.67
Oecetis	impact	48.00
Bezzia	impact	44.00
Baetis	impact	41.33
Centroptilum luteolum	impact	32.00
Paraleptophlebia submarginata	impact	32.00
Glossiphonia complanata	impact	24.00
Planaria torva	impact	24.00
Asellus aquaticus	impact	16.00
Cloeon simile	impact	16.00
Dendrocoelum lacteum	impact	16.00
Erpobdella octoculata	impact	16.00
Hydraenidae	impact	16.00
Leuctra	impact	16.00
Leuctra hippopus	impact	16.00
Oulimnius tuberculatus	impact	16.00
Piscicola geometra	impact	16.00
Proasellus meridianus	impact	16.00
Procloeon pennulatum	impact	16.00
Psychoda	impact	16.00
Serratella ignita	impact	16.00
Cottus gobio	impact	0.14
Salmo trutta	impact	0.07
Lampetra planeri	impact	0.01
Thymallus thymallus	impact	0.01
Gasterosteus aculeatus	impact	>0.01

Food web characterisation

Fishes

Dry mass was estimated using 60 individuals per species from control and impacted sites and species-specific conversions of wet to dry mass were extracted from http://fishbase.org/. Estimates were made with the following equation S1:

$$DM = a*WM \quad \text{(eq. 1)}$$

Where DM is dry mass (mg), a is a constant and WM is wet mass (mg).

Supplementary dry mass estimates were made using the following equation S2:

$$\text{Log}(DM) = \text{Log}(a) + (b)*\log(WM)$$

Where DM is dry mass (mg), a and b are constants and WM is wet mass (mg). Natural logarithms (ln) were used and constants were supplied by Edwards (unpublished).

Macroinvertebrates

The dry mass of macroinvertebrates M (dry mass [mg]) was determined from body length or head capsule width using length-mass regression equations (Table S2).

Table S2. HW = head-capsule width (mm); BL = total body length (mm); SL = shell length (mm). Morphologically similar taxa or higher taxonomic levels, shown in square parantheses, were used where equations were unavailable for taxa. The source of each equation is denoted by a letter at the end of each row, and are: 1) Meyer (1989); 2) Calow (1975); 3) Baumgäertner & Rothhaupt (2003); 4) Benke *et al.* (1999); 5) Woodward & Hildrew (2001); 6) Burgherr and Meyer (1997); 7) Towers *et al.* (1994); 8) Edwards *et al.* (2009); 9) Smock (1980).

taxa	y	x	regression equation	r^2	source
Agapetus fuscipes [Glossosoma]	ln(mg)	lnHW	y = 0.96+2.98x	0.71	1
Ancylus fluviatilis	log10(mg)	log10(SL)	y = -3.762 + 3.0x	0.99	2
Asellus aquaticus	ln(mg)	lnBL	y = -6.2+3.75x	0.69	3
Asellus meridianus [Asellus aquaticus]	ln(mg)	lnBL	y = -6.2+3.75x	0.69	3
Athripsodes [Oecetis spp.]	ln(mg)	lnHW	y = 1.913+3.3x	0.67	4
Baetis [Baetis spp.]	(mg)	HW	y = 1.2688*(x^3.326)	0.96	4
Baetis rhodani [Baetis spp.]	(mg)	HW	y = 1.2688*(x^3.326)	0.96	4
Baetis scambus [Baetis spp.]	(mg)	HW	y = 1.2688*(x^3.326)	0.96	4
Baetis vernus [Baetis spp.]	(mg)	HW	y = 1.2688*(x^3.326)	0.96	4
Bezzia [Bezzia sp.]	ln(mg)	lnBL	y = -4.13+1.12x	0.99	3
Caenis rivulorum [Caenis spp.]	ln(mg)	lnHW	y = -0.91+3.35x	0.63	3
Centroptilum luteolum [Baetis spp.]	(mg)	HW	y = 1.2688*(x^3.326)	0.96	4
Chironomid [Chrionomidae]	(mg)	HW	y = 2.7842*(x^2.835)	0.9	4
Dendrocoelum lacteum [Dugesia tigrina]	(mg)	BL	y = 0.0089*(x^2.145)	0.81	4
Dicranota sp.	ln(mg)	lnBL	y = -5.53+1.91x	0.54	5
Drusus annulatus [Limnephilidae]	ln(mg)	lnHW	y = 0.4109+3.1678(x)	0.83	1
Dysticidae sp. [Coleoptera, larvae]	ln(mg)	lnBL	y = -4.4518+2.4724	0.57	1

Elmis aenea [Adult Coleoptera]	ln(mg)	lnBL	y = -5.46+4.33x	0.78	6
Elmis aenea [Elmidae larvae]	ln(mg)	lnBL	y = -6.078+3.092x	0.83	7
Eloeophila sp [Diptera]	ln(mg)	lnBL	y = -6.21+2.52x	0.83	6
Erpobdella octoculata	Ln(mg)	LnBL	y = –3.20+2.22x	0.78	8
Gammarus pulex [Gammarus fossarum Koch]	Ln(mg)	Ln(BL)	y = y = –4·95 + 2·83(x)	0.9	6
Glossiphonia complanata	Ln(mg)	LnBL	y = -2.12+2x	0.64	8
Helobdella stagnalis	Ln(mg)	LnBL	y = -2.74+2.12x	0.62	8
Hydracarina [Hydracarina spp.]	Ln(mg)	LnBL	y = -2.202+1.66	0.48	3
Hydropsyche siltalai [Hydropsyche spp.]	(mg)	HW	y = 1.265*(x^2.747)	0.87	4
Hydroptilidae [Trichoptera, cased]	ln(mg)	lnHW	y = 1.30+3.62x	0.82	3
Hygrobia hermanni [Coleoptera, larvae]	ln(mg)	lnBL	y = -4.4518+2.4724	0.57	1
Ilybius [Coleoptera, larvae]	ln(mg)	lnBL	y = -4.4518+2.4724	0.57	1
Lepidostomata hirtum [Trichoptera, cased]	ln(mg)	lnHW	y = 1.30+3.62x	0.82	3
Leuctra spp [Leuctridae]	(mg)	HW	y = 0.8496*(x^3.201)	0.9	4
Limnephilus lunatus [Limnephilidae]	ln(mg)	lnHW	y = 0.4109+3.1678(x)	0.83	1
Limnius volkmari [Limnius larvae]	ln(mg)	lnHW	y = -8.71+4.53(x)	0.7	6
Niphargus aquilex [Gammarus fossarum Koch]	Ln(mg)	Ln(BL)	y = y = –4·95 + 2·83(x)	0.9	6
Oecetis [Oecetis spp.]	ln(mg)	lnHW	y = 1.913+3.3x	0.67	4
Oligochaeta	g		y = y = (πr2*1.05x)/4		9
Oreodytes sanmarkii [Hydroporus - dysticidae]	ln(mg)	lnBL	y = 0.0618*(x^2.502)	0.71	4
Oulimnius tuberculatus L [Limnius larvae]	ln(mg)	lnHW	y = -8.71+4.53(x)	0.7	6
Oxycera [Diptera]	ln(mg)	lnBL	y = -6.21+2.52x	0.83	6
Paraleptophlebia submarginata [Leptophebidae]	ln(mg)	lnHW	y = -0.83+4.25x	0.86	6
Piscicola geometra [Leech]	Ln(mg)	LnBL	y = -2.69+2.11x	0.62	8
Pisidium	(mg)	SL	y = 0.0163*(x^2.477))	0.87	4
Plectrocnemia [Plectrocnemia conspersa]	log10(ug)	log10HW	y = 2.58+2.80x		5

Polycelis tenuis [Dugesia tigrina]	(mg)	BL	y = 0.0089*(x^2.145)	0.81	4
Potamophylax latipennis [Limnephilidae]	ln(mg)	lnHW	y = 0.4109+3.1678(x)	0.83	1
Psychoda [Diptera]	ln(mg)	lnBL	y = -6.21+2.52x	0.83	6
Rhyacophila dorsalis	log10(µg)	log10HW	y = 1.55+3.21x	0.72	8
Serratella ignita [Serratella sp.]	(mg)	HW	y = 0.7255*(x^3.325)	0.72	4
Silo nigricornis [Goeridae]	ln(mg)	lnHW	y = 0.8613+3.576x	0.75	1
Simulium [Simulium sp.]	Ln(mg)	lnHW	y = y = 0·20 + 3·32(x)	0.93	6
Tanypod [Tanypodinae]	(mg)	HW	y = 2.1694*(x^2.623)	0.85	4
Tipula Yamatotipula [Tipula abdominalis (Say)]	ln(mg)	lnBL	y = y = –5·30 + 2·36(x)	0.93	9

Diatoms

The first 30 specimens of all common diatom species were measured and where species were encountered less frequently, all specimens in the count were measured (Table S3).

Table S3. Diatom biovoulmes were calculated using predefined shapes (after Hillebrand *et al.* 1999)

taxa	shape
Achnanthes clevei	prism on elliptic base
Achnanthes conspicua	prism on elliptic base
Achnanthes delicatula	prism on elliptic base
Achnanthes distincta	prism on elliptic base
Achnanthes grischuna	prism on elliptic base
Achnanthes helvetica	prism on elliptic base
Achnanthes hintzii	prism on elliptic base
Achnanthes lanceolata	prism on elliptic base
Achnanthes lapidosa	prism on elliptic base
Achnanthes lauenburgiama	prism on elliptic base
Achnanthes lenmermanii	prism on elliptic base
Achnanthes minutissima	prism on elliptic base
Achnanthes pediculus	prism on elliptic base
Achnanthes ploenensis	prism on elliptic base
Achnanthes pusilla	prism on elliptic base
Achnanthes silvahercynia	prism on elliptic base
Amphora aequalis	half elliptic prism
Amphora fogediana	half elliptic prism
Amphora inariensis	half elliptic prism
Amphora libyca	half elliptic prism
Amphora pediculus	half elliptic prism
Ampipleura pellucida	prism on elliptic base
Ampipleura rutilans	prism on elliptic base
Asterionella formosa	box
Aulacoseira granulata	cylinder
Caloneis bacillum	prism on elliptic base
Cocconeis disculus	prism on elliptic base
Cocconeis neodiminuta	prism on elliptic base
Cocconeis neothumensis	prism on elliptic base

Cocconeis pediculus	prism on elliptic base
Cocconeis placentula	prism on elliptic base
Cocconeis psedothumensis	prism on elliptic base
Cocconeis scutellum	prism on elliptic base
Cyclostephanos sp1	cylinder
Cyclotella comensis	cylinder
Cyclotella distinguenda	cylinder
Cyclotella meneghiana	cylinder
Cyclotella radiosa	cylinder
Cyclotella sp	cylinder
Cymbella affinis	half elliptic prism
Cymbella caespitosa	half elliptic prism
Cymbella minuta	half elliptic prism
Cymbella perpusilla	half elliptic prism
Cymbella prostrata	half elliptic prism
Cymbella pusilla	half elliptic prism
Cymbella silesiaca	half elliptic prism
Cymbella sinuata	half elliptic prism
Cymbella sp.	half elliptic prism
Denticula elegans	prism on elliptic base
Denticula kuetzingii	prism on elliptic base
Diatoma hyemalis	prism on elliptic base
Diatoma tenuis	prism on elliptic base
Diatoma vulgaris	prism on elliptic base
Diploneis oblongella	prism on elliptic base
Diploneis oculata	prism on elliptic base
Diploneis sp.	prism on elliptic base
Ellerbeckia arenaria	cylinder
Entomoneis paludosa	prism on elliptic base
Eunotia bilunaris	half elliptic prism
Eunotia intermedia	half elliptic prism
Fragilaria capucina undiff.	prism on elliptic base
Fragilaria exigua	prism on elliptic base
Fragilaria fasciculata	prism on elliptic base
Fragilaria virescens	prism on elliptic base
Frustulia rhomboide	prism on elliptic base
Gomphonema acuminatum	prism on elliptic base
Gomphonema agur	prism on elliptic base
Gomphonema angustatum	prism on elliptic base
Gomphonema angustum	prism on elliptic base
Gomphonema aquemineralis	prism on elliptic base
Gomphonema clavatum	prism on elliptic base
Gomphonema gracile	prism on elliptic base
Gomphonema minutiforme	prism on elliptic base
Gomphonema minutum	prism on elliptic base
Gomphonema olivaceum	prism on elliptic base
Gomphonema parvulum	prism on elliptic base
Gomphonema truncatum	prism on elliptic base

Gyrosigma acuminatum	prism on elliptic base
Gyrosigma attenuatum	prism on elliptic base
Gyrosigma nodiferum	prism on elliptic base
Gyrosigma scalproides	prism on elliptic base
Melosira lineata	cylinder
Melosira varians	cylinder
Meridion circulare	prism on elliptic base
Navicula aboensis	prism on elliptic base
Navicula atomus	prism on elliptic base
Navicula capitata var hungarica	prism on elliptic base
Navicula capitatoradiata	prism on elliptic base
Navicula cari	prism on elliptic base
Navicula caterva	prism on elliptic base
Navicula cf. densolineolata	prism on elliptic base
Navicula cincta	prism on elliptic base
Navicula clementis	prism on elliptic base
Navicula cryptocephala	prism on elliptic base
Navicula cryptotenella	prism on elliptic base
Navicula digitulus	prism on elliptic base
Navicula festiva	prism on elliptic base
Navicula gastrum	prism on elliptic base
Navicula goeppertiana	prism on elliptic base
Navicula gregoria	prism on elliptic base
Navicula halophila	prism on elliptic base
Navicula halophiloides.x. minuscula	prism on elliptic base
Navicula helensis	prism on elliptic base
Navicula ignota	prism on elliptic base
Navicula lanceolata	prism on elliptic base
Navicula lenzii	prism on elliptic base
Navicula luciadula	prism on elliptic base
Navicula margalithii	prism on elliptic base
Navicula menisculus	prism on elliptic base
Navicula minima	prism on elliptic base
Navicula phyllepta	prism on elliptic base
Navicula pupula	prism on elliptic base
Navicula pupula var mutata	prism on elliptic base
Navicula pygmaea	prism on elliptic base
Navicula radiosa	prism on elliptic base
Navicula recens	prism on elliptic base
Navicula reinhardtii	prism on elliptic base
Navicula schoenfeldii	prism on elliptic base
Navicula seminulum	prism on elliptic base
Navicula soehrensis var musciola	prism on elliptic base
Navicula spledicula	prism on elliptic base
Navicula striolata	prism on elliptic base
Navicula sublucidula	prism on elliptic base
Navicula subminuscula	prism on elliptic base

Navicula subrotunda	prism on elliptic base
Navicula tripunctata	prism on elliptic base
Navicula trivialis	prism on elliptic base
Navicula veneta	prism on elliptic base
Nitzschia acicularis	prism on elliptic base
Nitzschia agnita	prism on elliptic base
Nitzschia angustatula	prism on elliptic base
Nitzschia capitellata	prism on elliptic base
Nitzschia dissipata	prism on elliptic base
Nitzschia dubia	prism on elliptic base
Nitzschia flexa	prism on elliptic base
Nitzschia fonticola	prism on elliptic base
Nitzschia frustulum	prism on elliptic base
Nitzschia heufleriana	prism on elliptic base
Nitzschia intermedia	prism on elliptic base
Nitzschia linearis	prism on elliptic base
Nitzschia palea	prism on elliptic base
Nitzschia perminuta	prism on elliptic base
Nitzschia recta	prism on elliptic base
Nitzschia wuellerstorfi	prism on elliptic base
Opephora olsenii	prism on elliptic base
Pinnularia acoricola	prism on elliptic base
Pinnularia appendiculata	prism on elliptic base
Pinnularia lagerstedtii	prism on elliptic base
Pleurosigma attenuatum	prism on parallelogram base
Pseudostaurosira brevistriata	prism on elliptic base
Rhoicosphenia abbreviata	prism on elliptic base
Stauroneis smithii	prism on elliptic base
Staurosira construens	prism on elliptic base
Staurosira elliptica	prism on elliptic base
Staurosirella leptostauron	prism on elliptic base
Staurosirella leptostauron var. leptostauron	prism on elliptic base
Staurosirella pinnata	prism on elliptic base
Stephanodiscus hantzschii	cylinder
Stephanodiscus parvus	cylinder
Surirella angusta	prism on elliptic base
Surirella brebissonii	prism on elliptic base
Synedra ulna	prism on elliptic base
Tabellaria flocculosa	box
Tryblionella constricta	prism on elliptic base
Tryblionella levidensis	prism on elliptic base

Trivariate analysis

Trophic links from the literature (Table S4) were then assigned on the basis of this generality. For instance if the node *Agapetus fuscipes* was assigned the level 'genus' all trophic interaction involving the genus *Agapetus* would be assigned to *Agapetus fuscipes*. The assignment of pre-determined generality removes bias and allows this method to be reproduced exactly. The level of generality assigned to each node is given in Table 5. Examples of trivariate analysis are provided in the R package *Cheddar* (Hudson *et al.* 2012).

Table S4. Sources of feeding interactions from the primary literature

Source	System	Place
Gilliam *et al* (2011)	freshwater stream	UK
Layer *et al.* (2010)	freshwater stream	UK
Ledger *et al*. (2013)	experimental freshwater channels	UK
Brose *et al.* (2005)	freshwater lake	USA
Warren (1989)	experimental freshwater stream	UK
Becker (1990)	freshwater pond	UK
Jones *et al.* (1951)	freshwater stream	Europe
Northcott (1981)	freshwater river	UK
Hynes (1950)	freshwater lake	UK
Moore & Potter (1976)	laboratory experimental freshwater	UK
Iversen (1988)	freshwater stream	UK
Spänhoff *et al.* (2003)	freshwater stream	UK
Thomas (1962)	laboratory experimental freshwater	UK
Slack (1936)	freshwater stream	Europe
Clitherow *et al.* (2013)	freshwater stream	Europe
Maitland (1965)	freshwater river	UK
Lancaster *et al.* (2005)	freshwater river	UK
Rowan Dunn (1954)	freshwater river	Europe
Radforth (1940)	freshwater river	UK
Woodward *et al.* (2008)	freshwater stream	UK
Woodward *et al.* (2005)	freshwater lake	UK
Woodward unpublished	freshwater river	UK
Badcock (1949)	freshwater stream	UK
Mackereth (1957)	freshwater	unknown
Cook (1979)	freshwater stream	UK
Perkins unpublished	freshwater stream	UK
Townsend & Hildrew (1979)	freshwater river	UK

Tikkanen *et al.* (1997)	freshwater lake	UK
Harper-Smith *et al.* (2005)	freshwater stream	UK
Englund (2005)	freshwater stream	UK
N. Dewhurst & G. Woodward unpublished data	freshwater lake	Europe
Young & Procter (1986)	freshwater lake	USA
Mann & Blackburn (1991)	freshwater stream	UK
Warren, unpublished	experimental freshwater stream	Europe
Friday (Friday 1988)	freshwater lake	UK
Gee & Young (1993)	freshwater stream	UK
Elliott *et al.* (1988)	freshwater lake	UK
Fox (1978)	freshwater	UK
Harrison *et al.* (2005)	freshwater	UK
Hall *et al.* (2000)	freshwater stream	UK
Armitage & Young (1990)	freshwater stream	USA

Table S5. The taxonomic resolution (i.e. generality) assigned to each node in the networks to create links between nodes.

node	resolution
Achnanthes clevei	genus
Achnanthes conspicua	genus
Achnanthes helvetica	genus
Achnanthes hungarica	genus
Achnanthes lanceolata	genus
Achnanthes lanceolata abbreviata	genus
Achnanthes lanceolata bimaculata	genus
Achnanthes lanceolata lanceolata	genus
Achnanthes lanceolata rostrata	genus
Achnanthes minutissima	genus
Achnanthidium minutissimum	genus
Agapetus fuscipes	genus
Alboglossiphonia heteroclita	family
Amphipoda	exact
Amphora aequalis	genus
Amphora inariensis	genus
Amphora ovalis	genus
Amphora pediculus	genus
Amphora veneta	genus
Ancylus fluviatilis	family
Asellus aquaticus	family
Athripsodes	family
Baetis	genus
Baetis rhodani	genus
Baetis scambus	genus
Baetis vernus	genus
Bezzia	family
Caenis rivulorum	genus
Caenis robusta	genus

Centroptilum luteolum	genus
Chironomidae	family
Cloeon simile	genus
Cocconeis pediculus	genus
Cocconeis placentula	genus
Cocconeis pseudothumensis	genus
Coleoptera	exact
Cottus gobio	genus
Cyclotella	genus
Cyclotella meneghiniana	genus
Cyclotella radiosa	genus
Cymatopleura elliptica	genus
Cymatopleura solea	genus
Cymbella	genus
Cymbella cistula	genus
Cymbella proxima	genus
Dystiscidae	family
Dendrocoelum lacteum	family
Diatoma vulgaris	genus
Dicranota	genus
Diploneis oblongella	genus
Diploneis parma	genus
Diptera	exact
Drusus annulatus	genus
Elmis aenea	genus
Eloeophila	family
Encyonema silesiacum	genus
Ephemeroptera	exact
Erpobdella octoculata	genus
Fragilaria	genus
Fragilaria bidens	genus
Fragilaria capucina	genus
Fragilaria capucina gracilis	genus
Fragilaria capucina radians	genus
Fragilaria capucina rumpens	genus
Fragilaria construens venter	genus
Fragilaria elliptica	genus
Fragilaria leptostauron	genus
Fragilaria nitzschioides	genus
Fragilaria ulna	genus
Fragilaria vaucheriae	genus
Fragilariforma virescens	genus
Gammarus pulex	family
Gasterosteus aculeatus	genus
Glossiphonia complanata	family
Gomphonema	genus
Gomphonema angustum	genus
Gomphonema augur	genus
Gomphonema clavatum	genus
Gomphonema olivaceum	genus
Gomphonema parvulum	genus
Gyrosigma acuminata	genus

Gyrosigma attenuatum	genus
Hantzschia amphioxys	genus
Helobdella stagnalis	family
Hemerodromia	family
Hydracarina	family
Hydraenidae	genus
Hydropsyche siltalai	genus
Hydroptila	genus
Hydroptilidae	family
Hygrobia hermanni	genus
Ilybius	genus
Lampetra planeri	genus
Lepidostoma hirtum	genus
Leuctra	genus
Leuctra hippopus	genus
Leuctra inermis	genus
Limnephilidae	family
Limnephilus lunatus	genus
Limnius	genus
Earthworm	exact
Melosira varians	genus
Meridion circulare	genus
Navicula	genus
Navicula atomus	genus
Navicula bacillum	genus
Navicula cincta	genus
Navicula cryptonella	genus
Navicula exilis	genus
Navicula ignota	genus
Navicula lanceolata	genus
Navicula margalithii	genus
Navicula minima	genus
Navicula seminulum	genus
Navicula slesvicensis	genus
Neidium dubium	genus
Niphargus aquilex	family
Nitzschia	genus
Nitzschia amphibia	genus
Nitzschia capitellata	genus
Nitzschia dissipata	genus
Nitzschia fonticola	genus
Nitzschia frustulum	genus
Nitzschia heufleriana	genus
Nitzschia linearis	genus
Nitzschia palea	genus
Nitzschia recta	genus
Nitzschia sigmoidea	genus
Nitzschia sublinearis	genus
Oecetis	family
Oligochaeta	genus
Oreodytes sanmarkii	genus
Oulimnius tuberculatus	genus

Oxycera	family
Paraleptophlebia submarginata	genus
Phoxinus phoxinus	genus
Pinnularia	genus
Piscicola geometra	family
Pisidium	genus
Planaria torva	family
Planorbis	family
Polycelis tenuis	family
Potamophylax latipennis	genus
Proasellus meridianus	family
Procloeon pennulatum	family
Psammodictyon constrictum	genus
Pseudostaurosira brevistriata	genus
Psychoda	family
Pungitius pungitius	genus
Rhoicosphenia abbreviata	genus
Rhyacophila dorsalis	genus
Salmo trutta	genus
Scirtidae	family
Serratella ignita	genus
Silo nigricornis	genus
Simulium	genus
Simulium vernum	genus
Stauroneis	genus
Stauroneis smithii	genus
Staurosira construens	genus
Staurosira elliptica	genus
Staurosira pinnata	genus
Staurosirella lapponica	genus
Staurosirella leptostauron	genus
Staurosirella pinnata	genus
Surirella brebissonii	genus
Surirella capronii	genus
Synedra	genus
Synedra parasitica	genus
Synedra ulna ulna	genus
Tanypodinae	family
Thymallus thymallus	family
Tipula	genus
Trichoptera	exact
Undiff. centric diatom	exact
CPOM	exact
FPOM	exact

Leaf litter decomposition

Leaf breakdown rates were expressed as the exponential decay rate coefficient, *k* (after

Woodward *et al.* 2012) equation S3:

$$m_t/m_0 = e^{-kt}$$

where m_0 is the initial dry weight and m_t is the dry weight at time t.

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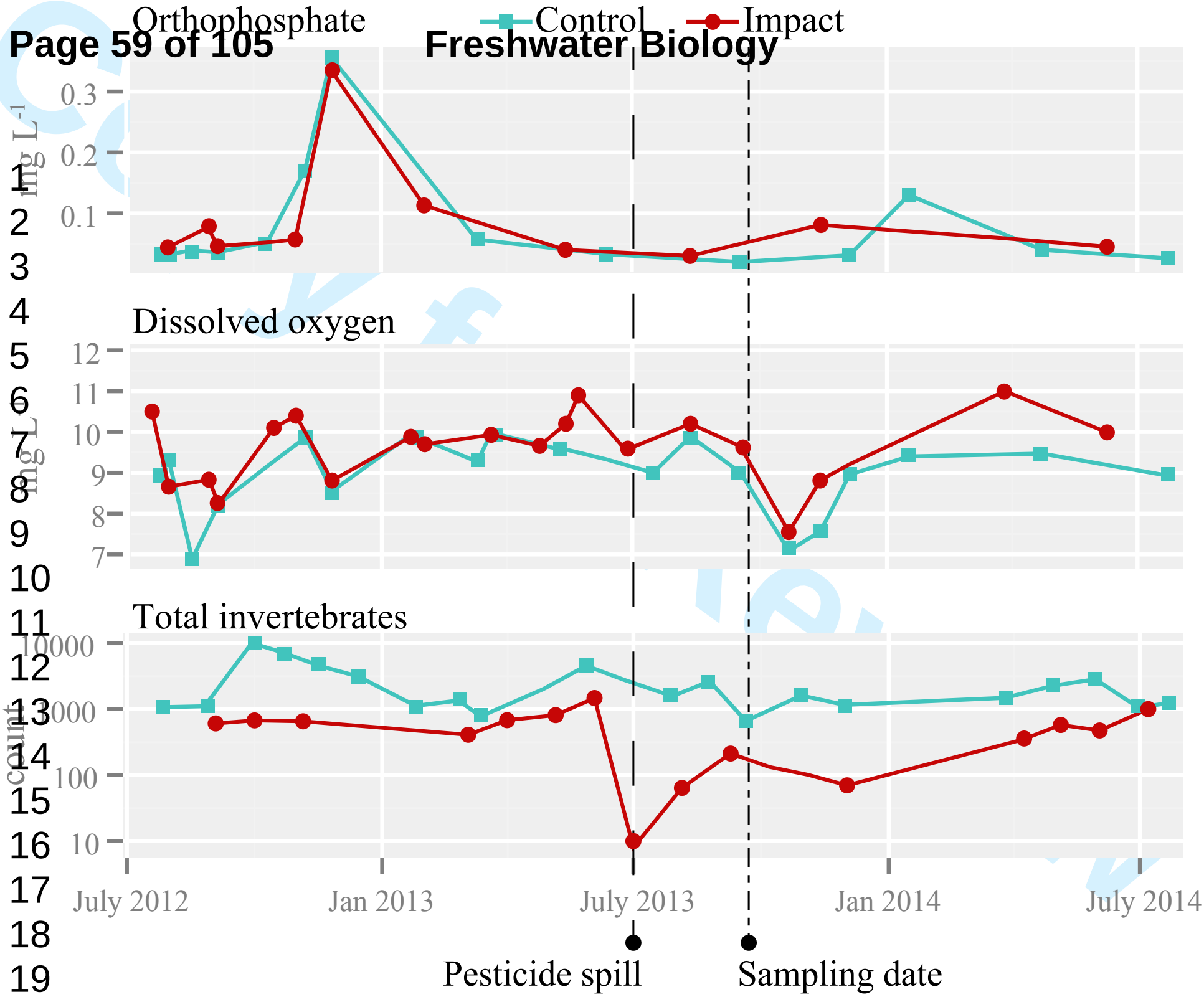
Orthophosphate
Control
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mg L-1
0.3
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Dissolved oxygen
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12
11
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9
8
7
Total invertebrates
count
10000
1000
100
10
July 2012
Jan 2013
July 2013
Jan 2014
July 2014
Pesticide spill
Sampling date

Title: Gene-to-ecosystem impacts of a catastrophic pesticide spill: testing a ~~novel~~ multilevel bioassessment approach in a large ~~natural~~river ecosystem

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Keywords: Pesticide, food web, functional gene abundance, biomonitoring, ecosystem function

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Summary

1. Pesticides can have ~~profoundly~~ strong deleterious impacts in fresh~~-~~waters, but understanding how these effects cascade through natural ecosystems, from microbes to apex predators, is limited because research that spans multiple ~~organisational~~ levels of biological organisation is rare.

2. We report how an accidental insecticide spill ~~in the River Kennet, UK,~~ altered the structure and functioning of a river across ~~different~~ levels ranging~~organisational levels,~~ from genes to ecosystems. We quantified the impacts on assemblages of microbes, diatoms,~~ invert~~ macroinvertebrates and fish and measured leaf-litter decomposition rates and microbial functional potential at upstream control and downstream impacted sites two months after the spill.

3. Both direct and indirect impacts were evident across multiple levels of organisation and taxa, from the base of the food web to ~~the~~ higher trophic levels. At the molecular level, ~~the abundance of bacterial functional genes associated with degrading organophosphates and ammonia oxidation were higher in the polluted sites. These~~ differences in functional gene abundance within the impacted sites reflected a combination of direct and indirect effects of the pesticide, via elevated microbial populations capable of utilising chlorpyrifos as a resource (i.e. direct effect) and oxidising ammonia released by decaying macroinvertebrate carcasses (i.e. indirect effect). ~~the processing of the pesticide and substrates from organic the breakdown of animal carcases, respectively.~~

4. At the base of the ~~autochthonous-based~~ food chains, diatom taxa found only in the impacted sites were an order-of-magnitude larger in cell-size than the largest comparable taxa in ~~the~~ control communities, following the near-extirpation of their consumers. ~~In the detrital-based food chains, p~~Population ~~b~~Population ~~b~~Biomass of the key ~~invertebrate~~ detritivore (*Gammarus pulex*) ~~decreased were~~ was markedly lower, ~~with~~ as was the rate of~~resultant~~

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Comment [MOG1]: Did I miss in the main text what indicator wa sused for this purpose? Also, consider replacing "processing" by a more specific term (e.g. degradation or what else is approriate given the indicator you chose).

Comment [s2]: This is unclear as written. Your sentence says that the taxa became larger, which could be read as increasing in number of species, body size or abundance. Please reword to clarify.

drops in litter decomposition rates in the impacted sites. This was partially compensated for, however, by increased elevated microbial driven breakdown, again suggesting another unexpected indirect food web effect of the toxic spill.

5. Although many species exhibited severe population crashes or local extirpation, total macroinvertebrate biomass and abundance were largely unaffected due to a compensatory increases ofelevation in small r-selected and less pesticide-sensitivetolerant taxa such as non-arthropods (e.g. oligochaetes), and/or thosetaxa which were inwith their a terrestrial n adult terrestrial aerial life-stage at the time of the spill (e.g. chironomids) life-stage that enabled them tomeaning they avoided contact with the polluted waters in the immediate aftermath of the spill (e.g. chironomids)and were therefore able to repopulate quickly. Mass-abundance scaling of trophic links between consumers and resources revealed extensive restructuring within the food web.

6. This case study shows that pesticides can affect both food web structure and ecosystem functioning, both directly and indirectly across multiple levels of biological organisation. It also demonstrates how such an integrated assessment approach, as adopted here, can elucidate these links between micro-biota, macroinvertebrates and fish, for instances, thus improving our understanding of the true spectrumrange of biological consequences of chemical contamination in natural ecosystems.

Introduction

Most lowland rivers in EuropeFreshwaters are exposed to multiplea cocktail of pesticides and other toxic chemicals at local to the global scales (Schinegger *et al.* 2011; Beketov *et al.* 2013; Stehle & Schulz 2015)(Schinegger *et al.* 2011; Beketov *et al.* 2013). Controlled eEcotoxicological experiments in the laboratory have revealed with great accuracy and

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Comment [MT4]: The key words life-stage were missing, it should now make sense!

Comment [MOG5]: Which links?

Comment [MOG6]: Also consider the recent paper on the global occurrence of insecticides by Stehle & Schulz in PNAS.

precision how these can affect the survival of target species (e.g. *G. pulex*; Xuereb *et al.* 2007)~~(e.g. *Gammarus pulex*; Xuereb *et al.* 2007) with great accuracy and precision in the laboratory (e.g. G. pulex; Xuereb *et al.* 2007)~~, and community- and ecosystem-level responses have been demonstrated in ~~experimental~~ micro- and mesocosm~~s~~ experiments (e.g. Van den Brink *et al.* 1995; Van Wijngaarden *et al.* 1996; Traas *et al.* 2004; Halstead *et al.* 2014)~~(e.g. Van den Brink *et al.* 1995; Van Wijngaarden *et al.* 1996; Traas *et al.* 2004; Halstead *et al.* 2014)~~ and field surveys (Chung, Wallace & Grubaugh 1993; Triebskorn *et al.* 2003; Malaj *et al.* 2014). In the last decade, new indices of community response have been proposed specifically to detect pesticide pollution (e.g. Liess & Ohe 2005; Schäfer *et al.* 2007; Liess, Schäfer & Schriever 2008)~~(e.g. Liess & Ohe 2005; Schäfer *et al.* 2007; Liess, Schäfer & Schriever 2008)~~ and to link community change to toxicants in ~~the~~ field data (e.g. Kefford *et al.* 2010)~~(e.g. Kefford *et al.* 2010)~~.

Despite these advances, a mechanistic understanding of both the toxic effects of pesticides (i.e. direct) and those mediated via the food web (i.e. indirect) across multiple ~~organisational~~ levels of biological organisation (i.e.~~,~~ from genes to ecosystems) is still limited in natural settings (Kohler & Triebskorn 2013). This ~~is~~~~might~~is likely ~~be~~ because there are relatively few opportunities to understand how pesticides affect whole rivers or lakes, due to the ~~inherent~~ logistical, ethical, and legal~~islative~~ difficulties in conducting such a study in a controlled manner. Here, we ~~aim to move towards~~ address~~ing~~ this research gap by quantifying the gene-to-ecosystem consequences of a major pesticide spill that caused widespread kills of ~~invert~~ macroinvertebrates over 15 km ~~of the~~in a large lowland r~~R~~iver ~~Kennet, a lowland chalk river, in the UK,~~ by combining citizen science biomonitoring data with a ~~comprehensive~~ suite of ~~more novel~~non-traditional measures of ecosystem impact.

~~Citizen science i~~Invertebrate data were collected by citizen scientists prior to, during and after the spill enabling before-after-control-impact (BACI) assessment. These data ~~helped~~ enabled the UK Environment Agency to identify chlorpyrifos as the cause of the catastrophic

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mortality following the spill. Chlorpyrifos is a widely used organophosphate pesticide (insecticide and acaricide), which attacks insect (and arachnid) nervous systems and is can also be also toxic to fishes and humans - as the cause of the catastrophicinvertebrate mortality event.

Since insects are core intermediate species in almost all loticstream food chainswebs, perturbations to their populations have the potential to ripple through the entire food web, as bottom-up effects on the fish assemblage and top-down effects on the microbial communities that drive a range of detrital processing and biogeochemical cyclesand biogeochemical processes, such as the nitrogen cycle. Specifically, chlorpyrifos can affect microbial, invert macroinvertebrate and fish populations, both directly and indirectly (see reviews by Barron & Woodburn 1995; Brock, Lahr & Van den Brink 2000; Giddings *et al.* 2014)[Raven & George 1989; Barron & Woodburn 1995; Van den Brink *et al.* 1995, 1996; Van Wijngaarden *et al.* 1996; also see reviews by Brock, Lahr & Van den Brink 2000; Giddings *et al.* 2014], food web structure (Traas *et al.* 2004) and can suppress invert macroinvertebrate-mediated detrital processing ratelitter breakdown (Maltby & Hills 2008). Placing the potentially subtle effects of pesticides within a coherent multilevel framework requires a combination of structural and functional measures from the microbial community at the base of the food web to apex predators. This has been partially achieved in some studies using mesocosms (e.g. Van den Brink *et al.* 1995; Van Wijngaarden *et al.* 1996; Kersting & Van den Brink 1997; Halstead *et al.* 2014), for instance, but rarely in natural settings (Kohler & Triebskorn 2013), and never in a manner that simultaneously captures molecular-level responses through to the full complexity of the food web in the same system.

Here lwWe present new data thate reveal how chlorpyrifos affected the structure and functioning of the wholeriver food web, based onusing several complementary approaches. including First, wWe used changes in the abundance of microbial populations based on specific functional gene loci to reveal how the genes or metabolic pathways of microbial

Comment [s7]: Do you really need all of these references to support the point? FWB tries to restrict reference strings to 3 or fewer references, unless it is essential to include more.

Comment [MOG8]: Please move most of this to the Methods section and merge the rest with the following paragraph. The goal is to develop a general case in the Introduction rather than using the section to introduce the particular case study.
...and there is a lot of redundancy between this paragraph and the numbered hypotheses that could be reduced. dls

communities are affected by the pesticide. Specifically, we investigated increases in the abundance of targeted functional genes, those responsible for the degradation of chlorpyrifos(Kwak *et al.* 2012), for example, measures of associated with organophosphate degradation and ammonia oxidisation which would suggest that microbes are both using chlorpyrifos as a resource (i.e. directly) and decomposing carcasses (i.e. indirectly), respectively. We measured microbial and macroinvertebrate activity across a range of substrates to provide a rapid assessment of the functional potential of a community thus further enhancing our understanding of the relationship between structure and function within the microbial portion of the food web. We also measure alterations in resource use ecosystem processes, in particular focusing on how the loss of keystone species, such as theg dominant detritivore, *Gammarus pulex*, could have a range of subtle yet potentially powerful indirect consequences. In addition, we useand "trivariate analysis" (*sensu* Cohen *et al.* 2009) to measure higher-level food-web responses, including changes in the size, structure and architecture of the food web.

To our knowledge, tThis study providescovers the most comprehensive collection of measures across multiple levels of organisation (genes, species, and higher-level measures) to be applied following a pesticide spill. Consequently, it provides a vital bridge between field and laboratory-based findings and highlights the advantages of using a holistic approach to understand chemical stressor impacts in natural ecosystems. This collection of measures has enabled us to test the following hypotheses:

We test the following hypotheses:

1. Microbial structure and function: tThe structure (assessed using the abundance of functional gene loci) and functional capacity of the microbial assemblage will change due to direct effects (i.e. the pesticide provides an novel additional substrate) and indirect effects (i.e. increased organic substrates are derived from decaying invert

Comment [MOG9]: Rationale not clear to me

Comment [MOG10]: This point is made repeatedly in the paper. I suggest highlighting it in the Discussion and abstract. But in the Introduction only mention the need for such a broad approach before saying in the last paragraph of the Introduction that you took such a broad approach.I 'm afraid that constantly hammering in how novel and unique your approach is will be counterproductive. Reviewers had expressed this worry before.

macroinvertebrates) of the pesticide.

2. ~~Community composition: e~~Compensatory mechanisms will be evident in the food web in the ~~immediate~~ aftermath of the spill, with less pesticide-sensitive, small, opportunistic, vagile, and ~~more r-selected~~fast-growing taxa (e.g. chironomids) ~~initially increasing~~ higher in abundance and/or biomass in the absence of larger, ~~more K-selected~~slow-growing taxa (e.g. *Gammarus pulex*), relative to control communities.

3. ~~Ecosystem function: l~~Leaf litter ~~decomposition rates~~breakdown will be impaired by the loss of key~~stone~~ detritivor~~ou~~s ~~invertebrates from the food web~~, with microbial activity hence accounting for a greater proportion of total litter breakdown.

4. ~~Trivariate analysis:~~ The food web will undergo extensive restructuring, particularly in terms of altered mass-abundance scaling relationships of the links between nodes. Local extirpations of intermediate species (e.g. herbivorous insects) will release basal species under top-down control (e.g. benthic algae) while suppressing bottom-up fluxes to ~~the~~ higher trophic levels (e.g. fish~~es~~).

Methods

Study site

The River Kennet is ~~designated as a Site of Special Scientific Interest (SSSI) and is~~ a lowland chalk tributary (catchment area 1200 km^2) of the River Thames in southern~~South~~ England, designated as a UK Site of Special Scientific Interest (SSSI). The river is groundwater-dominated, has ~~base-rich~~hard water ~~(mean annual~~ pH 7.6~~)~~ and is nutrient-rich ~~(Figure 1;~~

Comment [MOG11]: This could not be tested 2 months after the spill, could it? Please clarify/amend.

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Comment [MOG12]: Can you give alkalinity and conductivity as well in Table 1?

Table 1Table 1 (Fig. 1; Table 1), with aIts diverse flora and fauna is dominated by Gammaridae, Baetidae, Ephemerellidae, Simuliidae, and Chironomidae, which support an economically important sSalmonidae sportsgame fishery (Wright *et al.* 2002; 2004).

On 1 July 1st 2013, following their routine biomonitoring, the a citizen-science group (Action for the River Kennet, (ARK) reported a large-scale invert macroinvertebrate kill along a 15-km stretch of the river. On 25 July 5th 2013, an Environment Agency pollution incident team collected the first samples for, and detected, the organophosphate chlorpyrifos. This insecticide attacks the nervous system of insects by inhibiting acetylcholinesterase, and can be toxic to fish and meiofauna (Carr, Ho & Chambers 1997; DeLorenzo, Scott & Ross 1999). A cConcentrations of of 0.52-0.82µg L^{-1} were as recorded coming from the main tertiary sewage treatment works in Marlborough, Wiltshire, on 25 and 5 July, respectively 5th (Fig ure 1), likely probably resulting from a "down the drain" incident. Although tThe peak concentration was most likelyprobablylikely missed by the sampling team, but even thcis measured concentration is sufficient to be acutely toxic to arthropods (Giddings *et al.* 2014)(Raven & George 1989; Giddings *et al.* 2014), particularly over extended periods (i.e. >24 hours; Rubach, Crum & Van den Brink 2011). Chlorpyrifos was also detected at concentrations between 0.06-0.07-0.06 µg L^{-1} in repeat measures collected across the impacted study site on 5 July 5th. However, bBy 9 July 9th 2013 the pesticide was undetectable, indicating that this was a single pulse was received and that remained in the water column for just a few days.

Contribution of citizen scientists

Citizen scientists from ARK werehave been trained by the Riverfly Partnership toin the collection and identifyication of aquatic macroinvertebrates and had. They hadve collected data for multiple sites for several years prior to and following the spill (Fig ure S1). Dduring

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the current study, they collected one monthly kick sample (3-minutes duration) ~~using a standard hand net (1mm mesh), using following the Riverfly Monitoring Initiativeir standard protocol (http://www.riverflies.org),~~ from an upstream control and downstream impacted site ~~which complemented our own more intensive sampling~~ (Fig ~~ure~~ 1). A standard hand net (1-mm mesh) was used following the Riverfly Monitoring Initiative standard protocol (http://www.riverflies.org). The ~~invert~~ macroinvertebrates collected were identified live on the bank, without magnification, and abundance ranked per sample as: 0 = 0 individuals; 1-9 = 1; 10-99 = 2; 100-1000 = 3; >1000 = 4, for eight key groups: 1. ~~c~~Cased Trichoptera; 2. caseless Trichoptera; 3. Ephemeridae; 4. Ephemerellidae; 5. Heptageniidae; 6. Baetidae; 7. Plectoptera; 8. Gammaridae, which were summed to give a total score based on the number and diversity of the target taxa. These data provide a critical BACI element to the study, enabling us to track the impact of the spill through both space and time.

Mean annual water chemistry data were obtained for Environment Agency monitoring stations located 2.3 km ~~above~~ upstream and 2.7 km ~~below~~ downstream from the spill and were similar ~~in the two treatments~~across the study site (Table 1)(

Comment [MOG17]: This has not yet been introduced. Please delete or rearrange.

Comment [MOG18]: What devices were used? dissecting microscope? which magnification?

Comment [MOG19]: Size of the animals identified is obviously important. Please clarify.

Comment [MT20]: This refers to the Riverfly Monitoring Initiative citizen science protocol which requires no magnification and doesn't utilise body size information. This methodology has been successfully applied nationwide, see http://www.riverflies.org. Our more intensive methods use magnification equipment and we measure body size, described below.

Comment [MOG21]: How far?

Comment [MOG22]: Please reword. This was not a controlled experiment.

~~Table 1~~Table 1). These water chemistry data, combined with the ARK ~~invertebrate~~ monitoring data of macroinvertebrates, showed ~~indicate that there was~~ no evidence of organic pollution from the sewage treatment works, indicating ~~and~~ that ~~this could therefore not be ascribed~~sewage was an unlikely ~~the~~ cause of the ~~invert~~ macroinvertebrate mortality event (Fig ~~ure~~ S1).

Sampling protocol

~~We began~~ Comprehensive~~large scale~~ biological sampling began in September 2013, as soon as possible ~~as soon as was feasible~~ after the chlorpyrifos spill ~~was~~had been identified as the causal agent, using an experimental design comprising three upstream control and three downstream impacted reaches, each 50 m long~~in length~~, along a c~~a~~. 6 km river stretch (Fig ~~ure~~ 1). Sites were c~~approximately~~ 1 km apart, with similar channel forms and riparian surroundings. H~~In this study~~ere we present data from two control and two impacted reaches (Fig ~~ure~~ 1) for a suite of structural and functional ~~biotic measures~~indicators to test ~~the novel~~a multilevel bioassessment approach. ~~Depletion electrofishing, three Surber samples, a stone scrape and~~ Three sediment samples, a stone scrape, three Surber samples and depletion electrofishing were used to characterise ~~fish, invertebrates, diatoms and~~ microbial, diatom, macroinvertebrate and fish structural attributes, respectively. At each site, ~~ten~~ 10 ~~coarse (10mm) and ten~~ fine ~~mesh~~ (0.5mm) and 10 coarse-mesh (10mm) leaf litter bags were used to ~~assess~~ determine rates of ~~community and microbial~~ decomposition driven by microbes alone or by whole communities~~rates~~ (Woodward *et al.* 2012)~~after Woodward *et al.* 2012b)~~. ~~A and~~In addition, a sample of river water was collected and ~~then~~ incubated ~~over~~ with a range of substrates to ~~measure~~ assess microbial functional capacity.

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Microbial functional gene abundance

We used quantitative PCR (qPCR) to examine gene abundance for microbial functional and taxonomic marker genes. 16S rRNA gene abundance was used as a proxy for total bacterial abundance. Direct effects of the chlorpyrifos spill were examined using the organophosphate hydrolase gene (*opd*), which is responsible for the degradation of chlorpyrifos by bacteria; and bacterial populations containing this gene have previously been demonstrated to increase in abundance inat sites impacted by organophosphate impacted sites (Kwak *et al.* 2012). Indirect effects were examined by quantifying the abundance of N-cycling genes coding for enzymes involved in N-cycling: (nitrite reductase; (*nirS*;) and ammonia monoxygenase; (*amoA*-) from ammonia-oxidising archaea (AOA) and bacteria (AOB)) as these are most likely to reflect decomposition of dead arthropods in impacted sites. We hypothesised that decomposition of dead arthropods would result in an increased input of NH_4^+ from ammonification of organic N. We focused on *nirS* and *amoA* genes as both nitrification and denitrification pathways are important in removing N from systems and can be coupled when denitrifiers reduce the NO_3^- produced by the nitrifiers that oxidised NH_4^+. By focusing on functions of a range of functional populations, a change across all populations combined provides an indicator for community-level effects of chlorpyrifos on river microbes. Full details of DNA isolation, primer details and qPCR cycling conditions are available in the Microbial Ffunctional Ggene Aabundance section in the Ssupplementary Mmaterial.

Microbial functional potential

Open –water samples were collected from each site and returned to the laboratory in an ice-chilled cooler. Samples were allowed to settle (>10 min), after which a subsample of 100 µl[1] was aliquoted was pipetted into each well of a Biolog EcoPlate, which contained an individualsingle carbon substrate, including carbohydrates, polymers, fatty acids, and amino

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acids. Each well also contained the redox dye tetrazolium, which is reduced during microbial respiration, resulting in a measurable colour change. Each EcoPlate contains 31 substrates plus a no-substrate control in triplicate. Plates were incubated in the dark at 22°C for 5 days, after which colour change was quantified by measuring optical density at 600 nm using a Biotek HT absorbance reader (Biotek, Swindon, UK). For each EcoPlate, we calculated the substrate usage by subtracting the mean of the three no-substrate controls from each measurement. ~~Substrate u~~Usage was ranked across the substrates in each replicate, and the ranked optical densities were plotted to visualise broad changes across sites. ~~For each EcoPlate, we subtracted the mean of the three no-substrate controls from each measurement. Optical density was ranked across the substrates in each replicate, and the ranked optical densities were plotted to visualise broad changes across sites.~~

Comment [GW26]: Look for recent paper by Mulder using biolog plates – might be useful citation here?

Population abundance, community structure, and food web size-scaling

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Quantitative depletion electrofishing was undertaken, with population densities estimated using the R package FSA (Ogle 2012) and iterative Maximum Weighted Likelihood statistics (equation S1 and S2 in Supplementary Material; Carle & Strub 1978) ~~(equations S1 and S2; for additional equations and statistical methods see Carle & Strub 1978)~~. All fishes caught were identified to species and measured by fork length. For each species, individual dry mass was calculated ~~from length~~ using length-mass regression equations ~~generated from a sub-sample~~. Full details of fish dry mass estimation can be found in the Food web ~~(see equations S1 and S2)~~ characterisation section of the Supplementary Material.

Comment [MOG27]: Length?

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Invertebrates were collected (n = 3 samples per site) using a Surber sampler (0.0625 m^2, 335 µm mesh), preserved in 99.8% ethanol, and later sorted from debris, identified to the highest possible taxonomic resolution (usually species), and counted (Table S1). Dry masses of ~~invert~~ macroinvertebrates were determined from regressions of linear dimensions ~~(up to [60~~

Comment [MOG28]: And the minimum?

~~individuals measured per species)~~ using published equations (see Table S2); a subset of 60 individuals were measured per species per site, or every individual where abundance was below 60. ~~In our analyses w~~We distinguished between arthropods (i.e. insect larvae and Crustacea) and other taxa (i.e. Tricladida, Annelida and Mollusca) based on their sensitivity to chlorpyrifos (Raven & George 1989; Giddings *et al.* 2014).

Diatoms were scraped from 8.64 cm^2 of the upper surface of one cobble at each site using a toothbrush and 3.6 by 2.4 cm photographic slide as a flexible quadrat ~~and toothbrush~~, preserved using Lugol's iodine, and prepared using standard methods (Battarbee *et al.* 2001). A minimum of 300 diatom valves were identified to species per sample using the keys of Krammer & Bertalot (1986), Krammer *et al.* (1986), Krammer & Lange-Bertalot (1991a b)~~(1991a, b)~~ and abundances per unit area were determined as in Battarbee (1973). Linear dimensions were measured to the nearest 1 µm to estimate diatom biovolume (Table S3; Hillebrand *et al*. 1999). The first 30 specimens of all common (n >30) species were measured and where species were encountered less frequently, all specimens in the count were measured. Carbon content was estimated (Rocha & Duncan 1985) and then converted to dry mass (Sicko-Goad, Schelske & Stoermer 1984).~~as in (Battarbee (1973). Linear dimensions were measured to the nearest 1µm to estimate diatom biovolume (Table S3; after Hillebrand *et al*. 1999). The first 30 specimens of all common (n >30) species were measured and where species were encountered less frequently, all specimens in the count were measured. Carbon content was estimated (after Rocha & Duncan 1985) and then converted to dry mass (after Sicko-Goad, Schelske & Stoermer 1984).~~

We used these mass-abundance data from across the different taxa and trophic levels to construct whole-community 'trivariate food webs' - food webs ordinated by overlaying feeding links on the bivariate relationship between species mean body mass and their numerical abundance on a double logarithmic scale - ~~We used these mass-abundance data from across the different taxa and trophic levels to construct whole-community 'trivariate~~

~~food webs', which map feeding links into mass versus numerical (*MN*) abundance plots~~ to understand how chlorpyrifos alters food ~~-~~web structure ~~and substructure~~. Deviations in *MN* among species pairwise links can be used to identify alterations to biomass fluxes in the food web. For instance, altered consumer-resource feeding "link angles" can reveal rates of change in biomass, population production and population consumption between species-pairs, through to the food web as a whole (*sensu* Cohen *et al.* 2009), and these changes can help us to interpret direct and indirect effects of chlorpyrifos.

Comment [s29]: You'll probably want to explain here and in the legend to Fig. 2 that 'M' is individual body mass, not population biomass.

Comment [MOG30]: Please explain somewhat better for readers not familiar with the concept.

Trivariate webs were constructed for all sites. Feeding links were inferred from trophic interactions published in the literature ~~-~~ (Table S4). We assumed that if a trophic interaction between two species has been ~~observed~~reported in the literature and those same species ~~are~~were present at one of our sites, then that trophic interaction ~~is~~ also ~~present~~occurred, as has been validated in other ~~running water~~stream food webs (Layer *et al.* 2010; Layer, Hildrew & Woodward 2013) ~~(Layer *et al.* 2010; 2013)~~. In a few instances, feeding links were assigned on the basis of taxonomic similarity. For example, if a link had been established from the literature for at least one congener it was assumed that different species within the same genus fed upon the same resources and were consumed by the same consumers. ~~In some instances~~ It was necessary to extend this assumption to the family level in some instances where information in the primary literature was scarce (Table S5). This minimises bias between nodes where the quantity of directly observed information varies and allows the method to be reproduced exactly ~~-~~ (Gray *et al.* 2014) ~~(Gray *et al.* 2014)~~.

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Ecosystem functioning: leaf-litter decomposition

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At each site, the decomposition rate of leaf-litter was determined from leaf-packs containing 3.0 g (±0.3 g SD) black alder (*Alnus glutinosa* ~~(L.) Gaertn.~~) incubated in the river for 9 days. Coarse (150 mm by 100 mm, 10mm mesh) and fine (150 mm by 100 mm, 500 µm mesh)

Comment [s31]: Please tell us what the error term represents – SE? SD?

mesh-aperture bags were used to determine the fraction of decomposition contributed by microbes (mass loss from fine mesh bags) and ~~invert~~ macroinvertebrates (difference in mass loss from coarse and fine mesh bags). Leaf breakdown rates were expressed as the exponential decay rate coefficient, *k* (see equation S3; Woodward *et al.* 2012) ~~(see equation S3; after Woodward *et al.* 2012).~~

Data analysis

Trivariate statistics were calculated using the method of Cohen *et al* (2009) in the R package Cheddar (Hudson *et al.* 2012). We used link angles to estimate changes in potential biomass flux between a resource and its consumer. In summary, a link can be viewed as a vector from a resource to its consumer and, considering that ~~invert~~ macroinvertebrate taxa abundance and/or mass is predicted to decrease ~~within~~at impacted sites, a change in the angle of ~~invert~~ macroinvertebrate upper- and lower-links would indicate a potential change in biomass flux (Fig. ~~ure~~ 2).

Linear mixed effect models (LMM) were used to test for differences in mean annual water quality, with treatment and date as fixed and random ~~terms~~factors, respectively ~~[Results are presented in supplementary material]~~. Differences in ~~our~~ biotic response variables (link angles, species and community abundance and/or biomass, gene abundances and microbial capacity) between ~~treatments~~ control and impacted sites (i.e. condition) were tested using LMM with site and ~~treatment~~ condition as random and fixed factors, respectively. Where necessary a variance structure was used to account for unequal variance between sites in order to meet model assumptions (after Zuur *et al.* 2009). If data were not normally distributed they were Log_{10} transformed to meet the assumptions of the test. All LMM were performed using the nlme package in R (Pinheiro *et al.* 2011) and estimates were made using restricted maximum likelihood or, when testing for differences in group means (e.g. ~~invert~~

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Comment [MOG32]: Refer to Suppl. Mat.at the appropriate place in the Results section.

macroinvertebrate communities within and between treatments), using general linear hypotheses tests in the R package multcomp (Hothorn *et al.* 2014).

Comment [AJD33]: Worth citing R?

Results

ARK aquatic Macro invertebrate monitoring by citizen scientists

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Over six months wWithin control sites, *G. pulex* had the highest relative abundance compared to other taxa sampled by ARK citizen scientists (61%), followed by Baetidae (17%), Ephemerellidae (12%), cased Trichoptera (9%) and Plecoptera (1%). The pre-impact "riverfly"macroinvertebrate assemblage within the impacted site in the three months prior to the spill was similar but following the spill on July 1st 2013, there was a 99.5% reduction in total abundance relative to data from the previous month (Fig ure 3). By September, the time of our sampling date, total abundance had increased again, but was dominated by Ephemeroptera instead of *G. pulex*, the latter being the slowest taxa to recover, as recorded by the citizen scientists to recoverwith the latter being among the slowest of the four "riverfly" taxa to recover.

Comment [s34]: What 4 taxa are you referring to?

Microbial functional gene abundance and functional potential

Comment [GW35]: ALEX TO ALSO CHECK THIS SECTION
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Based on aAnalyses of gene abundances revealed that: Within the microbial community, populations of ammonia oxidisers (*amoA*), particularly AOBs, increased were by up to 3.20-fold higher174% (Fig ure 4 1c, t_2 = 4.99; p = 0.03), after the chlorpyrifos spill, and populations capable of usingutilising organophosphate (*oph*) as a resource degraders increasedwere by up to 7-fold24% higher(Fig ure 4 1e; t_2 = 6.14; p = 0.02), in impacted sites compared with control sites (Fig. 4a; t_2 = 6.14; p = 0.02). The large iIncreaseelevations in

Comment [MOG36]: Has this acronym been introduced above?
Comment [AJD37]: AOA and AOB are defined in the methods section.
Comment [MT38]: I prefer 3,174% as we use the same language when presenting the invert results
Comment [MOG39]: To my knowledge, this enumeration system doesn't exist in FWB. I suggest using letters a to p.

Yes, you will need to relabel the panels, and fix the references in the text accordingly – dls

Comment [s40]: If I understand your study design correctly, this wording isn't quite correct. I think you sampled microbes only after the spill (correct?). You can demonstrate a difference between C and I, but not an "increase" in the I section. I think that only the citizen science data can demonstrate increases. Please go through the text and reword as needed.
Comment [MT41]: Again I prefer 724%
Comment [AJD42]: I don't mind % instead of this. Not my idea to use "7-fold"

populationsthe abundance of ammonia oxidisers and organophosphate degradersboththese groupspopulations revealsuggests both direct (i.e. microbes utilised the insecticide as a resource) and indirect effects (i.e. microbes utilised ammonia fromreleased by decaying invert macroinvertebrates) of chlorpyrifos. However, there was no significant change difference in the total population abundance of bacteria (Fig.ure 4.1a), nor of populationsthe abundance of nitrite reducers (Fig.ure 4.1b) or ammonia- oxidising archaeaAOAs (Fig.ure 4a-1d).

The functional microbial assays showed impacted sites had higher overall substrate usage and a shallower rank abundance curve, indicating substantial functional changes in response to the spill. Mean overall carbon usage ofin the impacted sites differed from that inwas higher than the control sites (Fig.ure 4b.2; $t_2 = 4.2$, $p = 0.05$), with lower mean substrate usage in the latter). Differences among control and impacted sites suggested elevated rates of substrate usage of simple carbohydrates (e.g. glucose-1-phosphate, $t_2 = 4.4$, $p = 0.05$,- αalpha-D-lactose, $t_2 = 7.7$, $p = 0.02$) and amino acids in the impacted sites, with little difference in the usage of the more complex polymers (e.g. Tween 40).

Macroinvertebrate cCommunity compositionstructure and ecosystem functioning

Overall Total macroinvertebrate biomass and abundance did not significantly differ significantly between the control and impacted sites ($t_2 = -1.43$; $p = 0.29$; $t_2 = -2.11$; $p = 0.17$). However, tThe biomass of less pesticide-sensitive macroinvertebrate taxa considered less sensitive to pesticides was 97.2% lower than that of the sensitive arthropodsarthropod taxa within control sites (Table 2). Furthermore, However, total arthropod biomass was 92.9% lower within impacted sites thanwhen compared to control arthropod biomass in control sites and 80.4% lower thanrelative to biomass of less pesticide-sensitive invertebrate taxa within impacted sites (Table 2Table 2; Fig.ure 5). In addition, the biomass of

Comment [MOG43]: Please clarify how chlorpyrifos affects resource supply.

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Comment [MOG44]: This sentence seems contradictory to the following sentences. Please clarify.

macroinvertebrate taxa considered less sensitive to pesticides was 97.2% lower than that of the sensitive arthropods in control sites (Table 2), thus ~~less sensitive taxa~~ the former were partly compensating for the loss of ~~arthropods~~the latter within impacted sites. ~~Within the impacted sites there were decreases in~~ *G. pulex* biomass (99.6%) and abundance (99.2%) and *Baetis* biomass (18.7%) and abundance (95.6%) were lower (~~95.6%,~~ Fig ~~ure~~ 4c~~,~~; 4d), but ~~increases in~~ chironomid biomass (89.3%) and abundance (92.2%) and oligochaete~~a~~ biomass (85.4%) and abundance (94.5%) was higher in impacted sites compared to control sites (~~94.5%,~~ Table 2; Fig ~~ure~~ 5). M~~Fish~~ acroinvertebrate~~s~~ diversity was similar ~~across~~ between control and impacted sites (t_2 = -0.39; p = 0.74~~Table 3~~), as was also true for fish diversity ~~the invertebrates~~ (Table 3~~t_2 = -0.39; p = 0.74~~), whereas four taxa of large diatom~~s~~ ~~taxa~~ (*Cymatopleura solea*, *Cymatopleura elliptica, Gyrosigma attenuatum* and *Surirella caproni*) were present only in the impacted sites (Fig ~~ure~~ 4d~~-4~~). Microbial ~~mediated~~ decomposition was higher, whereas total decomposition mediated by both microbes and detritivores was lower, ~~with~~in the impacted sites (~~Table 2~~; Fig ~~ure~~ 4c~~-3~~), probably reflecting the decline of ~~the detritivore~~ *G. pulex* and partial compensation by increased microbial activity ~~consumers~~.

Trivariate analysis

Arthropod lower-link angles were less negative (i.e. shallower) than~~relative to~~ less pesticide-sensitive taxa in the control communities, ~~whereas these~~but ~~were~~ more negative (i.e. steeper) within the impacted communities (Table 2). This indicates altered mass-abundance scaling relationships of the links between nodes ~~as hypothesised (Fig ure 2)~~and a potential decrease in biomass flux from diatoms to arthropods within the impacted communities (Fig. 2). *G. pulex* and *Baetis* had the highest biomass and numerical abundance within the control macroinvertebrate community, respectively (Figs~~ure~~ 4c~~-3~~,~~-~~ 4d~~-4~~), and these species upper-link angles (i.e. to their predators) became ~~less negative~~shallower at impacted sites (Table 2), thus

Comment [s45]: Again, please use lower-case letters to designate the panels within a figure.

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Comment [GW46]: ditto

representingindicating a potential decrease in biomass flux to fishes from both the detritivorest and herbivorous food chains. To illustrate the direction of biomass flux through the network food web and the how one key species is connectioned of a key species to all the other taxas via relatively direct and short paths, we have constructed an example food chain with *G. pulex* as the focal species (Fig. ure 6). ; whichThis showedhighlights that even in this complex food web most species are only 1-2 links from all the others, highlighting the potential for perturbations to ripple rapidly through the network even in this complex food web. More commonly used wWhole-network metrics, such as the regression slope and intercept, showed no clear differences that could be ascribed to the pesticide spill (Table 3).

These gene-to-ecosystem results provide insights into previously unexpected phenomena, such as the increased gene abundance and increased functional capacity of the microbial community associated with both direct and indirect impacts of the pesticide, the appearance of large diatom taxa under reduced consumer densities, the suppression of ecosystem functioning due to the loss of a keystone detritivore and can provide plausible hypotheses for further testing.

Comment [MOG47]: Please omit this paragraph from the Results section. Possibly insert in the Discussion.

Discussion

The documented insecticide spill in the River Kennet affected multiple organisational levels, from individual genes, through to food web structure and an ecosystem processes. The location of pesticide-sensitive macroinvertebrate consumers relative to their resources in *MN* space shifted markedly, and the collapse in the population biomass of a previously dominant keystone detritivore, *G. pulex*, was especially notable. This was associated withresulted in dramatically impaired rates of invertebratedetritivore-mediated litter decomposition, with potential repercussions for the higher trophic levels. In this highly interconnected food web

Comment [s48]: I don't think that you rigorously linked the loss of Gammarus to the reduction of decomp rates.

(Fig. 6) , most species were separated by just 1-2 links[so]perturbations could potentially not only easily spread propagate quicklythrough species interactions, but they could also dissipate effectivelyrapidly. These small-world properties could confer resilience on the system as a whole, as alternative feeding paths provide an abundance of relatively direct "short-circuits" in the network food web (Fig ure 6). Various compensatory mechanisms and hystereses within the food web were evident following the spill, including increased elevated microbial decoemposer activity in the absence of invert macroinvertebrate detritivores (Fig ure 4c 3) and irruptions and growth of less pesticide-sensitive and *r*-selected taxa capable of exploiting recently vacated nichesnew resources (Fig ure 5). The functional potential of the microbial assemblage in particular increased was higher within the impacted sites, as did was the abundance of genes associated with organophosphate degradationuse and ammonia oxidation in the aftermath of widespread arthropod deaths (Fig ure 4 1a; 4 2b). Extended temporal sampling will likely reveal if the sewage treatment work is potentially confounding our interpretation of this result, although there is no suggestion this is the case, as water quality is essentially identical above and below the works (Table 1; Fig. S1).

It is [essentially unknown]exactlyMicrobes account for most of a river's biodiversity, drive key ecosystem processes and biogeochemical cyclesMicrobial biodiversity in natural ecosystems (Woodward, Gray & Baird 2013), even though these taxa account for most of a river's biodiversity, drives key ecosystem processes and biogeochemical cycles (e.g. nitrogen cycle) and both respond to and regulate changesinteracts with in higher trophic levels. Our qPCR assays revealed that the abundance of genes associated with processingthe turnover of organophosphate and ammonia increased was higher in polluted sediment, revealing both direct and indirect food web effects of the spill on , as a small first glimpse into the workings of the microbial activities"black box".

Strong links between changes in the structure and functioning of the microbial and invert macroinvertebrate community were evident, as revealed by the changes in decomposition

Comment [s49]: Did you show this?

Comment [s50]: Is it reasonable to think that there would be any trace of the ammonia from the dead invertebrates or the pesticide 2 months later? Many sewage treatment plants release ammonium, which in the absence of before-after data on the microbes, could be an alternative explanation for this observation. Consider softening the language.

Comment [MOG51]: Please tone down. There should be quite a bit of pertinent information on microbial biofilms even in rivers.

rates associated with these two major biotic drivers (Gessner & Chauvet 2002; Schäfer *et al.* 2007). The microbial community played a key role in maintaining ~~detrital processing~~litter decomposition following the ~~invert~~ macroinvertebrate losses~~extirpation~~, and microbial functional potential assessed by Ecoplate assays was also ~~increased~~ elevated at the impacted sites. ~~The~~ large-scale mortality of ~~invert~~ macroinvertebrates was likely to have released resources~~readily metabolised substrates~~ readily available for microbial use, ~~allowing~~promoting the proliferation of fast-growing ~~'weedy'~~ bacteria able to use a broad~~er~~ range of ~~the Ecoplate~~ substrates. Additional data from more extended ~~-~~ sampling will eventually help us to better understand the temporal dynamics of the recovery process, by providing deeper insights into the baseline variability. ~~Nevertheless,~~Even in the current absence of such additional data, ~~These~~our results clearly ~~reveal~~underline the potential of microbial ~~techniques as~~ bioindicators for~~in~~ assessing direct and indirect responses of river ecosystems to environmental impacts ~~at the base of the food web~~.

Employing a highly resolved network-based perspective provided further insight~~s~~ into both direct and indirect effects of the perturbation - from genes to species ~~individuals~~ and from food webs ~~species through~~ to the ecosystem as a whole - as we were able to connect structur~~al~~e and functional indicators across different levels of biological organis~~z~~ation, as well as ~~providing a deeper mechanistic~~improving understanding of the associated responses ~~and indicators~~. For instance, *G. pulex* and *Baetis* represented key nodes in the major detrit~~ivore~~~~al~~ and herbivor~~eous~~ food chains, respectively, as is the case in many lowland running waters (Woodward *et al.* 2008; Layer *et al.* 2010), and both populations collapsed in the impacted sites. Our broad ~~new~~ multilevel approach revealed how the loss of consumers could result in the release of their resources ~~(or~~and potential competitors~~)~~, and also how major conduits of energy and biomass flux to the ~~economically and ecologically important~~ species at the top of the food web, including ~~(e.g.~~ ecologically important and economically valuable~~d~~ fish species, such as trout~~,~~) could be compromised.

Comment [s52]: Ok, but I guess I'd be surprised if much of this material persisted for 2 months, considering rapid decomp of invertebrate tissue during the summer, and wash-out of soluble DOM and nutrents.

Microcosm and mesocosm experiments have described ecosystem-level responses to, and recovery from, combined pesticide and nutrient additions (Traas *et al.* 2004; Halstead *et al.* 2014)~~(e.g. Traas *et al.* 2004; Halstead *et al.* 2014)~~, and observational field--based research has demonstrated ~~a study of a stream in the US treated with pesticides reported~~ that recovery of the ~~invert~~ macroinvertebrate community and leaf~~, litter processing~~decomposition was related to aerial mobility of repopulating taxa (Chung *et al.* 1993). Our study represents a novel ~~integrated~~ approach, ~~that~~ integrating~~s~~ a broad range of assessment metrics at multiple levels and this has helped us to better understand~~ing~~ the ~~c~~effects of a pesticide ~~contamination~~ spill in a natural setting. ~~bridging the gap between experimental and previous observational field based research~~. ~~It Our approach~~The same approach is also more widely applicable ~~also comparable~~ to ~~other studies which have shown how interactions within freshwater food webs exposed to~~assessments of effects caused by other stressors, such as acidification and eutrophication, where interactions within food webs ~~have been found to can modulate~~have a ~~bearing on~~can shape both the ecosystem impact and the rate and trajectory of recovery (e.g. Ledger & Hildrew 2005; Layer *et al.* 2010; Rawcliffe *et al.* 2010). ~~As such~~Thus, ~~it the~~such an approach ~~highlights~~ offers ~~how~~ a way ~~we can~~to move beyond ~~a~~ partial taxonomic or trait-based views to one that ~~more~~ explicitly incorporates species interactions ~~with~~in ~~the wider~~ food webs~~:~~ and ecosystem processes in river bioassessment ~~i.e. this provides a means of shifting from autecological, node-based approaches towards more [synecological], network-based biomonitoring~~ (Gray *et al.* 2014)~~(Gray *et al.* 2014)~~.

~~In addition,~~Our study also highlights the value of citizen science in biomonitoring and bioassessment~~is highlighted~~, as it enabled us to place the ~~more~~ detailed ~~intensive~~ data specifically and intensively collected after the toxic spill in the context of a ~~much~~ wide~~r~~ before-and-after-control-and-impact (BACI) ~~-~~style "natural experiment", which would have otherwise been impossible to employ in the search for causal relationships. Mobile Ephemeroptera (*Baetis* and Ephemerellidae, both active swimmers with an aerial adult

Comment [MOG53]: This sounds to me like there isn't much information, whereas in reality there is plenty. Please rephrase accordingly.

Comment [MOG54]: Consider deleting this statement.

Comment [MOG55]: Most of traditional river bioassessment is in fact based on synecological approaches, namely on macroinvertebrate community structure.

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~~terrestrial~~ life-stage that coincided with the pollution) repopulated the ~~river~~impacted sites more quick~~ly~~er than *G. pulex* (Fig.~~ure~~ 3), as did the often opportunistic ~~more *r*-selected~~ chironomid species~~s~~ and less sensitive non-arthropod taxa ~~including~~ such as oligochaetes (Fig.~~ure~~ 5). The~~is~~e responses echo~~es~~ tho~~s~~e ~~responses~~ of small *r*-selected taxa~~. which also~~ preceding~~ed~~ the recovery of larger *K*-selected species in previous ~~pesticide spill contamination field~~ studies on pesticide ~~contamination~~ contamination (Chung *et al.* 1993; Liess & Schulz 1999; Beketov *et al.* 2008)~~ (Raven & George 1989; Chung *et al.* 1993)~~.

Comment [MOG56]: There should must be a lot more recent studies.

It has been hypothesised that ecological inertia can operate within freshwater food webs, creating 'community closure' or ~~altering~~ furthering recovery trajectories ~~such~~ that ~~they~~ are not simple reversals of ~~the~~ impacts (e.g. Ledger & Hildrew 2005; Layer *et al.* 2011; 2013)~~(e.g. Ledger & Hildrew 2005; Layer *et al.* 2011; Layer, Hildrew & Woodward 2013)~~. ~~Our initial data suggest these systems are relatively resilient: i.e. both the impact and the recovery phase can move quickly~~be short in duration~~, so long as sufficient alternative nodes and links are retained within the affected food web. Nonetheless, i~~Impacts on key nodes can alter important aspects of food~~ web structure and associated processes, such that although the latter might operate at similar rates, they may be driven by microbes and *r*-selected taxa instead of ~~the larger~~ *K*-selected taxa, as has been reported in response to pesticide contamination (Chung *et al.* 1993) and other stressors (Hladyz *et al.* 2011)~~.~~ Our initial data demonstrate that, while the R. Kennet's ~~ecosystem~~ecological structure and functioning were significantly ~~impacted~~altered by the toxic spill, ~~but that~~ there were many alternative nodes and links ~~retained~~ within the ~~affected~~ food web ~~suggesting that the system is relatively resilient~~that could help confer some level of resilience even in the face of catastrophic population losses.

Future work will require ~~more~~ well co-ordinated laboratory and field ~~based experiments~~ investigations based on ~~that share~~matching methodologies to ~~develop a mechanistic~~improve understanding of the links between ~~the~~ microbiota and ~~macrobiota~~ larger organisms before, if

ever, one can be used as a proxy for the other (e.g. Triebskorn *et al*. 2003). Nonetheless, our study represents a proof-of-concept as to how these vastly different metrics might be linked and, a Also, as more data are generated both spatially and temporallyover time, the potential time × t treatment interactions and any potential underlying effects of the sewage treatment works can also be more thoroughly explored. Additional metrics based on techniques, for instance, such as next-generation sequencing (e.g. Rosi-Marshall *et al.* 2013)(e.g. Rosi-Marshall et al. 2013) or, and measures of whole-ecosystem respiration (e.g. Young, Matthaei & Townsend 2008)(e.g. Young, Matthaei & Townsend, 2008), could be incorporated to gain a clearer view ofcapture the full extent of the impacts and recovery trajectories more fullyy of recovery.

Although they have only coveringed only a subsetpart of the spectrum of responses reported here, several other multimetric bioassessments studies have also shown parts of a comparable pictureyielded similarcomparable results, including how pesticides 1) can indirectly release prey species from predation (Papst & Boyer 1980), 2) constrain consumer populations through loss of resources (Brazner & Kline 1990), affect the micro and macrobiota structure and functioning of microbial and invertebrate aquatic communities in mesocosms (Downing *et al.* 2008; Relyea 2008; Halstead *et al.* 2014) or, alter the structure and functioning of natural stream communities (Chung *et al.* 1993; Schäfer *et al.* 2007). Results from ecent correlational workstudies has also suggested that changes across at multiple trophic levels may be related to contamination from organic chemicals contaminants (mostly pesticides) at the continental scale (Malaj *et al.* 2014). Despite this and the worldwide use of, and predicted projected increase in, pesticides application, studies of their effects at the ecosystem-level are rare in natural settings (Kohler & Triebskorn 2013), but with thisThe present study we contributes to bridging this research gap.

To the best of our knowledge, this study represents the most comprehensive diverse collection set of measures across multiple levels of biological organisation to have been

Comment [MOG57]: This paper may also be useful in this context, as well as in the Introduction: Rosi-Marshall, E. J., Royer, T. V. 2012. Pharmaceutical compounds and ecosystem function: an emerging research challenge for aquatic ecologists. Ecosystems 15: 867-880.

Comment [MOG58]: Recast correct? Or animal communities because some of the cited studies address vertebrates?

Comment [MOG59]: Only pesticides in that study? or just insecticides?

Comment [MT60]: As stated – organic chemical contaminants including (and mostly) pesticides

Comment [MOG61]: See also various papers by Rosi-Marshall, for example (e.g. in Ecol. Appl.)

Comment [MOG62]: This paragraph seems superfluous. The points have been made and an additional summary is unnecessary. I suggest deleting it, which in my eyes actually make sthe paper stronger.

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~~applied following a pesticide spill. We have been able to demonstrate both direct and indirect effects of the pesticide by combining structural and functional measures and integrating ecological and molecular approaches from a food-web perspective. By applying multiple metrics in this way the added information gained from the links between them will help to develop causation and refine predictions of perturbations in complex systems, and studies such as these could provide invaluable data for parameterising future predictive network-based models of stressor impacts (Gray *et al.* 2014).~~

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Acknowledgments

This study is a contribution from the Imperial College Grand Challenges in Ecosystems and the Environment initiative. We thank: The Natural Environment Research Council (NERC) for funding (NE/L008491/1); Royal Society University Research Fellowship supporting TB; and Queen Mary University of London for funding CG; Action for the River Kennet including Charlotte Hitchmough, Harry Forbes, Kevin Light and Joe Phillips among others, Savernake Flyfishers, John and Rob Hounslow, Marlborough College and Ivor Dunbar for their continued support, data, assistance in the field and access to sites; and the Environment Agency, specifically Adam Hilliard, John Sutton and Jonathan Baxter for their dedication to the project; Manon Czuckermand and Kris Hart for their dedication in the field and laboratory; William Beaumont from the Games & Wildlife Conservation Trust for his teams logistical support; and Lawrence Hudson for his help in the illustration of the R. Kennet food web.

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Comment [MOG63]:
1) Is Barron et al. a book chapter or a journal article? Please adjust format of reference accordingly. Either one would be incomplete.
2) I made a number of editorial corrections in the Reference list. Please double check to ensure they are not lost when the paper is relinked with your Endote file!
3) Complete Brock et al. (2000)
4) Complete/update Gray et al. (2014)
5) 2 references added:
Young R.G., Matthaei C.D., Townsend C.R. (2008) Organic matter breakdown and ecosystem metabolism: functional indicators for assessing river ecosystem health. *Journal of the North American Benthological Society* **27**, 605-625.
Rosi-Marshall E.J., Kincaid D.W., Bechtold H.A., Royer T.V., Rojas M. & Kelly J.J. (2013) Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms. *Ecological Applications* **23**, 583-593.

Comment [MT64]:
Dursban®

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Tables

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Table 1. Locations of upstream control and downstream impacted sites as well as of water chemistry monitoring stations of the Environment Agency (EA). Mean and range, in brackets, of annual water chemistry concentrations from Environment Agency monitoring data are shown from sites located between control and impacted reaches. Oxidised nitrogen (oxidised N) is the sum of nitrate (NO_3^-) and nitrite (NO_2^-).

Site	Condition	Latitude, Longitude
A	Control	51°4170'N, 1°7536'W
EA Control	Control	51°4163'N, 1°7325'W
C	Control	51°4235'N, 1°7165'W
D	Impacted	51°4227'N, 1°6982'W
EA Impact	Impacted	51°4227'N, 1°6982'W
F	Impacted	51°4269'N, 1°6650'W
Water chemistry	**EA Control**	**EA Impacted**
Alkalinity (mg L^{-1})	250 (187-262)	243 (189-254)
Conductivity (µS cm^{-1})	626 (449-738)	609 (492-686)
Oxidised N (mg L^{-1})	6.6 (4.4-7.5)	6.8 (4.4-7.6)
Dissolved oxygen (mg L^{-1})	9.0 (6.9-10.0)	9.6 (6.9-10.9)
Temperature (°C)	11.0 (5.7-14.4)	11.1 (5.7-14.5)
pH	7.6 (7.4-7.8)	7.9 (7.4-8.1)
Ortho-phosphate (mg L^{-1})	0.08 (0.02-0.36)	0.08 (0.02-0.34)

Table 2. General linear model tests of the biomass (mg) and abundance of arthropods and other macroinvertebrates (Tricladida, Annelida and Mollusca, which are considered to be less sensitive to chlorpyrifos than arthropods) per sample; *Baetis*, *G. pulex* (i.e. *K*-selected taxa), chironomid and oligochaete (i.e. *r*-selected taxa) biomass and abundance; arthropod-resource and other-resource trivariate lower-link angles, *Baetis* and *G. pulex* upper-link angles and both total and microbial leaf-litter breakdown rate between control (C) and impacted (I) sites. Significant *p* values (<0.05) are highlighted in bold.

Log_{10} (biomass +1)	Estimate	Std. Error	*z* value	*p*
C:arthropods - C:other	1.62	0.09	17.53	**<0.001**
I:arthropods - I:other	-0.73	0.12	6.00	**<0.001**
C:arthropods - I:arthropods	1.17	0.23	5.19	**<0.001**
C:other - I:other	-1.17	0.25	-4.73	**<0.001**
Log_{10} (abundance +1)	-	-	-	-
C:arthropods - C:other	1.28	0.19	6.82	**<0.001**
I:arthropods - I:other	-0.05	0.19	0.25	0.99
C:arthropods - I:arthropods	0.56	0.24	2.37	0.06

C:other - I:other	-0.76	0.24	-3.23	**0.005**
Log_{10} (biomass +1)				
C:*Baetis* - I:*Baetis*	0.62	0.16	4.00	**<0.001**
C:*G. pulex* - I:*G. pulex*	2.30	0.15	15.82	**<0.001**
C:chironomids - I:chironomids	-0.93	0.15	-6.38	**<0.001**
C:oligochaetes - I:oligochaetes	-0.81	0.15	-5.49	**<0.001**
Log_{10} (abundance +1)				
C:*Baetis* - I:*Baetis*	1.21	0.24	4.98	**<0.001**
C:*G. pulex* - I:*G. pulex*	2.31	0.22	10.63	**<0.001**
C:chironomids - I:chironomids	-1.14	0.22	-5.24	**<0.001**
C:oligochaetes - I:oligochaetes	-1.12	0.23	-4.92	**<0.001**
Invertebrate-resource lower-link angles				
C:arthropods - C:other	-0.08	0.02	-3.8	**<0.001**
I:arthropods - I:other	0.2	0.02	10.35	**<0.001**
C:arthropods - I:arthropods	-0.32	0.24	-1.36	0.44
C:other - I:other	-0.04	0.24	-0.18	>0.99
Baetis and *G. pulex* upper-link angles				
C:*Baetis* – I:*Baetis*	-103.71	24.3	-4.27	**<0.001**
C:*G. pulex* – I:*G. pulex*	-62.8	25.73	-2.44	**0.03**
Leaf litter decomposition (k)				
I:total - C:total	-0.05	0.01	-6.57	**<0.001**
I:microbial - C:microbial	0.01	0.002	5.75	**<0.001**

Table 3 Properties of the trivariate food webs at control and impacted river sites.

	Site A	Site C	Site D	Site F
Property	Control	Control	Impacted	Impacted
Number of nodes	68	60	64	73
Number of fish species	4	4	5	3
Number of macroinvertebrate taxa	35	23	20	32
Number of diatom taxa	29	33	39	38
Number of links	837	635	739	1060
Linkage density	11.96	10.41	11.37	14.13
Directed connectance	0.17	0.17	0.17	0.19
Trivariate regression slope	-0.98	-0.67	-0.92	-0.95
Trivariate regression intercept	1.29	1.26	1.58	1.35

~~Table 1. Locations for of upstream control and downstream impacted sites and as well as of Environment Agency water chemistry monitoring stations of the Environment Agency (EA). Mean and range, in brackets, of annual water chemistry concentrations from Environment Agency monitoring data are shown from sites located between control and impacted reaches are shown.~~

Site	Condition	Latitude, Longitude
A	Control	51°4170'N, 1°7536'W
EA Control	Control	51°4235'N, 1°7165'W
C	Control	51°4227'N, 1°6982'W
D	Impacted	51°4227'N, 1°6982'W
EA Impact	Impacted	51°4170'N, 1°7536'W
F	Impacted	51°4163'N, 1°7325'W

Water chemistry	EA Control	EA Impacted
Oxidised N ([mg l^{-2}])	6.14 [4.35-7.547]	6.82 [4.435-7.657]
Dissolved oxygenO ([mg l^{-1}])	9.04 [6.89-10.09.98]	9.657 [6.89-10.9]
Temperature ([mg $l^{-3}C^{-1}$])	11.02 [5.7-14.4]	11.14 [5.7-14.5]
pH [mg l^{-1}]	7.64 [7.4-7.8]	7.92 [7.4-8.1]
Ortho-phosphate ([mg l^{-1}])	0.083 [0.02-0.36]	0.08 [0.02-0.34]

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Comment [MOG65]: I sthis NO3- plus NO2-? Please clarify

Comment [s66]: The oxidised N values are very high; please double-check that they are correct - dls

Comment [MT67]: Double checked and these are the units the EA use. This is also comparable to other chalk systems I work in

Comment [MOG68]: Please replace brackets by parentheses.

Table 2. General linear model tests of the biomass and abundance of arthropods = arth and non-arthroped = other macroinvertebrates (Tricladida, Annelida and Mollusca, which i.e. groups are considered to be less sensitive to chlorpyrifos than arthropods to chlorpyrifos – Tricladida, Annelida and Mollusca) biomass and abundance, *Baetis*, *Gammarus pulex*, chironomid and oligochaeta biomass and abundance (i.e. *K*- versus *r*- selected taxa), arthropod resource and other-resource trivariate lower link angles, *Baetis* and *G. pulex* upper-link angles and both total and microbial leaf litter breakdown rates between control (C) and impacted (I) sites. Significant *p* values (<0.05) are highlighted in bold.

Log_{e} (biomass +1)	Estimate	Std. Error	*t* value	*p*
C:arthropods - C:other	1.62	0.09	17.53	**<0.001**
I:arthropods - I:other	-0.73	0.12	6.00	**<0.001**
C:arthropods - I:arthropods	1.17	0.23	5.19	**<0.001**
C:other - I:other	-1.17	0.25	-4.73	**<0.001**
Log_{e} (abundance +1)	-	-	-	-
C:arthropods - C:other	1.28	0.19	6.82	**<0.001**
I:arthropods - I:other	-0.05	0.19	0.25	0.99
C:arthropods - I:arthropods	0.56	0.24	2.37	0.06
C:other - I:other	-0.76	0.24	-3.23	**0.005**
Log_{e} (biomass +1)	-	-	-	-
C:Baetis - I:*Baetis*	0.62	0.16	4.00	**<0.001**
C:G. pulex - I:*G. pulex*	2.30	0.15	15.82	**<0.001**
C:chironomids - I:chironomids	-0.93	0.15	-6.38	**<0.001**
C:oligochaetes - I:oligochaetes	-0.81	0.15	-5.49	**<0.001**

Comment [s69]: Please give the units of measurement here

Comment [s70]: This is confusing as written; which taxa are K-selected and which are r-selected. Please rewrite to clarify.

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Comment [MOG71]: Be consistent in use of either English or Latin (e.g. oligochaetes/chironomids or Oligochaeta/Chironomidae)

I oligochaetes a				
Log_{10} (abundance +1)	-	-	-	-
C. Baetis - I. *Baetis*	1.21	0.24	4.98	**<0.001**
C. G. pulex - I. *G. pulex*	2.31	0.22	10.63	**<0.001**
C: chironomids - I: chironomids	-1.14	0.22	-5.24	**<0.001**
C: oligochaetes a - I: oligochaetes a	-1.12	0.23	-4.92	**<0.001**
Invertebrate resource lower-link angles				
C: arthropods - C: other	-0.08	0.02	-3.8	**<0.001**
I: arthropods - I: other	0.2	0.02	10.35	**<0.001**
C: arthropods - I: arthropods	-0.32	0.24	-1.36	0.44
C: other - I: other	-0.04	0.24	-0.18	>0.99
Baetis and *G. pulex* upper-link angles				
C. Baetis - I. *Baetis*	-103.71	24.3	-4.27	**<0.001**
C. G. pulex - I. *G. pulex*	-62.8	25.73	-2.44	**0.03**
Leaf litter decomposition (k)	-	-	-	-
I: total - C: total	-0.05	0.01	-6.57	**<0.001**
I: microbial - C: microbial	0.01	0.002	5.75	**<0.001**

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Table 3. Properties of the trivariate food webs associated with control and impacted stream sites.

Property	Site A Control	Site C Control	Site D Impacted	Site F Impacted
Number of Nodes	68	60	64	73
Number of Fish Species	4	4	5	3
Number of macroInvertebrate Taxa	35	23	20	32
Number of Diatom Taxa	29	33	39	38
Number of Links	837	635	739	1060
Linkage Density	11.96	10.41	11.37	14.13
Directed Connectance	0.17	0.17	0.17	0.19
Trivariate Regression Slope	-0.98	-0.67	-0.92	-0.95
Trivariate Regression Intercept	1.29	1.26	1.58	1.35

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Figure legends

Comment [MOG72]: Please arrange a s describe din the instructions for authors.

s

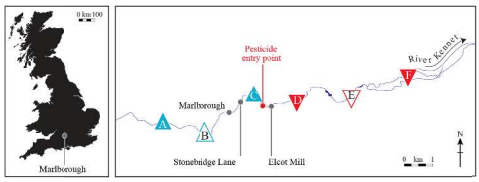
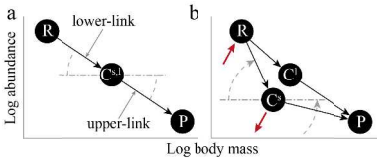


Fig.ure 1. River- Kennet (UK) with study sites A-C (upward pointing triangles = control) and D-F (downward pointing triangles = impacted) ~~highlighted~~. Data for sites A, C, D and F (filled triangles) are presented here. ~~and ARK routine aquatic riverfly invertebrate M~~monitoring data for aquatic macroinvertebrates were collected by citizen scientists upstream (i.e. control site) ~~from~~ at Stonebridge Lane ~~(an upstream control site)~~ and downstream at Elcot Mill (~~a downstream~~ i.e. impacted site) of Marlborough sewage treatment works, where the pesticide entered the river.

Comment [MOG73]: What do red and blue triangles vs circles stand for? Also say a word about Marlborough.

Comment [s74]: I echo Mark's question of the significance of triangles vs. circles. Also, I remind you that you will have to pay to print this in colour in the paper copy, so if you are unwilling to pay, you'll need to recast this and other figures in greyscale, at least for the paper version.



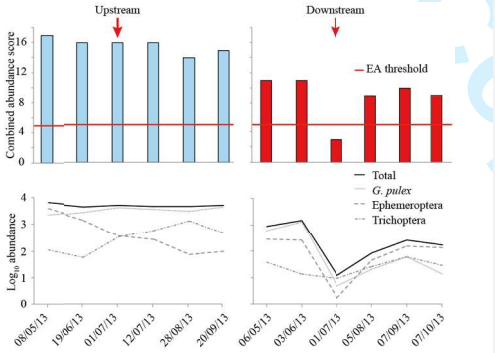
Fig~~ure~~ 2. (a~~1~~) ~~The~~ Location of consumers sensitive to pesticide~~s sensitive~~ (C^s) and less sensitive to pesticide~~s sensitive~~ (C^l) ~~consumers~~ in relation to the~~ir~~ consumer resources (R) and predators (P) as viewed on a double-logarithmic ~~in Log~~scale of body mass ~~(M)~~ versus ~~Log~~ abundance ~~(N) space~~. (b2) Changes within the food web following pesticide exposure can be assessed by using link angles ~~which are~~as a proxy for changes in potential biomass flux ~~across~~within the ~~network~~food web: a predicted decrease in C^s *MN* following pesticide exposure and an increase in R *MN* due to the release from top-down consumer control can be assessed using the C^s link angles in relation to C^l and control data; a decrease in C^s lower-link angles would indicate a potential reduction in biomass flux between R-C^s; an increase in C^s upper-link angle w~~e~~ould indicate a potential reduction in biomass flux to P and hysteresis within the network whereby P is yet to be impacted by the loss of C^s, or that P has increased reliance on other resources, or a combination~~s~~ of the two.

Comment [MOG75]: Is the size of the grey arrow indicating the angle on the right of panel 2 correct?

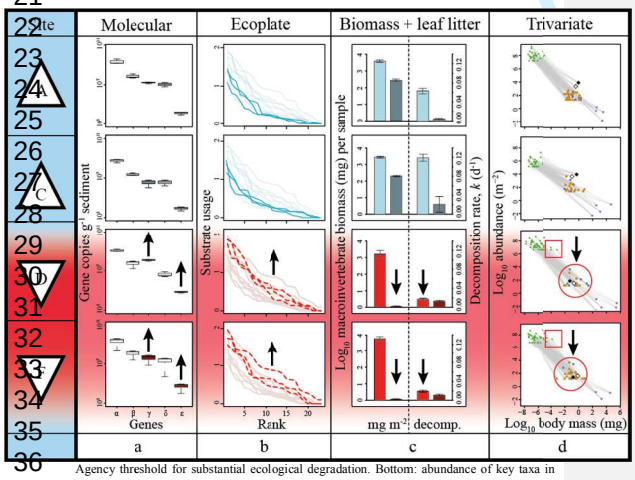
Comment [MOG76]: Please double check correct format in instructions for authors and if correct replace 1 and 2 in figures by a and b.

Yes, panels in figures should be labelled with lower-case letters - dls

Comment [s77]: To avoid possible confusion with population biomass


Upstream
Downstream
Combined abundance score
EA threshold
Total
G. pulex
Ephemeroptera
Trichoptera
Log10 abundance
08/05/13
19/06/13
01/07/13
12/07/13
28/08/13
20/09/13
06/05/13
03/06/13
01/07/13
05/08/13
07/09/13
07/10/13

Fig~~ure~~ 3. Top: ~~ARK routine a~~Aquatic macroinvertebrate monitoring data collected by citizen scientists show macroinvertebrate scores before and after the toxic spill (~~red~~ arrows), based on ~~a sum of the~~total abundance of the target taxa. The red line represents an Environment Agency threshold for substantial ecological degradation. Bottom: abundance of key taxa in relation to scores collected from an upstream control at Stonebridge Lane and a downstream impacted site at Elcot Mill (see ~~Fig. Figure 1~~Fig. 1).



Fig~~ure~~ 4. Vertical arrows indicate notable differences between ecological data from control sites A and C and from impacted sites D and F two months after the toxic spill, error bars represent standard error. (a) ~~Ecological data from control sites A & and C and from impacted~~

Comment [MOG78]:
-use subscript for 10 in log10
-use italics for G. pulex

Comment [MOG79]: I suggest:
- -deleting microbiota and macrobiota (partly because macrobiota is not an established term)
- replacing 1 to 4 at the top by molecular to trivariate at the bottom
- Enumerate individual panels from a to p and adjust legend accordingly
- Inserting a blank space before sed (y-axis label of column 1), spelling out sediment, and adding "Gene". Thus "Gene copies g^{-1} sediment"
- Use notation 10^3 etc. for y-axes of panels in first column
- Column 2, y-axis label: macroinvertebrate biomass
- Column 3: better use 4 bars in each panel and clarify which axis corresponds to which bar; also give unit for k: i.e. "Decomposition rate, k (d^{-1})"
- What's the correct notation for the log values, as shown for the y-axis of column 2 or 4? Please harmonize

sites D & and F two months after the toxic spill. 1) Molecular results from microbial qPCR assays targeting the (ae) 16S rRNA (microbial abundance), (bf) *nirS* (nitrite reductase) (ce) *amoA* (ammonia monooxygenase) AOB (ammonia oxidising bacteria), (dg) *amoA* (ammonia monooxygenase) AOA (ammonia oxidising archaea), (ee) *opd* (organophosphorus hydrolase) genes. Results show gene copy number per gram of sediment on a $\log_{10}$ scale. (b2) Ecoplate microbial functional potential on 31 carbon substrates (x-axis) and their usage (y-axis; measured as optical density at 600 nm after 5 days of incubation at 22 °C as defined in the Methods). Ecoplate microbial functional potential on 31 carbon substrates (x-axis) and their usage (y-axis). (c3) Biomass of macroinvertebrates (lighter shading) and a keystone detritivore, (*Gammarus pulex* (darker shading), and leaf litter breakdown rates by all consumers (light shading) and microbes only (dark shading). (d4) Trivariate mass-abundance food webs: green circles = algae (large species found only in the impacted sites highlighted), yellow symbols = arthropods (decreased relative to controls), blue symbols = other macroinvertebrates, black filled diamond = *G. pulex*, black open diamond = *Baetis*, pink symbols = fishes.

Comment [MOG80]: What do the individual panels show?

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Comment [MOG81]: Please give some more information

Comment [s82]: In addition to the issues that Mark raised, I note the following problems that will need attention:

-for the panels that show error bars, please tell us what the error bars represent (SE? SD? CI?)

-to avoid confusion in labelling, please use something other than lower-case letters to indicate qPCR categories (lower-case letters are going to be used to label the panels) – perhaps lower-case Greek letters? (α, β, γ, δ, ε)

-please tell us what the vertical arrows mean

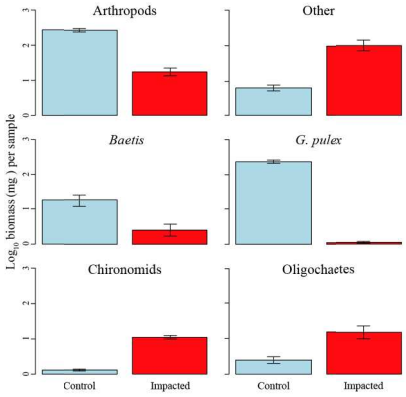
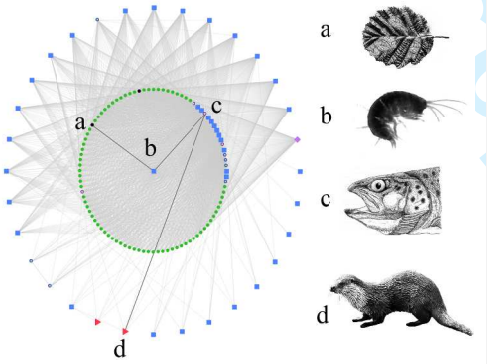


Figure 5. Macroinvertebrate mean biomass (per sample with standard error) ~~across the treatment~~at control and impacted sites in the River Kennet.

Comment [MOG83]:
1)Please spell out Control and Impacted
2) Subscript 10 in log10
3) Report units
4) Arthropods instead of Athropod
5) Chironomidae and Oligochaeta or English terms (chironomids, oligochaetes) in both cases (and as above)

...and please tell us what the error bars represent
-also please increase the font size on tic- and axis-labels - dls



Fig~~ure~~ 6. Aggregated network for the River~~-~~ Kennet food web, highlighting an exemplar food chain from the basal resource to the apex predator ~~is highlighted~~; a = coarse ~~detritus~~ particulate organic matter (~~CPOM, e.g. such as~~ e.g. leaf litter), b = *Gammarus pulex*, c = brown trout, *Salmo trutta*, d = Eurasian otter, *Lutra lutra*. The two concentric circles of nodes represent the shortest food web distances to or from *G. pulex* – those in the inner circle are a single link removed from *G. pulex*, those in the outer circle are separated by two links in the shortest path.~~-~~ Here, all species are ~~no more than~~at most 2 links away from *G. pulex*, although longer food chains are present in the network, as shown by a-b-c-d. Symbols for nodes represent different trophic elements: green circles ~~represent~~ = producers, blue squares ~~=~~ macroinvertebrates, purple diamonds ~~=~~ vertebrate ectotherms, red triangles~~.~~ = endotherms, black circles~~.~~ = abiotic resources. Light blue and light purple circles ~~represent~~ = cannibalistic nodes of ~~invert~~ macroinvertebrates and vertebrate ectotherms, respectively.

Comment [MOG84]: Why not just say leaf litter?

Comment [s85]: Do you need to credit the source of the organism illustrations, or get permission to use them?