Synchronismes et antagonismes dans les relations entre environnement agricole, biodiversité, et fonctions écologiques dans les zones tampons humides artificielles

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Synchronisms and antagonisms in the relationships between agricultural environment, biodiversity, and ecological functions in constructed wetlands

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Titre : Synchronismes et antagonismes dans les relations entre environnement agricole, biodiversité, et fonctions écologiques dans les zones tampons humides artificielles

Mots clés : Biodiversité - Amphibiens - Invertébrés aquatiques - Zones tampons humides artificielles - Pesticides - Fonctions écologiques

Résumé : Les contaminants d'origine agricole, incluant les pesticides et les nitrates, peuvent être transférés jusqu'à l'hydrosphère, et ainsi avoir des effets néfastes sur les organismes et sur les écosystèmes aquatiques. Les Zones Tampons Humides Artificielles (ZTHAs) peuvent être implantées dans le paysage agricole pour réduire le transfert des contaminants d'origine agricole dans l'hydrosphère grâce à des propriétés épuratoires naturelles. Cependant, bien que leur but premier soit de réduire la pollution du milieu aquatique, paradoxalement, les ZTHA peuvent constituer des milieux intercepteurs et concentrateurs de pesticides et nitrates, avec les répercussions négatives que ces contaminants peuvent engender sur les organismes aquatiques, faisant des ZTHA des potentiels pièges écologiques pour la faune aquatique.

Par l'étude d'un site pilote situé en Seine-et-Marne (France) et sujet à un suivi de la qualité de l'eau depuis 2012, la présente thèse vise à évaluer le potentiel pour une ZTHA agricole à agir comme un piège écologique pour les amphibiens et les invertébrés aquatiques autochtones.

Au travers d'un ensemble de suivis écologiques et écotoxicologiques multi-niveaux et *in situ*, les résultats obtenus tendent à montrer que le risque induit par les flux de contaminants d'origine agricole dans la ZTHA est important pour les amphibiens, et que des effets négatifs sub-cellulaires, comportementaux, et écologiques s'exercent sur la faune aquatique. Ce travail permet de mieux comprendre les impacts potentiels des flux de contaminants d'origine agricole sur la faune aquatique dans les ZTHA.

Title: Synchronisms and antagonisms in the relationships between agricultural environment, biodiversity, and ecological functions in constructed wetlands

Keywords: Biodiversity - Amphibians - Aquatic invertebrates - Constructed wetlands - Pesticides - Ecological functions

Abstract: Agrochemicals, including pesticides and nitrates, can be transferred to the hydrosphere, with adverse effects on organisms and aquatic ecosystems. Constructed wetlands (CWs) can be implemented in the agricultural landscape to reduce the transfer of agrochemicals to the hydrosphere through their natural purification properties. However, although their primary aim is to reduce pollution of the aquatic environment, paradoxically, CWs can act as interceptors and concentrators of pesticides and nitrates, with the negative repercussions that these contaminants can have on aquatic organisms, making CWs potential ecological traps for aquatic fauna.

By studying a pilot site located in Seine-et-Marne (France) and subject to water quality monitoring since 2012, the present thesis aims to assess the potential for an agricultural CW to act as an ecological trap for amphibians and aquatic invertebrates.

Through a series of multi-level, *in situ* ecological and ecotoxicological monitoring studies, the results obtained tend to show that the risk induced by agrochemical fluxes in the CW is notable for amphibians, and that negative sub-cellular, behavioral, and ecological effects are exerted on aquatic fauna. This work provides a better understanding of the potential impacts of agricultural contaminant fluxes on aquatic fauna in CWs.

Avant-propos en français

Dans un contexte de pression croissante sur les milieux aquatiques, liée aux activités agricoles intensives, la préservation de la qualité de l'eau représente aujourd'hui un enjeu majeur pour les sociétés humaines. L'usage des pesticides et des intrants azotés, bien qu'ayant permis une augmentation significative des rendements agricoles au cours des dernières décennies, engendre également des impacts écologiques durables, comme la dégradation de la biodiversité, l'eutrophisation des masses d'eau, et la contamination des nappes phréatiques. Proposées comme des « solutions fondées sur la Nature », les Zones Tampons Humides Artificielles (ZTHA) apparaissent comme des dispositifs innovants, capables de limiter le transfert des contaminants d'origine agricole vers l'hydrosphère tout en créant de nouveaux habitats pour la faune. La présente thèse s'inscrit dans ce contexte, à l'interface entre l'écologie, l'écotoxicologie, les sciences de l'environnement et les sciences naturalistes, en se focalisant principalement sur l'étude de la ZTHA agricole de Rampillon, située dans le département de la Seine-et-Marne, ouvrage construit en 2010 pour répondre à la fois aux enjeux de préservation de la qualité de l'eau et de conservation de la biodiversité dans les paysages agricoles, et vise précisément à considérer la question du rôle des ZTHA agricoles pour la biodiversité : sont-elles des milieux de vie durablement favorables ou des pièges écologiques ?

D'un point de vue purement informatif, j'ai pu valoriser ces travaux de thèse auprès de la communauté scientifique sous la forme de plusieurs communications orales et d'un poster, au sein-même de mon centre de recherche d'accueil (INRAE), au cours de colloques ou séminaires nationaux (SEFA, FIRE, Rencontres Biosefair), et internationaux (SEFS13). J'ai produit une plaquette informative sur la biodiversité de la ZTHA de Rampillon, sur la base de travaux naturalistes antérieurs réalisés avant mon arrivée à INRAE, et sur la base de mes propres observations sur le terrain. J'ai eu l'opportunité de présenter cette plaquette et, de manière plus générale, la biodiversité de la ZTHA de Rampillon au cours de visites de la zone (Projet ALFAwetlands, visites d'équipe). J'ai également encadré un stage de M2, en collaboration avec le Muséum National d'Histoire Naturelle, pendant 6 mois, et j'ai réalisé des interventions auprès de stagiaires de 3ème et de 2nde dans les locaux d'INRAE.

Résumé long en français

Le terme biodiversité, qui naît dans les années 80 (Sarkar, 2021), renvoie à la diversité de la vie dans tous ses aspects, incluant les gènes, les populations, les communautés d'espèces, les dynamiques de flux biotiques, les interactions entre les organismes, les processus écologiques et les fonctions écosystémiques (Leenhardt et al., 2022; Wake & Vredenburg, 2008). Le concept de biodiversité englobe trois niveaux de diversité : (i) la diversité écologique, faisant référence à la diversité des écosystèmes et des paysages, (ii) la diversité spécifique, faisant référence à la diversité des espèces et des taxons, et (iii) la diversité intraspécifique, faisant référence à la diversité individuelle et génétique (MNHN & OFB [Ed], 2003; Swingland, 2013).

La biodiversité connaît actuellement une crise majeure que certains qualifient de « Défaunation de l'Anthropocène » (Dirzo et al., 2014). Cet événement écologique de grande ampleur est principalement lié aux pressions anthropiques qui sont, au moins en partie, responsables de la surexploitation des ressources naturelles, de la modification des paysages et des habitats, du changement climatique, de l'invasion d'espèces exotiques, de la surexploitation des espèces (par exemple, par la chasse, la pêche, la sylviculture, le braconnage), l'apparition de maladies et les pollutions chimiques qui, *in fine,* « compromettent la capacité d'adaptation des écosystèmes aux changements globaux » (Blann et al., 2009; Dirzo et al., 2014; Escher & Marchant, 2019; Leenhardt et al., 2022; Pievani, 2014).

L'intensification de l'agriculture, qui vise à assurer une production alimentaire suffisante pour répondre à la demande de la croissance de la population humaine, joue un rôle important dans la perte de biodiversité (Dudley & Alexander, 2017; Kehoe et al., 2017). Actuellement, les terres agricoles couvrent 47 % et 45 % de la surface de l'Europe (Eurostat, 2021; Herzog et al., 2012) et de la France (Couleaud et al., 2021), respectivement. Cinquante pour cent des espèces européennes (Eurostat, 2022) sont devenues très dépendantes des milieux agricoles, les habitats semi-naturels ayant été éradiqués par l'intensification et la spécialisation des pratiques agricoles (Dudley & Alexander, 2017; Herzog et al., 2012). Cette dépendance fait que les espèces vivantes bénéficient, mais aussi subissent, les effets de la gestion des terres par les activités agricoles (Le Roux et al., 2012). Selon Dudley & Alexander (2017), l'agriculture exerce une influence négative sur la biodiversité de quatre manières, à savoir (i) la conversion des écosystèmes naturels, (ii) l'intensification de la gestion, (iii) le rejet de polluants et (iv) les impacts de la chaîne de valeur (par exemple, l'utilisation de l'énergie et des transports, les déchets alimentaires). Bien que la nécessité de modifier les modèles agricoles actuels soit désormais bien établie (Demonet et al., 2013; Gross & Charbonnier, 2014), la surface des terres utilisées

pour les pâturages et les cultures continue d'augmenter à l'échelle mondiale (*HYDE*, 2024; Liu et al., 2022).

En lien avec l'intensification de l'agriculture, les pesticides, incluant les herbicides, les fongicides, les insecticides, les molluscicides et autres, sont utilisés pour protéger les cultures contre les maladies et les ravageurs (EFSA, 2022), et les nitrates sont utilisés pour optimiser les rendements des cultures (Gao et al., 2022; Khajuria & Kanae, 2013). La toxicité des pesticides et des nitrates pour les organismes non ciblés, y compris les humains, est étudiée depuis les années 1940 pour les nitrates (Wilson, 1943), et les années 1950 pour les pesticides (Conley, 1949; DeWitt, 1956b). De nos jours, les pesticides sont omniprésents dans l'environnement et constituent un risque environnemental à l'échelle mondiale (Tang et al., 2021), et les concentrations de nitrates dans les écosystèmes aquatiques ne cessent d'augmenter en raison des activités humaines (Banerjee et al., 2023a). Les pesticides, en particulier, peuvent affecter la biodiversité à tous les niveaux d'organisation biologique, jusqu'aux écosystèmes et aux fonctions associées (Geiger et al., 2010; Isenring, 2010; Leenhardt et al., 2022; McMahon et al., 2012). Le déclin mondial des insectes dû aux pesticides est considéré comme pouvant mettre en péril la prospérité humaine (van der Sluijs, 2020). En outre, les néonicotinoïdes sont soupçonnés d'être à l'origine d'une immunodépression de la biodiversité mondiale affectant les abeilles, les poissons, les chauves-souris, les oiseaux et les amphibiens (Mason, 2013).

Les amphibiens et les macroinvertébrés benthiques font partie de la faune aquatique. Ils sont essentiels pour le bon fonctionnement des écosystèmes. En particulier, les amphibiens contribuent à l'équilibre structurel et fonctionnel des écosystèmes grâce à leur rôle dans les réseaux trophiques, les flux d'énergie, la lutte contre les ravageurs et les maladies, et l'ingénierie des écosystèmes, par la modification physique de l'habitat, comme la bioturbation ou le « pâturage » du biofilm par exemple (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2018; Hocking & Babbitt, 2014; Verburg et al., 2007; Whiles et al., 2006). Les amphibiens peuvent, ou devraient, être considérés comme des sentinelles environnementales en raison de leur sensibilité à diverses pressions environnementales (Roy, 2002; N. Yang et al., 2023). En parallèle, du fait de leur remarquable diversité taxonomique et écophysiologique, les invertébrés aquatiques jouent également un rôle important dans l'écosystème, à travers leur contribution à de nombreuses fonctions écosystémiques telles que le soutien des réseaux trophiques, le cycle des nutriments et la purification de l'eau (Collier et al., 2016; Murkin & Wrubleski, 1988; Prather et al., 2013; Wallace & Webster, 1996). Certains taxons d'invertébrés aquatiques, comme les bivalves, les crustacés et les odonates, entre autres, sont également considérés comme des sentinelles pour détecter la dégradation de la qualité de l'environnement (Damásio et al., 2010; Datto-Liberato et al., 2024; Marmonier et al., 2013). Ainsi, compte tenu de leur biomasse, de leur rôle écologique et de leur sensibilité à la pollution, y compris aux pressions agrochimiques, les amphibiens et les invertébrés aquatiques sont des taxons pertinents pour étudier l'impact des produits agrochimiques sur l'environnement.

Les amphibiens et les invertébrés aquatiques subissent plusieurs pressions, notamment les effets négatifs de l'intensification de l'agriculture et de l'utilisation de produits agrochimiques. Les amphibiens sont actuellement les vertébrés les plus menacés, avec 40,7 % d'espèces menacées, principalement, plus ou moins directement, en raison des conséquences des activités anthropiques (Collins & Storfer, 2003; IPBES, 2019; Luedtke et al., 2023), notamment le changement climatique, la perte d'habitat, les maladies et les contaminants (Collins, 2010; Cushman, 2006; Nyström et al., 2007; Rollins-Smith, 2009). En particulier, l'agriculture intensive est l'un des principaux facteurs de déclin de la diversité des amphibiens, principalement en raison de la perte et de la dégradation de l'habitat (Collins & Storfer, 2003; Curado et al., 2011; Gallant et al., 2007; Houlahan & Findlay, 2003; Luedtke et al., 2023; J. W. Ribeiro et al., 2018). Il a été suggéré que les pesticides et les nitrates jouent un rôle dans le déclin des amphibiens (Alford & Richards, 1999; Bishop et al., 1999; C. Davidson, 2004; Fellers et al., 2004; Hamer et al., 2004; Sparling et al., 2010). Parallèlement, les invertébrés aquatiques peuvent également présenter des proportions inquiétantes d'espèces menacées (Collier et al., 2016; Sayer et al., 2025). Chez les insectes aquatiques, par exemple, les taxons odonates, plécoptères, trichoptères et éphéméroptères sont notamment en déclin à l'échelle mondiale, avec des proportions d'espèces en déclin de 37 %, 35 %, 44 % et 37 % respectivement, les plécoptères affichant un taux d'extinction stupéfiant de 19 % (Sánchez-Bayo & Wyckhuys, 2019). L'intensification de l'agriculture apparaît comme l'une des principales menaces pour les invertébrés aquatiques en raison de la perte d'habitat, affectant par exemple 61 % des espèces menacées d'odonates dans le monde (Sánchez-Bayo & Wyckhuys, 2019; Sayer et al., 2025). Au sein des paysages agricoles, les invertébrés aquatiques ont également tendance à souffrir d'une érosion accrue des sols, liée à une augmentation des charges de sédiments en suspension, et de l'eutrophisation par exemple (Burdon et al., 2013; Campbell et al., 2009; Euliss & Mushet, 1999; Gleason et al., 2003; Matthaei et al., 2010b). Enfin, les pesticides et les nitrates sont également incriminés dans le déclin des populations d'invertébrés aquatiques (Beketov et al., 2013; Nessel et al., 2021; van der Sluijs, 2020; Yamamuro et al., 2019).

En milieu agricole, la vulnérabilité des amphibiens et des invertébrés aquatiques aux produits agrochimiques va dépendre de plusieurs facteurs. Cette vulnérabilité dépend ainsi des espèces, en raison de l'existence de différences interspécifiques de sensibilité (Adams, Leeb, Roodt, et al., 2021), du stade de vie (Greulich & Pflugmacher, 2003; Kulkarni et al., 2013), des traits bio-écologiques, tels que la taille, le sexe, les modalités de reproduction, le spectre trophique, la relation au substrat, entre autres, qui vont influencer la manière dont les organismes sont exposés (Awkerman et al., 2024; EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2018; Huang et al., 2022; Ippolito et al., 2012), le temps et la concentration d'exposition au(x) contaminant(s) (Ashauer et al., 2006), la nature de la combinaison de molécules (également appelée « cocktails ») , et le contexte environnemental dans lequel les espèces sont exposées. Ainsi, dans le milieu naturel, l'interaction complexe de tous ces facteurs, la dynamique de l'exposome, et les spécificités phénologiques des espèces détermineront leur vulnérabilité aux produits agrochimiques, et donc, le risque généré par les pressions agrochimiques sur les amphibiens et les invertébrés aquatiques (Awkerman et al., 2024; Buss et al., 2021; Chiu et al., 2016; EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2018; Vormeier et al., 2023).

Dans des conditions de risque élevé, les produits agrochimiques peuvent avoir des effets néfastes sur les amphibiens et les invertébrés aquatiques, à tous les niveaux d'organisation biologique (Sánchez-Bayo & Mann, 2011). Ces effets peuvent d'abord se manifester aux niveaux biologiques les plus fins, c'est-à-dire aux niveaux cellulaire et subcellulaire, incluant les biomolécules, les enzymes, les molécules porteuses d'informations génétiques (c'est-à-dire l'ADN, l'ARN), les gènes, les organites, et enfin la cellule dans son ensemble (Ezemonye & Tongo, 2010; Josende et al., 2015; Knapik & Ramsdorf, 2020; Sparling et al., 2015). En perturbant les structures et les fonctions des gènes, des enzymes, des organites et de la cellule entière, et donc en perturbant les processus génétiques et biochimiques associés, des effets peuvent se produire à des niveaux biologiques supérieurs. Ainsi, des altérations des fonctions nerveuses, musculaires, endocriniennes, immunitaires et métaboliques peuvent affecter les différents tissus et organes de l'organisme, conduisant finalement à des effets négatifs sur l'individu, sur le développement, et donc sur les caractéristiques morpho-anatomiques, sur le système nerveux, et donc sur le comportement, sur la fonction de reproduction, sur le système immunitaire et sur le métabolisme énergétique de l'organisme, pouvant conduire à la mort (Agostini et al., 2020; Bonfanti et al., 2004; Coors et al., 2008; Langlois et al., 2010; A. P. Moore & Bringolf, 2018; Ortiz et al., 2004). La réduction de la valeur sélective des amphibiens et des invertébrés dans l'environnement naturel, ou même les effets létaux des produits agrochimiques, peuvent avoir des répercussions à des niveaux biologiques plus élevés, c'est-à-dire, les niveaux écologiques, comprenant les populations, les communautés, les écosystèmes et les fonctions écologiques associées, par des effets tels que la réduction de la biomasse, la perturbation des relations intraspécifiques et interspécifiques, la structure des communautés, les réseaux trophiques et, en fin de compte, la perturbation des fonctions de l'écosystème telles que la décomposition de la litière ou le cycle des nutriments (Auber et al., 2011; Boone & James, 2003; Brosed et al., 2016; Hamer et al., 2004; Relyea, 2009; Schäfer et al., 2007).

Malgré l'impact significatif de l'intensification agricole et des produits agrochimiques sur la faune aquatique, les masses d'eau agricoles (c'est-à-dire les masses d'eau lentiques ou lotiques, naturels ou artificiels, en milieu agricole), notamment les cours d'eau, les fossés, les étangs et les zones humides, peuvent constituer des abris pour la faune aquatique, qu'il s'agisse des amphibiens (J. M. R. Baker & Halliday, 1999; Knutson et al., 2004) ou des invertébrés aquatiques (Cereghino et al., 2008; Davis & Bidwell, 2008; Hill et al., 2016; Ruggiero et al., 2008; Verdonschot et al., 2011; Williams et al., 2004). Néanmoins, les mares et les zones humides souffrent de l'intensification de l'agriculture, de la pollution, du changement climatique et de la croissance de la population humaine (Czech & Parsons, 2022; Finlayson & Spiers, 1999; Heath & Whitehead, 1992; Indermuehle et al., 2008; Oertli et al., 2008; Wood et al., 2003), et le rythme et l'intensité de leur déclin sont alarmants (N. C. Davidson, 2014; Fluet-Chouinard et al., 2023; Levy, 2015; Thinzilal, 2013). La disparition des mares en France, principalement due à l'intensification de l'agriculture (Arntzen et al., 2017), menace l'avenir des communautés d'amphibiens dans les paysages agricoles (Curado et al., 2011). De plus, les produits agrochimiques, qui pénètrent dans l'hydrosphère principalement par pulvérisation, ruissellement ou par les systèmes de drainage (Meite et al., 2018; Tournebize et al., 2017), sont responsables d'une contamination globale des masses d'eau (Leenhardt et al., 2022). Ainsi, des mares du monde entier ont été suggérés comme étant pollués par des pesticides (Frank et al., 1990; Miglioranza et al., 2002; Uddin et al., 2013), y compris des mares françaises (Chaumet et al., 2021; Sarrazin et al., 2022). Ainsi, bien que les mares agricoles puissent être des habitats appropriés pour la faune aquatique, elles peuvent intercepter divers produits agrochimiques, qui peuvent être responsables d'effets délétères sur cette faune (Goessens et al., 2022; Leenhardt et al., 2022; Xiao et al., 2024).

Pour réduire les pressions chimiques sur les eaux de surface et souterraines, une solution a été mise au point : les zones tampons humides artificielles (ZTHA), qui sont des bassins de rétention ou des étangs végétalisés ou non ayant une fonction tampon. Elles ont été développées pour la première fois à la fin du 20ème siècle en Europe et en Amérique du Nord pour tirer parti de la capacité de biodégradation des plantes (Hammer, 1989; Shutes, 2001). Récemment, l'Union internationale pour la conservation de la nature (UICN) a introduit le concept de solutions fondées sur la nature (*Nature-based solutions*, NbS, en anglais) pour relever les défis sociétaux mondiaux (Cohen-Shacham et al., 2016). Selon l'UICN, les solutions fondées sur la nature sont « des actions visant à protéger, gérer durablement et restaurer les écosystèmes naturels ou modifiés qui répondent aux défis sociétaux de manière efficace et adaptative, tout en assurant le bien-être humain et des bénéfices pour la biodiversité » (Cohen-Shacham et al., 2016). En tant que solution basée sur la nature et interface paysagère, les ZTHA agricoles sont conçus pour atténuer les contaminants provenant du ruissellement et du drainage. Dans une certaine mesure, elles permettent la remédiation des eaux contaminées par les nitrates et les pesticides (Gersberg et al., 1983; Tournebize et al., 2017; Vymazal & Březinová, 2015), par le biais de processus biologiques (ex, métabolisation par les plantes et les bactéries), et des processus physiques et chimiques (par exemple, hydrolyse, photolyse, séquestration des sédiments) (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Gregoire et al., 2009; Overton et al., 2023), tout en étant capable d'agir comme des milieux supports de biodiversité (Zhang et al., 2020). En raison de leurs capacités de purification, les ZTHA peuvent également agir comme des pièges écologiques, qui « se produisent lorsque les animaux préfèrent par erreur des habitats où leur fitness est plus faible que dans d'autres habitats disponibles à la suite d'un changement environnemental rapide » (Hale & Swearer, 2016). Ce potentiel est lié à leur rôle d'intercepteur de contaminants, susceptible d'affecter les espèces sauvages qu'elles abritent (Piha, 2006; Stillway et al., 2019; Zhang et al., 2020), notamment les amphibiens (Sievers et al., 2018), mais aussi les invertébrés aquatiques (Duchet et al., 2018). Dans certains contextes, qui restent à découvrir, les ZTHA pourraient agir soit comme des milieux habitables, soit comme des pièges écologiques pour la faune aquatique. Bien que le potentiel de piège écologique des ZTHA soit reconnu dans des conditions contrôlées (Duchet et al., 2018; Sievers et al., 2018), les preuves empiriques *in situ* manquent et un travail de terrain holistique ciblé est nécessaire pour évaluer l'état écologique des ZTHA agricoles en particulier.

La zone tampon humide artificielle agricole française de Rampillon (la ZTHA de Rampillon, ou *constructed wetland of Rampillon*, CWR, en anglais) (Seine-et-Marne, France), qui est soumise à une pression agrochimique chronique, apparaît comme une opportunité pour aborder cette question. La ZTHA de Rampillon a été construite en 2010 pour atténuer les nitrates et les pesticides provenant du drainage et du ruissellement des zones agricoles, tout en soutenant la faune aquatique. La ZTHA a été largement étudiée dans des travaux antérieurs afin d'évaluer son potentiel à concilier le double enjeu de la qualité de l'eau et de la conservation de la biodiversité dans les paysages agricoles (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Lebrun et al., 2019; Letournel, Pages, et al., 2021; Mander et al., 2021; Tournebize et al., 2017). La ZTHA est caractérisée par un niveau notable de richesse spécifique (Letournel, Pages, et al., 2021), suggérant un possible rôle d'abri pour la biodiversité au sein d'un environnement relativement hostile. Cependant, en raison de son rôle d'intercepteur de produits agrochimiques, la CWR pourrait plutôt agir comme un piège écologique pour la faune aquatique. Ainsi, malgré le gain net de biodiversité qui peut être induit par certaines solutions basées sur la nature, certaines, comme la ZTHA agricole de Rampillon, pourraient être responsables d'une réduction de la valeur sélective des organismes vivant dans ces environnements.

Dans le contexte des problématiques de conservation de la faune aquatique dans les paysages agricoles, la présente thèse visait, par une approche holistique, à déterminer si la ZTHA agit comme un environnement sûr dans la matrice agricole ou comme un piège écologique pour la faune aquatique, en se concentrant sur les amphibiens et les invertébrés aquatiques. Pour répondre à cette question, nous avons adopté une approche de biosurveillance spatio-temporelle, multi-taxons, multi-niveaux et multi-réponses (voir Figure 1) visant à :

- (i) identifier les périodes à risque pour la communauté d'amphibiens par l'étude des synchronismes entre les flux agrochimiques et les périodes de vulnérabilité de la communauté,
- (ii) évaluer les effets des pesticides sur les caractéristiques enzymatiques et morphologiques de deux espèces d'amphibiens indigènes, le Crapaud commun (*Bufo bufo*) et la Grenouille verte (*Pelophylax sp.*), en utilisant une méthode non invasive, à savoir l'écouvillonnage buccal,
- (iii) évaluer les effets d'un mélange de pesticides représentatif de la pression agrochimique moyenne de la ZTHA sur les caractéristiques enzymatiques et comportementales d'une espèce d'invertébré aquatique, *Gammarus fossarum*, dans des mésocosmes,
- (iv) évaluer les effets des flux agrochimiques sur la structure de la communauté de macroinvertébrés benthiques indigènes et sur une fonction écosystémique associée, la dégradation de la litière.

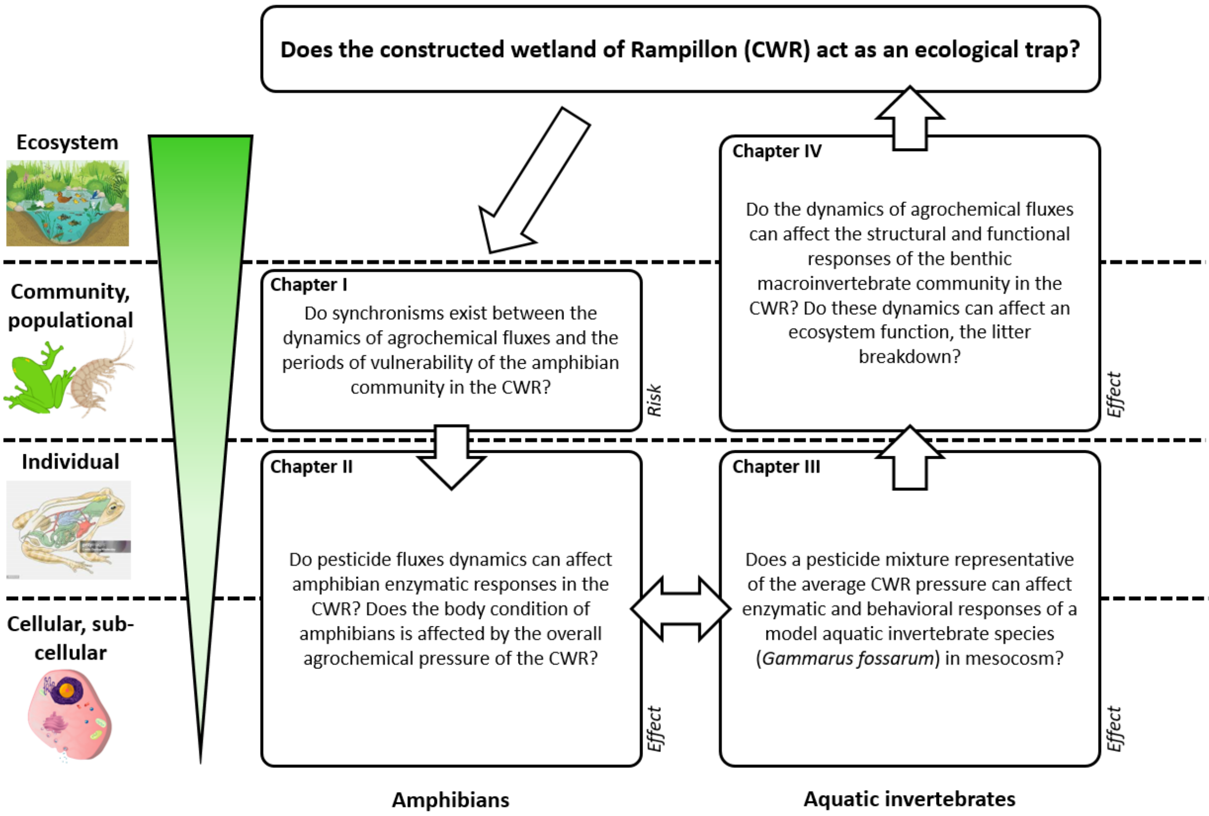

Figure 1 Diagramme présentant le cadre conceptuel de la thèse avec la question centrale, générale, et les différentes questions spécifiques à la recherche.

Le projet de thèse SynBioTox se concentre sur la zone tampon humide artificielle agricole de Rampillon (ZTHA) située dans le département de la Seine-et-Marne en France, à environ 60 km au sud-est de Paris (48°32′19,5″N ; 3°03′46,7″E). La ZTHA de 5.600 m^2, construit en 2010, et situé en dérivation du ru « le ru des gouffres », collecte les eaux de ruissellement et de drainage d'un bassin versant agricole de 355 hectares soumis à une rotation intensive des cultures, principalement du blé, du maïs et des betteraves (85 % de l'assolement moyen). La ZTHA atténue la pollution pesticide et azotée avant l'infiltration directe dans la nappe phréatique des calcaires de Champigny qui alimente en eau 1,5 million d'habitants de l'Île-de-France (Tournebize et al., 2012). Le CWR intercepte en moyenne 40% des écoulements moyens, soit 800 000 m^3 par an. La CWR est équipée depuis 2012 pour surveiller les flux agrochimiques à l'aide de trois stations de mesure hydrologique (SMH) situées en amont et en aval de la ZTHA, et à la sortie de la ZTHA et du bassin versant. Chaque SMH comprend une mesure de débit (sonde Doppler) couplée à un système d'échantillonnage composite contrôlé par le volume d'eau qui permet de mesurer les flux entrants et sortants de pesticides et de nitrates. La stratégie d'échantillonnage donne accès à des concentrations de flux bihebdomadaires adaptées au calcul des flux de polluants entrants et sortants (mais pas à la variabilité instantanée des concentrations). Il existe un gradient de contamination de l'amont vers l'aval dû à l'action épuratrice de la ZTHA (Lebrun et al., 2019; Letournel, Pages, et al., 2021). Une période de 10 ans de suivi en continu (2012-2022) et d'analyse de 531 pesticides et de leurs métabolites a montré que la CWR intercepte 40-700 g de pesticides par an, selon les années et les conditions hydrologiques (Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Letournel, Pages, et al., 2021). La concentration moyenne en pesticides des eaux entrantes est d'environ 1 µg/L de janvier à décembre, avec des pics de concentration d'environ 10 µg/L observés en mai et juin, suite à l'épandage d'herbicides et de fongicides qui a lieu d'avril à juin (il s'agit de concentrations issues d'échantillons composites d'eau, basées sur des prélèvements multiples sur des périodes de 1 à 3 semaines, en fonction de l'hydrologie, il s'agit donc de concentrations moyennes, et non de valeurs maximales). Sur l'année, les concentrations en nitrates sont supérieures au seuil de 30,1 mg/L, correspondant à une mauvaise classe de qualité écologique (Ministère de la transition écologique et solidaire, 2019) (C. Chaumont, non publié). La diminution moyenne des concentrations due aux capacités épuratoires de la ZTHA est d'environ 37 % selon les propriétés des différentes molécules des pesticides (50 % sont des herbicides), et d'environ 11 mg/L pour la concentration en nitrates (Letournel, Pages, et al., 2021). Les principaux pesticides retrouvés dans la mare sont les herbicides métamitron, quinmerac, mésotrione, métolachlore, éthofumesate, terbuthylazine, bentazone, isoproturon, nicosulfuron, imazamox, glyphosate et son métabolite AMPA ainsi que l'insecticide thiaméthoxam, le fongicide tébuconazole et le molluscicide métaldéhyde (justifiant en partie le choix des pesticides pour le mélange étudié dans les mésocosmes). La ZTHA a été largement étudiée dans des travaux antérieurs pour évaluer son potentiel à concilier le double enjeu de la qualité de l'eau et de la conservation de la biodiversité dans les paysages agricoles (Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Lebrun et al., 2019; Letournel, Chaumont, et al., 2021; Letournel, Pages, et al., 2021; Mander et al., 2021; Tournebize et al., 2012, 2017). La ZTHA est subdivisée en trois sous-bassins : un bassin de sédimentation d'un mètre de profondeur, un bassin végétalisé intermédiaire de 30 centimètres colonisé par des roseaux (*Phragmites australis*), des joncs (Juncaceae) et des carex vrais ou carex (*Carex sp.*), et un bassin final d'environ un mètre de profondeur. Des inventaires faunistiques antérieurs et des observations opportunistes ont montré que la ZTHA avait le potentiel d'abriter un niveau notable de biodiversité (Letournel, Pages, et al., 2021).

Tout d'abord, pour étudier les synchronismes entre la dynamique des communautés d'amphibiens, et donc les périodes de vulnérabilité de la communauté d'amphibiens, et la dynamique agrochimique, ainsi que la diversité de cette communauté, nous avons réalisé des inventaires faunistiques dans la CWR et dans des étangs de comparaison, entre 2021 et 2022. Nous avons également utilisé des références naturalistes (i.e. des guides herpétologiques) pour reconstruire la phénologie des espèces présentes dans la ZTHA, et des données de flux agrochimiques en continu pour étudier spécifiquement les synchronismes entre la communauté d'amphibiens et les produits agrochimiques. Deuxièmement, pour étudier les effets des pesticides sur les activités enzymatiques et l'état corporel du Crapaud commun (*B. bufo*) et de la Grenouille verte (*Pelophylax sp.*), nous avons utilisé une approche non-invasive, l'écouvillonnage buccal, pour collecter la salive des espèces étudiées et effectuer des mesures morphométriques, incluant notamment des mesures de la longueur totale et du poids. Les enzymes étudiées chez le Crapaud commun et la Grenouille verte étaient l'acétylcholinestérase, intervenant dans la neurotransmission, la β-galactosidase et la β-glucosidase, intervenant dans la nutrition, la glutathion S-transférase, intervenant dans la détoxication de l'organisme, les phosphatases acide et alcaline, et les peroxidases, impliquées dans l'immunité non-spécifique et étudiés en tant que biomarqueurs d'exposition. Pour cette étude, la méthode utilisée pour l'analyse statistique a été celle des modèles additifs généralisés (GAM) afin d'évaluer les relations entre les traits biologiques étudiés et les pressions exercées par les pesticides, en raison des relations non linéaires attendues entre ces deux groupes de variables. Troisièmement, pour étudier les effets du mélange de pesticides (aclonifen, bentazone, chloridazon, S-métolachlore, glyphosate, chlorotoluron, métazachlore, boscalid, époxiconazole et tebuconazole, total de 64,5 µg/L), formé en laboratoire, sur *G. fossarum*, nous avons utilisé des mésocosmes extérieurs implantés directement dans la ZTHA, pour étudier les réponses biologiques de *G. fossarum* dans des conditions semi-réalistes (c'est-à-dire, en intégrant les conditions climatiques locales de la ZTHA dans la compréhension des réponses étudiées, en particulier). Dans les mésocosmes, le mélange a été ajouté, une seule fois, et sa toxicité a été suivie dans le temps grâce à une méthodologie de biosurveillance active. Nous avons effectué 4 séries d'exposition, d'une semaine chacune, dans les mésocosmes. Les gammares ont été mis en cage pendant une semaine dans des microcosmes, au sein des mésocosmes, avec un renouvellement de la population après chaque semaine d'exposition à différents niveaux de concentration de pesticides. Nous avons étudié leurs réponses enzymatiques et leurs réponses comportementales, incluant le taux de survie, l'activité locomotrice et reproductive, ainsi que le taux d'alimentation. Nous avons utilisé des régressions linéaires simples pour étudier les relations entre les traits biologiques mesurés chez *G. fossarum* (activités enzymatiques, traits comportementaux), et les pressions de pesticides, ainsi qu'entre les traits biologiques et les facteurs confondants naturels (température, nitrite, etc.). Enfin, pour étudier les effets des flux agrochimiques sur la structure et le fonctionnement de la communauté de macroinvertébrés benthiques, une étude spécifique a été développée pour suivre les réponses structurelles et fonctionnelles de la communauté, telles que plusieurs indices de diversité et de sensibilité, et la dégradation de la litière. Nous avons utilisé des sacs à litière pour réaliser des inventaires faunistiques et déterminer le taux de décomposition de la litière en fonction des niveaux d'exposition aux produits agrochimiques, sur 4 mois de mesures, avec des sessions de mesures espacées de 15 jours. La méthode utilisée pour l'analyse statistique a été celle des modèles linéaires généralisés (GLM) afin d'étudier, principalement, les effets des pressions exercées par les pesticides sur la communauté de macroinvertébrés benthiques et les réponses en termes de fonctionnement.

Dans le cadre de cette thèse, un total de 10 mares a été sélectionné et étudié, dont la ZTHA, et 9 mares de comparaison, toutes situées en Ile-de-France. L'étude de ces mares avait pour but d'apporter un certain recul sur les différents résultats obtenus pour la ZTHA. Elles ont été sélectionnées sur la base de la consultation de données naturalistes, et sur la base de prospections de terrain, afin de s'assurer de la présence d'amphibiens notamment.

Premièrement, l'évaluation du risque pour l'intégrité de la communauté d'amphibiens vivant dans la ZTHA, en lien avec les dynamiques de flux de pesticides et nitrates, a permis de mettre en évidence des synchronismes entre les dynamiques temporelles des niveaux de contamination dans la ZTHA avec les périodes où la communauté d'amphibiens est la plus vulnérable. Sur 10 ans de suivi de la qualité chimique de l'eau au sein de la ZTHA de Rampillon, les scientifiques en charge de l'étude de la zone ont déterminé qu'en moyenne les flux de pesticides et de nitrates s'élevaient respectivement à 1 µg/L et 55-60 mg/L au cours de l'année, avec des pics de concentrations d'environ 10 µg/L pour les pesticides, et de plus de 100 mg/L pour les nitrates durant les mois de mai et juin. Plus précisément, au sein de la ZTHA de Rampillon, les périodes où les plus fortes concentrations en pesticides et nitrate sont mesurées correspondent également aux périodes (i) où le plus d'espèces d'amphibiens sont présentes simultanément dans l'eau, (ii) à fort enjeu écologique, c'est-à-dire les périodes où les amphibiens se reproduisent, où les juvéniles se développent, correspondant donc au moment où les jeunes amphibiens sont le plus à même d'être sujet aux propriétés que certains pesticides empruntent aux perturbateurs endocriniens, etc. Ces synchronismes temporels entre les périodes de vulnérabilité de la communauté d'amphibiens et les dynamiques des flux de pesticides et nitrates dans la ZTHA, en lien avec les pratiques agricoles saisonnières sur le bassin versant, sont à l'origine d'un risque écologique marqué pour la communauté d'amphibiens, particulièrement aux mois de mai et juin, où les transferts des fongicides du sol vers l'hydrosphère interviennent majoritairement couplés à ceux d'herbicides due à des pluies printanières.

Dans un second temps, des modifications significatives des niveaux d'activités enzymatiques mesurées chez les Crapaud commun et la Grenouille verte ont été identifiées, en lien avec la contamination en pesticides, dans le cadre du suivi basé sur le recueil de salive par écouvillonnage buccal, en prenant en considération l'ensemble des sites étudiés (incluant la ZTHA et les 5 mares de comparaison étudiées spécifiquement pour cet axe de recherche, avec un gradient de contamination compris entre 0 et environ 40 µg/L de pesticides en fonction des mares considérées). Cela signifie que les enzymes étudiées sont influencées par les pesticides en milieu naturel chez les amphibiens. De plus, puisqu'il a été possible de réaliser deux sessions d'échantillonnage distinctes séparées d'un mois (l'une fin mai, et l'autre fin juin) et hétérogènes en termes de niveaux de contamination en pesticides pour la Grenouille verte au sein même de la ZTHA, des effets directs de la dynamique temporelle des flux de pesticides dans la ZTHA ont été mis en évidence sur les enzymes étudiées chez cette espèce. Plus précisément, des effets significatifs des pesticides ont été mis en évidence sur l'acétylcholinestérase, la β-galactosidase, la β-glucosidase, la glutathione S-transférase, et les peroxidases, pouvant se répercuter sur certains traits individuels. Concernant la condition corporelle des amphibiens, approximation de leur état de santé global, aucune différence significative n'a été observé entre les différentes mares, quel que soit leur niveau de contamination. Paradoxalement, cela ne signifie pas que les pesticides n'ont aucun effet sur ce trait. En effet, beaucoup de facteurs environnementaux et intrinsèques aux individus étudiés (génétique) sont en mesure d'influencer la condition corporelle des amphibiens, et peuvent masquer des effets des pesticides. Des investigations plus poussées sont requises pour aller plus loin dans cette analyse. Cet axe souligne le caractère précoce des réponses enzymatiques, i.e., les enzymes sont des indicateurs très réactifs aux changements des conditions de l'environnement (ici, en lien avec la contamination en pesticides en particulier). Cela démontre également que certaines fonctions enzymatiques, chez les amphibiens, peuvent être perturbées par les dynamiques de flux de pesticides, en lien avec la temporalité des pratiques agricoles et de l'hydrologie du bassin versant, avec de possibles répercussions sur l'état de santé global des organismes aquatiques.

Troisièmement, nous avons pu montrer que l'exposition au cocktail de pesticides en mésocosmes extérieurs est responsable de modifications comportementales et biochimiques chez le Gammare. En effet, le cocktail de pesticides étudié a entraîné, chez le Gammare, une augmentation du taux d'amplexus (*i.e., l'activité de reproduction du Gammare*) et de l'activité locomotrice (*i.e., la quantité de déplacement du Gammare par unité de temps*). Ces deux effets comportementaux peuvent correspondre à une réponse au stress induit par le cocktail de pesticides utilisé : l'augmentation du taux de reproduction peut correspondre à une stratégie visant à prévenir les effets létaux du cocktail, délétères pour la survie de la population (plus se reproduire a pour conséquence d'endiguer le déclin de la population), tandis que l'augmentation de l'activité locomotrice peut correspondre à une stratégie d'évitement de la pression chimique. Des effets significatifs ont été détectés également au niveau biochimique (i.e., au niveau des enzymes). Par exemple, l'enzyme chitobiase, qui intervient dans la mue chez les crustacés et les insectes notamment, est partiellement inhibée par le cocktail de pesticides. Étant donné que la mue permet au Gammare de croître, l'inhibition de la chitobiase par les pesticides dans le milieu naturel est susceptible de perturber leur croissance, et donc de perturber la dynamique et la structure de la population autochtone.

À une autre échelle, le suivi basé sur l'utilisation des sacs à litière démontre les effets néfastes que peuvent avoir les flux de contaminants d'origine agricole sur les communautés de macroinvertébrés dans les ZTHA. Des effets négatifs significatifs des flux de pesticides dans la ZTHA ont effectivement pu être mis en évidence dans la structure de la communauté de macroinvertébrés benthiques autochtones, avec notamment une baisse de diversité de la communauté, et avec la mise en évidence de la présence d'espèces plus tolérantes aux pressions chimiques. Ces résultats tendent à démontrer l'existence d'une trajectoire écologique spécifique de la communauté de macroinvertébrés benthiques dû à la présence de contaminants. De plus, un effet négatif sur la fonction écologique étudiée et associée aux invertébrés aquatiques, la dégradation de la litière, souligne le risque que représente la vie en ZTHA agricole. En combinaison avec les nitrates, les pesticides peuvent avoir des effets déstructurant forts sur les communautés d'invertébrés aquatiques, et être responsables d'une augmentation de la proportion d'espèces tolérantes, réduisant ainsi la diversité de l'écosystème.

L'ensemble de ces résultats tendent à montrer que la ZTHA de Rampillon agit comme un piège écologique pour la faune aquatique. La ZTHA de Rampillon est effectivement une infrastructure intéressante et attractive pour les amphibiens et les invertébrés aquatiques, qui y trouvent des ressources en abondance (nourriture, habitats, stabilité), et où ils peuvent se reproduire. Cependant, en contrepartie, la ZTHA de Rampillon, en tant que milieu intercepteur de pesticides et nitrates, a le potentiel de générer des effets néfastes sur ces organismes. Le projet présenté ici met en lumière le risque notable auquel fait face la communauté d'amphibiens, en lien avec ses périodes de vulnérabilité et les dynamiques de flux de pesticides et nitrates dans la ZTHA. De plus, des effets négatifs des pesticides ont été identifiés chez les amphibiens et les macroinvertébrés benthiques, du niveau cellulaire (enzymes), jusqu'à la l'écosystème (dégradation de la litière, en tant que fonction écologique). D'ailleurs, certains macroinvertébrés, dont les crustacés, ont une vie aquatique permanente et sont donc soumis à une pression diffuse et continue. Il est cependant important de notifier que la ZTHA de Rampillon, malgré son rôle de piège écologique, agit comme un support de biodiversité ; de nombreuses espèces, sans la ZTHA, ne seraient pas présentes dans le bassin versant. Ce projet met ainsi en évidence toute la complexité de la question du potentiel des ZTHA agricoles à concilier à la fois les enjeux de protection et de préservation de la qualité de l'eau et les enjeux de conservation de la biodiversité en paysage agricole.

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List of abbreviations

AChE	Acetylcholinesterase
BC	Body condition
CA	Correspondence analysis
CAS	CAS registry number, Chemical abstracts service
CDNB	1-chloro-2,4-dinitrobenzene
CPB	Citrate-phosphate buffer
CW	Constructed wetland
CWR	Constructed wetland of Rampillon
DTNB	5,5′-dithio-bis-(2-nitrobenzoic acid)
EFSA	European food safety authority
GAL	β-galactosidase
GAM	Generalized additive model
GLM	Generalized linear model
GLU	β-glucosidase
GST	Glutathione S-transferase
HMS	Hydrological measurement station
INERIS	Institut national de l'environnement industriel et des risques
IUCN	International union for conservation of nature
LOEC	Lowest observed effect concentration
MNHN	Muséum national d'histoire naturelle
NAG	Chitobiase
NbS	Nature-based solution
NOEC	No observed effect concentration
OFB	Office français de la biodiversité
PAC	Acid phosphatase
PAL	Alkaline phosphatase
PCA	Principal component analysis
PEROX	Peroxidase(s)
PPDB	Pesticides properties database
SD	Standard deviation
SPEAR	Species at risk
TU	Toxic unit
ZTHA	Zone tampon humide artificielle

I. General introduction

I. General introduction

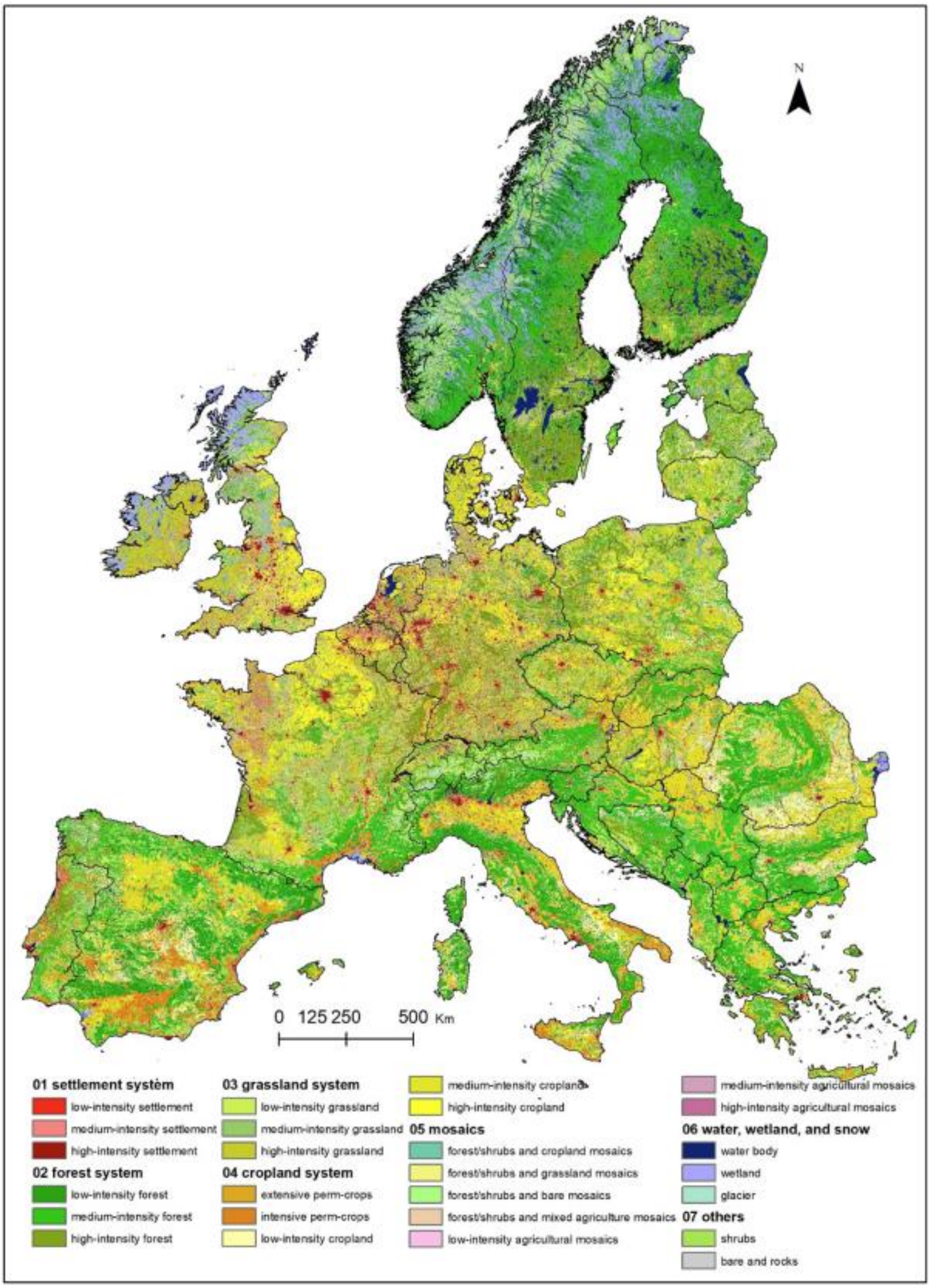
Biodiversity is declining mainly due to anthropogenic activities. One of the major anthropogenic causes of the biodiversity crisis is agricultural intensification, responsible for habitat loss and degradation, endangering populations of living organisms, and, through a cascading effect, threatening the structural and functional equilibrium of ecosystems. As one of the causes of the degradation of habitat quality, the substances used in the context of agricultural intensification, namely agrochemicals (i.e., in particular pesticides and nitrate), can be transferred to the hydrosphere, and thus disturb aquatic fauna by affecting a wide range of biological traits. The water quality of the majority of lentic agricultural waterbodies is therefore impacted, generating a risk for the aquatic fauna that find shelter in these hydrosystems. Agricultural constructed wetlands have the role of mitigating, part of the agrochemical pollution thanks to their natural purification properties. Paradoxically, these constructed wetlands by acting as agrochemical interceptors, can in turn become low quality habitats, and ecological traps for aquatic fauna.

The present thesis, entitled "*Synchronisms and antagonisms in the relationships between agricultural environment, biodiversity, and ecological functions in constructed wetlands*", focuses on the role played by constructed wetlands for aquatic fauna in the agricultural landscape. It investigates the potential of agricultural constructed wetlands to act as ecological traps for aquatic fauna through the study of synchronisms and antagonisms between chemical and bio-ecological dynamics within a pilot site located in France. In this general introduction, we thus introduce the general context and the different concepts used in this thesis by presenting: (i) the role of agricultural intensification and agrochemicals in biodiversity decline, (ii) the effects of agrochemicals on aquatic fauna, from sub-cellular to ecosystem responses, (iii) the ability of ponds and wetlands to support aquatic fauna, but with a risk to be under agricultural pressure, and then to be contaminated by agrochemicals, (iv) the potential of agricultural constructed wetlands to act as ecological traps. We then present the thesis objectives, and the main study site, the agricultural constructed wetland of Rampillon.

1. The role of agricultural intensification and agrochemicals in biodiversity decline

The term biodiversity, coined in the mid-1980s (Sarkar, 2021), refers to the diversity of life in all of its aspects including genes, populations, communities of species, biotic flow dynamics, interactions between organisms, ecological processes, and ecosystem functions (Leenhardt et al., 2022; Wake & Vredenburg, 2008). The concept of biodiversity encompasses three levels of diversity, namely (i) the ecological diversity, (ii) the specific diversity, and (iii) the genetic diversity, referring, respectively to (i) the diversity of ecosystems and landscapes, (ii) the diversity of species and taxa, and (iii) the diversity within species, i.e., the diversity of individuals (MNHN & OFB [Ed], 2003; Swingland, 2013). Resulting from billions years of evolution, biodiversity is currently facing its sixth extinction crisis, following those of the Ordovician, Devonian, Permian, Triassic, and Cretaceous geological periods (Ceballos & Ehrlich, 2010). The current extinction crisis is sometimes referred to as the "Anthropocene defaunation" (Dirzo et al., 2014). This large-scale ecological event is mainly related to anthropogenic pressures which are, at least partly, responsible for natural resource over-exploitation, landscape and habitat modifications, climate change, exotic species invasion, species over-exploitation (e.g., through hunting, fishing, forestry, poaching), disease outbreaks, and chemical pollutions, which, *in fine*, "compromises the ecosystems adaptation ability to global changes" (Blann et al., 2009; Dirzo et al., 2014, 2014; Escher & Marchant, 2019; Leenhardt et al., 2022; Pievani, 2014).

The intensification of agriculture, aiming to ensure optimal food production to meet the demands of human population growth, has an important role in biodiversity loss (Dudley & Alexander, 2017; Kehoe et al., 2017). Currently, agricultural land covers 47% and 45% of the surface of Europe (Eurostat, 2021; Herzog et al., 2012) and France (Couleaud et al., 2021), respectively (Fig. 1). Fifty percent of all European species (Eurostat, 2022) have become highly dependent on agricultural environments, since semi-natural habitats have been eradicated by the intensification and specialization of farming practices (Dudley & Alexander, 2017; Herzog et al., 2012). This dependency results in living species benefiting from, but also suffering from, the effects of land management through agricultural activities (Le Roux et al., 2012). According to Dudley & Alexander (2017), agriculture exerts a negative influence on biodiversity through four ways, namely (i) natural ecosystems conversion, (ii) management intensification, (iii) release of pollutants, and (iv) value chain impacts (e.g., energy and transport use, food waste). Although the necessity to change actual agricultural models is now well established (Demonet et al., 2013; Gross & Charbonnier, 2014), the land surface used for grazing and cropland continues to increase globally (*HYDE*, 2024; Liu et al., 2022). However, in some cases, agricultural landscapes can benefit biodiversity (Hartel et al., 2010; A. A. Moore & Palmer, 2005; Schmitt & Rákosy, 2007), thanks to landscape heterogeneity and attentive management actions (Guerra & Aráoz, 2015; Hartel et al., 2010; Knutson et al., 2004). In other cases, croplands may have mitigating effects on biodiversity, by, for example, increasing species abundance but negatively affecting some fitness[1] parameters (e.g., in amphibians) (Gray et al., 2004). Nonetheless, agriculture intensification remains undeniably one of the largest driver of biodiversity loss worldwide (Attwood et al., 2008; Dudley & Alexander, 2017; J. M. H. Green et al., 2019).



[1] The fitness of an individual, or a genotype, a central concept in evolutionary biology and ecology, refers to the number of viable and fertile offspring that this individual can produce, or that each individual of this genotype can produce on average, compared to other genotypes. In simplified terms, it can be summarized as the product of the survival and fecundity of an individual or genotype (Sæther & Engen, 2015; Tirard et al., 2022; Wadgymar et al., 2024).

Fig. 1 Distribution of land systems in Europe (Dou et al., 2021). This map shows the importance of agricultural land in Europe.

Linked with the intensification of agriculture, pesticides, including herbicides, fungicides, insecticides, molluscicides, and others, are used to protect crops from diseases and pests (EFSA, 2022), and nitrate is used to optimize crop yields (Gao et al., 2022; Khajuria & Kanae, 2013). The toxicity of pesticides and nitrate in non-target organisms, including humans, is studied since the 1940's for nitrate (Wilson, 1943), and the 1950's for pesticides (Conley, 1949; DeWitt, 1956a). Nowadays, pesticides are ubiquitous in the environment and pose an environmental risk at a global scale (Tang et al., 2021), and nitrate concentrations in aquatic ecosystems are continuously increasing due to human activities (Banerjee et al., 2023b). Pesticides, in particular, can affect biodiversity, at all levels of biological organization, up to ecosystems and associated functions (Geiger et al., 2010; Isenring, 2010; Leenhardt et al., 2022; McMahon et al., 2012). The global decline of insects due to pesticides is considered to be able to jeopardize human prosperity (van der Sluijs, 2020). Additionally, as a current high-profile example, neonicotinoids are suspected to be responsible for global biodiversity immune suppression affecting bees, fish, bats, birds, and amphibians (Mason, 2013).

Thus, with varying responses depending on the taxon, the intensification of agriculture is able to affect a wide range of taxa, mainly through habitat loss and habitat degradation by agrochemicals, as illustrated in Fig. 2. These taxa include microorganisms (J. E. Fox et al., 2007; Mandal et al., 2020), plants (Chamorro et al., 2016; Egan et al., 2014; Storkey et al., 2012; Uematsu et al., 2010), terrestrial and aquatic invertebrates (Attwood et al., 2008; Beketov et al., 2013; Brittain et al., 2010; Egan et al., 2014; Euliss & Mushet, 1999; Gleason et al., 2003; Grab et al., 2019; Hart et al., 2006; Matthaei et al., 2010a; Rix et al., 2017; Sánchez-Bayo, 2021; Thomas, 2016; Wagner, 2020), fish (Burkhardt-Holm et al., 2005; Dembkowski & Miranda, 2012; Reis et al., 2016; Sayer et al., 2025), amphibians (Agostini et al., 2020; Arntzen et al., 2017; Curado et al., 2011; Gallant et al., 2007; Jeliazkov et al., 2014; Renoirt et al., 2024; J. W. Ribeiro et al., 2018), reptiles (Doherty et al., 2020; Driscoll, 2004; R. Ribeiro et al., 2009; Simbula et al., 2021; Tingley et al., 2019), mammals (Balestrieri et al., 2019; J. M. H. Green et al., 2019; Park, 2015), and birds (Donald et al., 2006; Hart et al., 2006; Y. Li et al., 2020; Newton, 2004; Scharlemann et al., 2005).

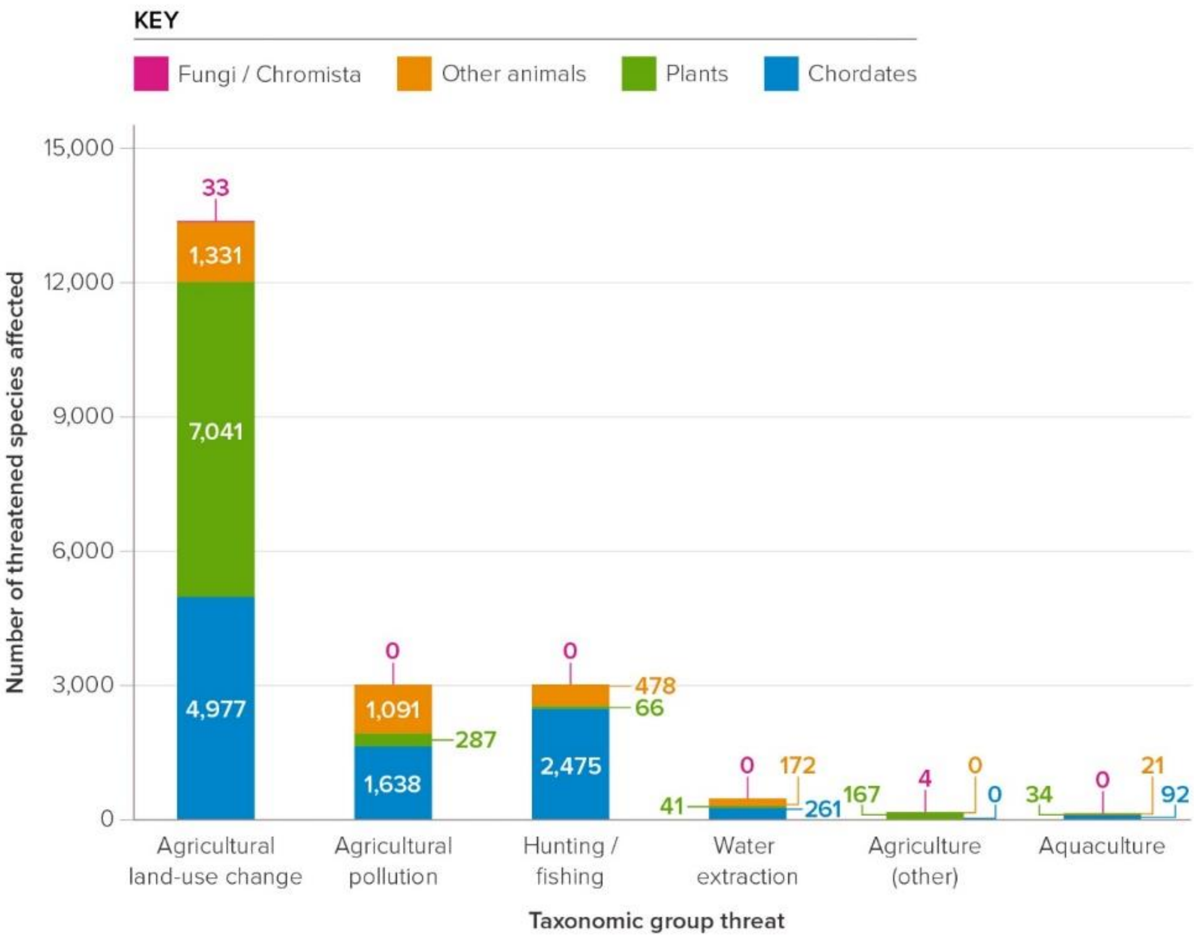


Fig. 2 Influence of various anthropogenic activities, including agricultural land-use change, and agricultural pollution, on the number of threatened species affected (Tilman & Williams, 2021).

To name just a few, and since they will be the subject of this thesis, we will focus more specifically on amphibians and aquatic invertebrates, that are part of the aquatic fauna. Although their existence as such is a primary and sufficient criterion for justifying the desire to know and protect them, amphibians and aquatic invertebrates are also essential to the proper functioning of ecosystems. The notion of ecosystem function corresponds to "the role of organisms within an ecological system", or "to the overall processes that sustain an ecological system" (Jax, 2005). In the same way that the organs that make up an organism, through their position within the organism, and through their specialization in very specific functions, contribute to making the organism a coherent, functional whole, in the environment, species, through their place and role in the environment (i.e., their ecological niche), participate in processes that influence the biotic and abiotic components of the environment, thus contributing to the functioning of ecosystems. In particular, amphibians contribute to the structural and functional equilibrium of ecosystems through their role in food webs, energy flows, pest and disease control, and ecosystem engineering, through physical habitat modification, such as bioturbation or grazing for instance (EFSA, 2018; Hocking & Babbitt, 2014; Verburg et al., 2007; Whiles et al., 2006). Amphibians can, or should be considered environmental sentinels due to their sensitivity to various environmental pressures (Roy, 2002; N. Yang et al., 2023). In parallel, because of their remarkable taxonomic and ecophysiological diversity, aquatic invertebrates also play an important role in the ecosystem, through their contribution to many ecosystem functions such as trophic networks support, nutrient cycling, and water purification (Collier et al., 2016; Murkin & Wrubleski, 1988; Prather et al., 2013; Wallace & Webster, 1996). Some aquatic invertebrate taxa, as Bivalvia, Crustacean, and Odonata, among others, are also considered sentinels for detecting environmental quality degradation (Damásio et al., 2010; Datto-Liberato et al., 2024; Marmonier et al., 2013). Thus, given their biomass, ecological roles and their sensitivity to pollution, including agrochemical pressures, amphibians and aquatic invertebrates are relevant taxa for studying the impact of agrochemicals on the environment.

Amphibians and aquatic invertebrates are suffering from several pressures, including the negative effects of agricultural intensification and agrochemical use. Amphibians are currently the most threatened vertebrates with 40.7% threatened species, mainly, more or less directly, due to consequences of anthropogenic activities (Collins & Storfer, 2003; IPBES, 2019; Luedtke et al., 2023), including climate change, habitat loss, diseases, and contaminants (Collins, 2010; Cushman, 2006; Nyström et al., 2007; Rollins-Smith, 2009). In particular, intensive agriculture is one of the strongest factors of amphibian diversity decline, mainly due to habitat loss and degradation (Collins & Storfer, 2003; Curado et al., 2011; Gallant et al., 2007; Houlahan & Findlay, 2003; Luedtke et al., 2023; J. W. Ribeiro et al., 2018). In fact, increasing arable surfaces leads to the drop of suitable woodland or grassland habitats (B. H. Green, 1990; Guerry & Hunter, 2002) and hinders dispersal (Arntzen et al., 2017; Jeliazkov et al., 2019). In addition, water quality within agricultural landscapes strongly influences amphibian individuals and communities, through individual fitness, and population and community structure modifications (Battaglin et al., 2016; García-Muñoz et al., 2019; Jeliazkov et al., 2014; Jordan et al., 2016). Pesticides and nitrate have been suggested to play a role in the decline of amphibians (Alford & Richards, 1999; Bishop et al., 1999; C. Davidson, 2004; Fellers et al., 2004; Hamer et al., 2004; Sparling et al., 2010). In parallel, aquatic invertebrates may also be showing worrying proportions of species at risk (Collier et al., 2016; Sayer et al., 2025). In aquatic insects, for example, the Odonata, Plecoptera, Trichoptera and Ephemeroptera taxa are notably declining worldwide, with proportions of species in decline of 37%, 35%, 44%, and 37% respectively, and the Plecoptera exhibiting a staggering extinction rate of 19% (Sánchez-Bayo & Wyckhuys, 2019). To varying degrees depending on the taxon, aquatic invertebrates suffer particularly from habitat degradation and destruction, water pollution, overexploitation, invasive species, and diseases (Collier et al., 2016; Sánchez-Bayo & Wyckhuys, 2019; Sayer et al., 2025), that, *in fine*, endanger the sustainability of the ecosystem functions they support (Collier et al., 2016; Macadam & Stockan, 2015). The intensification of agriculture appears as one of the main threats for aquatic invertebrates due to habitat loss, affecting, for example, 61% of threatened species of odonates worldwide (Sánchez-Bayo & Wyckhuys, 2019; Sayer et al., 2025). Within agricultural landscapes, aquatic invertebrates also tend to suffer from increased soil erosion, linked with increased suspended sediment loads, and eutrophication for example (Burdon et al., 2013; Campbell et al., 2009; Euliss & Mushet, 1999; Gleason et al., 2003; Matthaei et al., 2010a). Finally, pesticides and nitrate are also incriminated for the decline of aquatic invertebrate populations (Beketov et al., 2013; Nessel et al., 2021; van der Sluijs, 2020; Yamamuro et al., 2019). This induced decline may be attributable to the sensitivity of amphibians and aquatic invertebrates to agrochemicals, the effects of which are highly variable, as detailed below.

2. Effects of agrochemicals on aquatic fauna: from sub-cellular to ecosystem responses

Amphibians and aquatic invertebrates appear to be particularly sensitive and vulnerable to agrochemical pressures. This sensitivity is linked to some of their ecophysiological traits, as the highly vascularized pelvic spot in amphibians (Smith et al., 2007), and respiratory modalities in both taxa (i.e., dermic, integumental, or branchial respiration) (Buchwalter et al., 2002; Rico & Van den Brink, 2015). In addition, as poikilothermic organisms, the increased respiratory and metabolic rates of amphibians and aquatic invertebrates can favor oral diffusion and inhalation of chemicals (Camp & Buchwalter, 2016; EFSA, 2018; Halsey & White, 2010). The uptake of pesticides can result in bioaccumulation in amphibians (Smalling et al., 2015; Venne et al., 2008), and aquatic invertebrates (Katagi, 2010; Katagi & Tanaka, 2016), ultimately leading to physiological impairments. Although the acquisition of resistance and tolerance mechanisms to pesticides have been described in amphibians (Boyd et al., 1963; Cothran et al., 2013; Hua et al., 2013, 2015; Jones et al., 2024) and aquatic invertebrates (Becker et al., 2020; Shahid et al., 2018), the involvement of these phenomena in maintaining the integrity of wild populations exposed in agricultural environments remains largely understudied. Yet, many species of amphibians and aquatic invertebrates occur, reproduce, and migrate within agricultural landscapes (Cereghino et al., 2008; Guan & Wu, 2021; Guerry & Hunter, 2002; Semlitsch, 2008; Verdonschot et al., 2011), making them subject and vulnerable to agrochemicals in agricultural environments (J. M. R. Baker & Halliday, 1999; Bishop et al., 1999; Davis & Bidwell, 2008; EFSA, 2018; Verdonschot et al., 2011).

In agricultural environments, the vulnerability of amphibians and aquatic invertebrates to agrochemicals will depend on several factors. This vulnerability thus depends on species, due to the existence of inter-specific differences in sensitivity (Adams, Leeb, Roodt, et al., 2021; Liess & Ohe, 2005; Rico & Van den Brink, 2015), life stage (Greulich & Pflugmacher, 2003; Kulkarni et al., 2013), bio-ecological traits, such as size, sex, reproductive modalities, trophic spectrum, relation to substrate, among others, that will influence the way organisms are exposed (Awkerman et al., 2024; EFSA, 2018; Huang et al., 2022; Ippolito et al., 2012; Rico & Van den Brink, 2015), time and concentration of exposure to the contaminant(s) (Ashauer et al., 2006), nature of the combination of molecules (also known as "mixtures", or "cocktails")[2], and the environmental context in which species are exposed. Thus, in the natural environment, the complex interplay of all these factors, the dynamics of the exposome[3], and the phenological specificities of species will determine their vulnerability to agrochemicals, and thus, the risk generated by agrochemical pressures on amphibians and aquatic invertebrates (Awkerman et al., 2024; Buss et al., 2021; Chiu et al., 2016; EFSA, 2018; Vormeier et al., 2023) (cf. Chap. I). Phenological characteristics of species, such as species timing, duration of larval development, proportion of each different life stage in the life cycle of the species considered, will indeed have an influence on the critical exposure periods of the species, and thus on its vulnerability to pollution (see Awkerman et al., 2024; EFSA, 2018).

In high risk conditions, agrochemicals can have adverse effects on amphibians and aquatic invertebrates, at all levels of biological organization (Sánchez-Bayo & Mann, 2011) (see Fig. 3 for an overview of the potential effects of agrochemicals on amphibians and aquatic invertebrates, at all levels of biological organization, and see Appendix 1-2 for the complete list of corresponding references). These effects may first manifest themselves at the finest biological levels, i.e., cellular and sub-cellular levels, including biomolecules, enzymes (cf. Chap. II & III), molecules carrying genetic information (i.e., DNA, RNA), including genes, organelles, and finally the cell as a whole (Ezemonye & Tongo, 2010; Josende et al., 2015; Knapik & Ramsdorf, 2020; Sparling et al., 2015). By disrupting the structures and functions of genes, enzymes, or-

[2] Effects of combinations of molecules, also known as "mixtures", or "cocktails", are difficult to predict, due to the unpredictable nature of the effects and toxicity that this mixture of molecules will have, given the existence of diverse interactions between molecules composing the mixture, such as synergistic, antagonistic, additive, neutralizing effects, among others (Rizzati, 2016; Weisner et al., 2021).

[3] The term "exposome" refers to "the cumulative measure of environmental influences and associated biological responses throughout the lifespan, including exposures from the environment, diet, behavior, and endogenous processes" (Miller & Jones, 2014).

ganelles and the whole cell, and thus by disrupting the associated genetic and biochemical processes, effects on higher biological levels can occur. In this way, impairments in nervous, muscular, endocrine, immune, and metabolic functions can affect the organism's various tissues and organs, ultimately leading to negative effects on the individual, on development, and thus on morpho-anatomical traits (cf. Chap. II), nervous system, and thus on behavior (cf. Chap. III), reproductive function (cf. Chap. III), immune system, and on the organism's energy metabolism, potentially leading to death (Agostini et al., 2020; Bonfanti et al., 2004; Coors et al., 2008; Langlois et al., 2010; A. P. Moore & Bringolf, 2018; Ortiz et al., 2004). Reduced fitness of amphibians and invertebrates in the natural environment, or even lethal effects of agrochemicals, can have repercussions at higher biological levels, i.e., ecological levels, including populations, communities, ecosystems, and associated ecological functions, through effects such as biomass reduction, disruption of intra- and inter-specific relationships, community structure (cf. Chap. IV), trophic networks, and, ultimately, disruption of ecosystem functions such as litter breakdown (cf. Chap. IV), or nutrient cycling (Auber et al., 2011; Boone & James, 2003; Brosed et al., 2016; Hamer et al., 2004; Relyea, 2009; Schäfer et al., 2007) (Fig. 3, Appendix 1-2).

Because of the precocity of their responses (i.e., a few hours to a few days), cellular and subcellular traits are early biomarkers of exposure, providing an early warning of the risk posed by chemical pressures. At the opposite, ecological responses, which are much more time-delayed than cellular and sub-cellular responses, help to account for the long-term, integrated, and therefore, proven effects of agrochemicals on aquatic fauna and ecosystems (Sánchez-Bayo & Mann, 2011). A holistic approach, combining the study of the effects of pressures generated by agrochemicals on aquatic fauna at different levels of biological organization, can provide a better understanding of the links that are established between these different levels, and enable a more integrated apprehension of the potential impacts of agrochemicals on aquatic ecosystems.

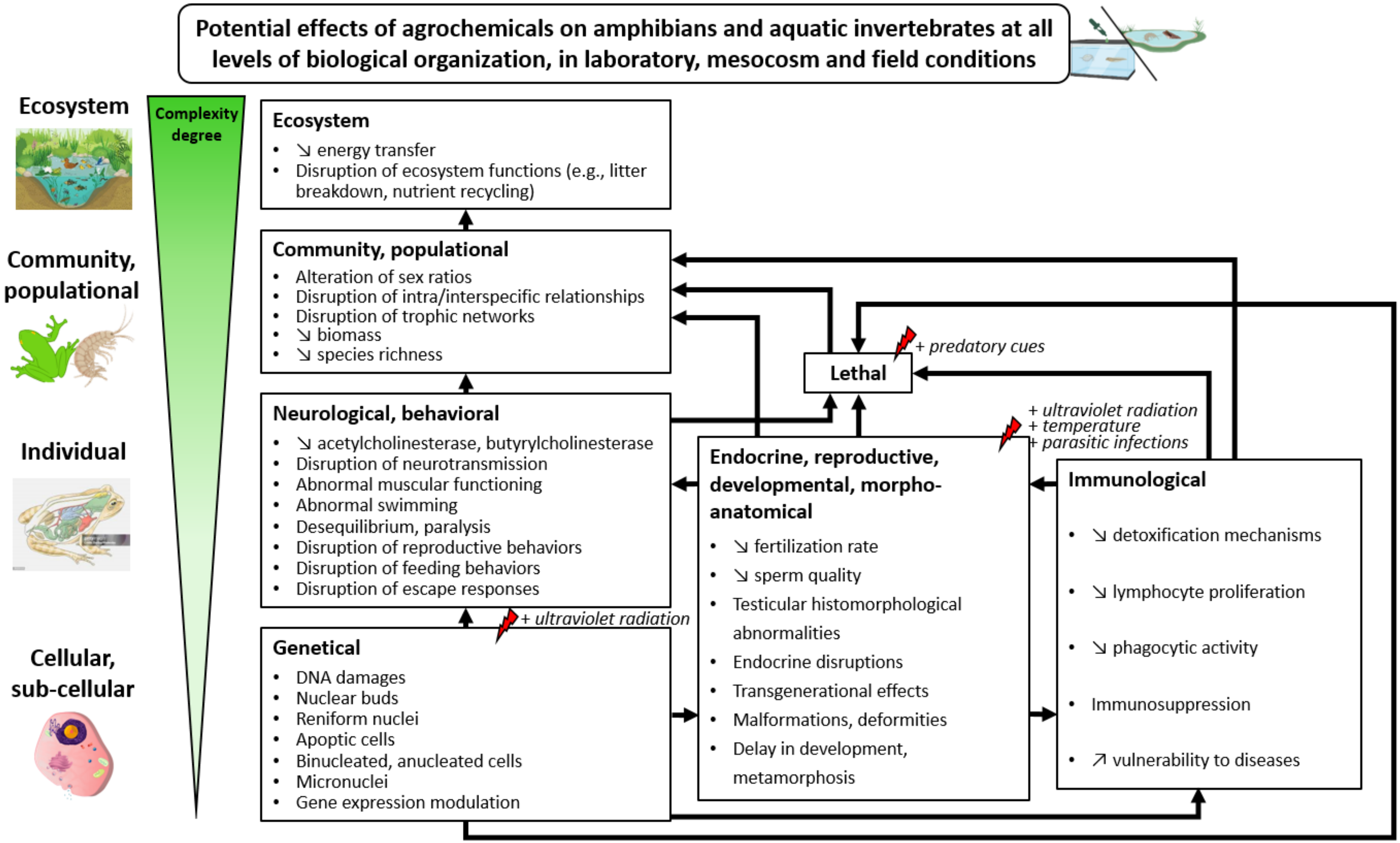

Potential effects of agrochemicals on amphibians and aquatic invertebrates at all levels of biological organization, in laboratory, mesocosm and field conditions
Ecosystem
Community, populational
Individual
Cellular, sub-cellular
Complexity degree
Ecosystem
• ↘ energy transfer
• Disruption of ecosystem functions (e.g., litter breakdown, nutrient recycling)
Community, populational
• Alteration of sex ratios
• Disruption of intra/interspecific relationships
• Disruption of trophic networks
• ↘ biomass
• ↘ species richness
Neurological, behavioral
• ↘ acetylcholinesterase, butyrylcholinesterase
• Disruption of neurotransmission
• Abnormal muscular functioning
• Abnormal swimming
• Desequilibrium, paralysis
• Disruption of reproductive behaviors
• Disruption of feeding behaviors
• Disruption of escape responses
+ ultraviolet radiation
Genetical
• DNA damages
• Nuclear buds
• Reniform nuclei
• Apoptic cells
• Binucleated, anucleated cells
• Micronuclei
• Gene expression modulation
Lethal
+ predatory cues
+ ultraviolet radiation
+ temperature
+ parasitic infections
Endocrine, reproductive, developmental, morpho-anatomical
• ↘ fertilization rate
• ↘ sperm quality
• Testicular histomorphological abnormalities
• Endocrine disruptions
• Transgenerational effects
• Malformations, deformities
• Delay in development, metamorphosis
Immunological
• ↘ detoxification mechanisms
• ↘ lymphocyte proliferation
• ↘ phagocytic activity
• Immunosuppression
• ↗ vulnerability to diseases

Fig. 3 A non-exhaustive overview of the potential effects of agrochemicals on amphibians and aquatic invertebrates at all levels of biological organization, in laboratory, mesocosm, and field conditions. The black arrows represent proven or hypothetical links between the various biological traits presented, at the different levels of biological organization, specifically in the context of the study of agrochemical effects on amphibians and aquatic invertebrates. The red thunderbolts represent factors that can have synergistic effects with agrochemicals. See Appendix 1-2 for the complete list of references (+ 200 references) (A. Michel).

Linking agrochemical exposure with responses of populations, communities, or ecosystems, still remains challenging, given:

(i) the complex interplay of environmental conditions on biota,

(ii) the complexity of organisms' responses connected across all levels of biological organization, due to hierarchical effects and emergence (see Cohen & Harel (2007); for examples illustrating the consequences of emergence, particularly in the context of the study of the effects of agrochemicals, see Forbes & Galic (2016); Leenhardt et al. (2022); Mann et al. (2009); Mcdowell (2014); Piha (2006); Schmidt (2004)),

(iii) the existence of complex mixture effects between molecules (Flores et al., 2014; Rizzati, 2016; Weisner et al., 2021),

(iv) the behavioral ability of amphibians and aquatic invertebrates to avoid agrochemical pressures (Ensabella et al., 2003; Lauridsen & Friberg, 2005; Leeb, Kolbenschlag, et al., 2020; Moreira-Santos et al., 2019; Schulz & Liess, 1999; Takahashi, 2007; Wojtaszek et al., 2005),

(v) or to acquire tolerance and resistance mechanisms to it (Becker *et al.*, 2020; Boyd *et al.*, 1963; Cothran *et al.*, 2013; Hua *et al.*, 2013, 2015; Lambert & Donihue, 2020; Shahid *et al.*, 2018), and,

(vi) the existence of direct, as well as indirect agrochemical effects (Leenhardt et al., 2022; Mann et al., 2009; Sánchez-Bayo, 2021), as observed in trophic networks for example (Bundschuh et al., 2012; Groner & Relyea, 2011; Sánchez-Bayo, 2021) (see Fig. 4).

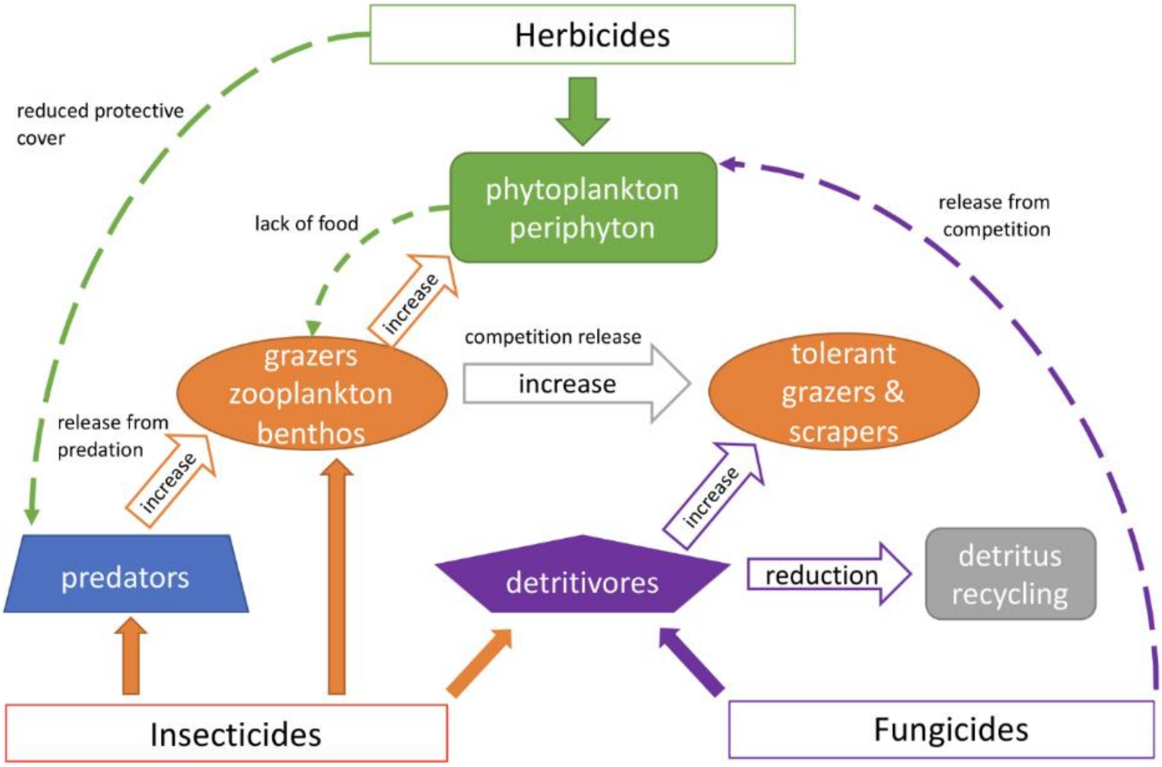


Fig. 4 Indirect effects of pesticides on insects and other arthropods in aquatic ecosystems (Sánchez-Bayo, 2021). In his review, the author highlights the complexity of the indirect effects that pesticides can have, by disrupting trophic relationships between species of different trophic groups.

In this thesis, we were able to address mainly (i) and (iii). Despite the complexity of understanding in detail the impact of agrochemicals on aquatic fauna, they represent a significant risk to the functions and services that freshwater ecosystems provide (Chagnon et al., 2015; Leenhardt et al., 2022). It should be noted that, intermediate approaches between controlled laboratory conditions and uncontrolled fieldwork conditions, namely semi-controlled approaches in mesocosms, can offer a good compromise between repeatability and experimental realism, to understand better the responses of organisms to defined factors.

3. Ponds and wetlands are shelters for aquatic fauna

Despite the significant impact of agricultural intensification and agrochemicals on aquatic fauna, agricultural water bodies (i.e., lentic or lotic bodies of water, natural or artificial, in agricultural environments), including streams, ditches, ponds, and wetlands, can provide shelters for aquatic fauna, whether amphibians (J. M. R. Baker & Halliday, 1999; Knutson et al., 2004), or aquatic invertebrates (Cereghino et al., 2008; Davis & Bidwell, 2008; Hill et al., 2016; Ruggiero et al., 2008; Verdonschot et al., 2011, 2011; Williams et al., 2004). The distinction between hydrosystems known as “ponds” and “wetlands” has long been blurred. Richardson et al. (2022) proposed the following functional definition of ponds: “Ponds are small and shallow waterbodies with a maximum surface area of 5 ha, a maximum depth of 5 m, and < 30% coverage of emergent vegetation. Ponds will have light penetration to the sediments if water clarity permits and can be permanent or temporary and natural or human-made”. Wetlands are characterized by a larger surface area and a greater degree of vegetation cover (Richardson et al., 2022). Ponds and wetlands are crucial for the many ecosystem services they provide, including habitats and biodiversity support, landscape and ecosystem diversity, climate regulation, water quality processing, microbial activities, and hydrological, and hydraulic regulation (Bobbink et al., 2006; De Groot et al., 2018; Denny, 1994, 1997; Son, Jinkwan et al., 2014). Thus, agricultural ponds, in particular, in addition of providing those services, are considered as crucial landscape features for amphibians and aquatic invertebrates, to such an extent that some shelter rare animal taxa (Biggs et al., 2007; Wood et al., 2003). Ponds are also important for amphibians and aquatic invertebrates as they reinforce the landscape connectivity, a key factor in the persistence of amphibian and aquatic invertebrate populations (Barta et al., 2024; Moor et al., 2024; R. Ribeiro et al., 2011; Schofield et al., 2018).

Nevertheless, ponds and wetlands are suffering from agriculture intensification, pollution, climate change, and human population growth (Czech & Parsons, 2022; Finlayson & Spiers, 1999; Heath & Whitehead, 1992; Indermuehle et al., 2008; Oertli et al., 2008; Wood et al., 2003), and the rate and intensity of their decline are alarming (N. C. Davidson, 2014; Fluet-Chouinard et al., 2023; Levy, 2015; Thinzilal, 2013). Pond loss in France, mainly due to the intensification of agriculture (Arntzen et al., 2017), threatens the future of amphibian communities in agricultural landscapes (Curado et al., 2011). Moreover, agrochemicals, that enter into the hydrosphere mainly by spraying, flowing, or drainage systems (Meite et al., 2018; Tournebize et al., 2017), as illustrated in Fig. 5, are responsible for an overall contamination of water bodies (Leenhardt et al., 2022). As a result, ponds all around the world have been suggested to be polluted with pesticides (Frank et al., 1990; Miglioranza et al., 2002; Uddin et al., 2013), including French ones (Chaumet et al., 2021; Sarrazin et al., 2022). Thus, although agricultural ponds can be suitable habitats for aquatic fauna, they can intercept various agrochemicals, that can be responsible for deleterious effects on this aquatic fauna, as illustrated previously in Fig. 3 (Goessens et al., 2022; Leenhardt et al., 2022; Xiao et al., 2024).

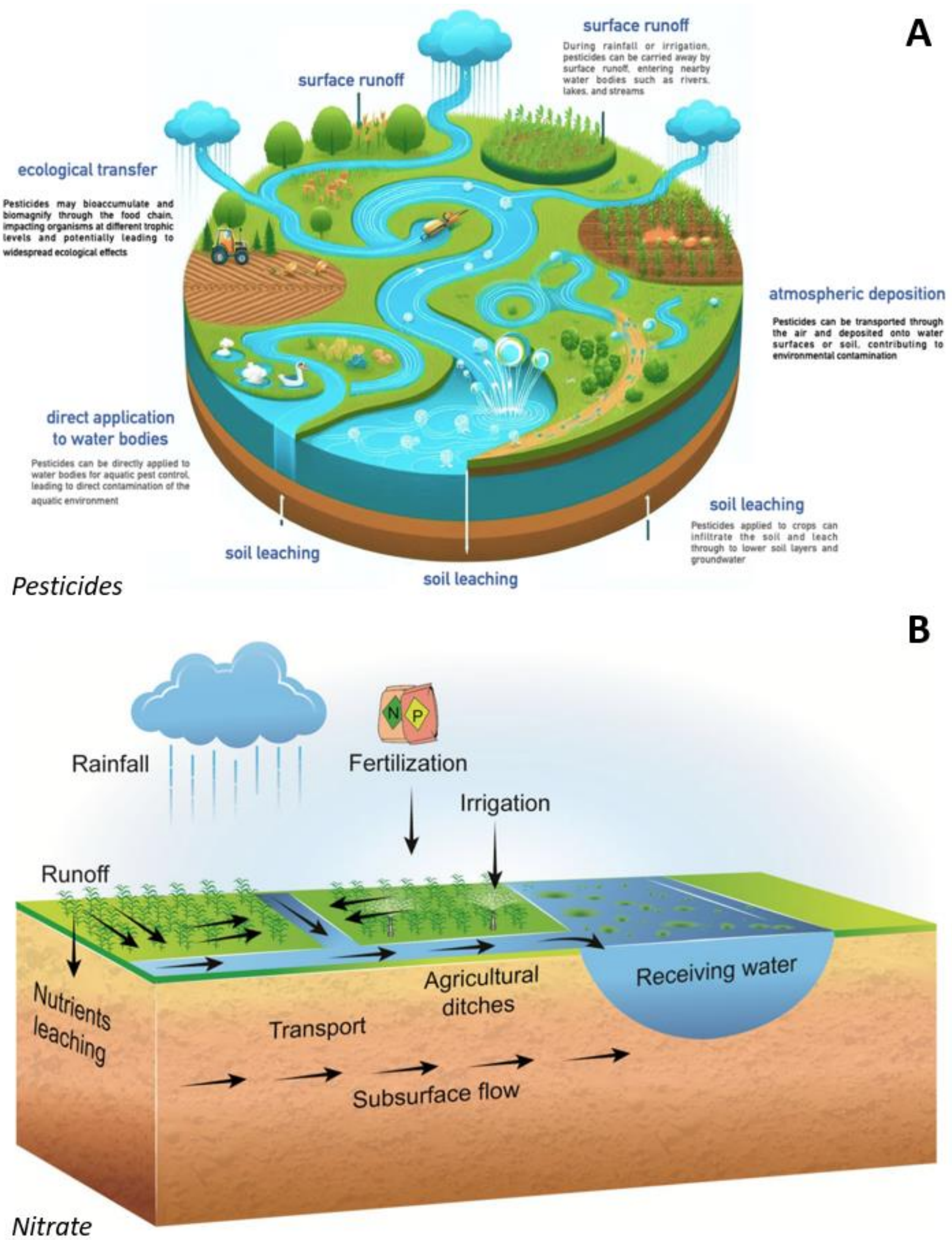


Fig. 5 Overview of (A) pesticides, and (B) nitrate transfer routes into the aquatic environment (Ortiz-Martínez et al., 2024; Xia et al., 2020). Note that drainage is not mentioned, although it may be a preferred transfer route in intensively drained areas.

To reduce chemical pressures on running and underground water, a solution has been developed into Constructed Wetlands (CWs), which are living, or non-living (i.e., with, or without plants) retention basins or ponds with a buffer function. They have been first developed in the end of the 20th century in Europe and North America to take advantage of the biodegradation ability of plants (Hammer, 1989; Shutes, 2001). Recently, the International Union for Conservation of Nature (IUCN) introduced the concept of Nature-based Solutions (NbS) to address global societal challenges (Cohen-Shacham et al., 2016). According to the IUCN, nature-based solutions are "actions to protect, sustainably manage and restore natural or modified ecosystems that address societal challenges effectively and adaptively, simultaneously providing human well-being and biodiversity benefits" (Cohen-Shacham et al., 2016). As a nature-based solution and landscape interface, agricultural CWs are conceived to mitigate contaminants originating from runoff and drainage. To a certain extent, they enable the remediation of nitrate- and pesticide-contaminated waters (Gersberg et al., 1983; Tournebize et al., 2013, 2017; Vymazal & Březinová, 2015), through biological (e.g., metabolization by plants and bacteria), and physical and chemical processes (e.g., hydrolysis, photolysis, sediment sequestration) (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Gregoire et al., 2009; Overton et al., 2023) (Fig. 6), while being able to support biodiversity (Zhang et al., 2020).

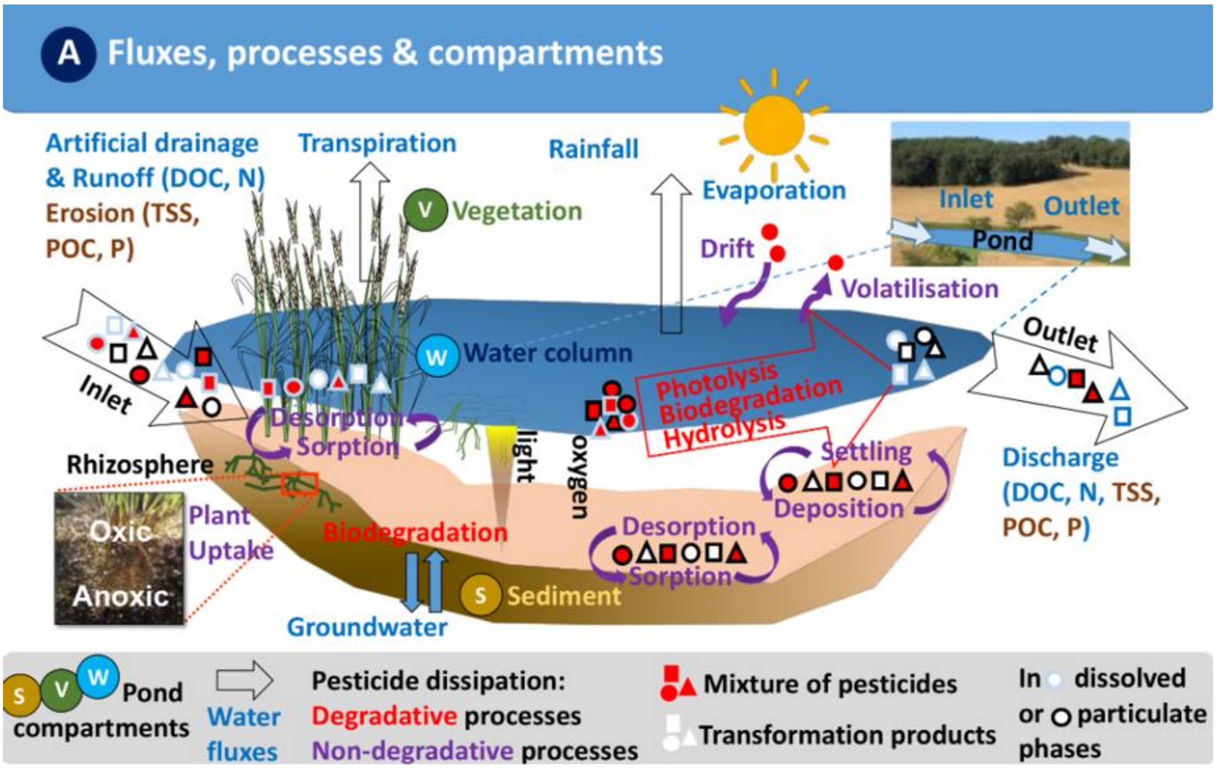


Fig. 6 Fluxes and processes of pesticide dissipation in the different compartments of ponds (Imfeld et al., 2021). TSS: total suspended solids, DOC: dissolved organic carbon, N: nitrogen, P: phosphorus, POC: particulate organic carbon.

In particular, agricultural CWs seem to contribute promoting amphibians (Letournel, Pages, et al., 2021; Rannap et al., 2020; Strand & Weisner, 2013), freshwater coleopterans (Becerra-Jurado et al., 2014), and odonates (Huikkonen et al., 2020; Letournel, Pages, et al., 2021) in agricultural landscapes. Some agricultural CWs are able to support macroinvertebrate diversity at a similar level than natural wetlands (Becerra-Jurado et al., 2010). Thus, the creation of ponds and wetlands in agricultural landscapes is often considered beneficial as it can permit to increase species diversity (Cereghino et al., 2008; Thiere et al., 2009). Moreover, constructed wetlands may reinforce the network of ponds in which it is embedded (Préau et al., 2022; Y. Qu et al., 2024), which is crucial for maintaining the integrity of aquatic fauna populations. Those hydroecosystems have thus an "important role in environmental health, water quality management, and biodiversity conservation" (Denny, 1997), even though, as suggested by certain authors, their conservation "should not be at the expense of natural wetlands" (Li et al., 2013; Ma et al., 2004), especially since they have the potential for acting as ecological traps.

4. Can agricultural constructed wetlands act as ecological traps? Thesis objectives

Due to their purification abilities, CWs may also act as ecological traps, that "occur when animals mistakenly prefer habitats where their fitness is lower than in other available habitats following rapid environmental change" (Hale & Swearer, 2016). This potential is linked with their role of contaminant interceptor, likely to affect the wild species they shelter (Piha, 2006; Stillway et al., 2019; Zhang et al., 2020), including amphibians (Sievers et al., 2018), and aquatic invertebrates as well (Duchet et al., 2018). In some contexts, which remain to be discovered, CWs could act either as habitable shelters or as ecological traps for aquatic fauna. Although the potential for CWs to act as ecological trap is recognized in controlled conditions (Duchet et al., 2018; Sievers et al., 2018), empirical *in situ* evidence is missing and holistic targeted fieldwork is needed to assess the ecological status of agricultural CWs specifically.

The French agricultural constructed wetland of Rampillon (CWR) (Seine-et-Marne, France), which is subject to agrochemical pressure, appears as an opportunity to address this issue. The CWR has been constructed in 2010 to mitigate nitrate and pesticide coming from drainage and runoff of agricultural area, while supporting aquatic fauna. The CWR has been extensively studied in previous works to assess its potential in reconciling the dual issues of water quality and biodiversity conservation in agricultural landscapes (Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Lebrun et al., 2019; Letournel, Chaumont, et al., 2021; Letournel, Pages, et al., 2021; Mander et al., 2021; Tournebize et al., 2012, 2017). The CWR is characterized by a noticeable level of species richness (Letournel, Pages, et al., 2021), suggesting a possible role of shelter for biodiversity within a relatively hostile environment. However, due to its role of agrochemical interceptor, the CWR may instead act as an ecological trap for aquatic fauna. Thus, despite the net gain in biodiversity that may be induced by certain nature-based solutions, some, like the agricultural constructed wetland of Rampillon, could be responsible for a reduction in the fitness of the organisms living in those environments.

In the context of aquatic fauna conservation issues in agricultural landscapes, the present thesis aimed, through a holistic approach, to determine whether the CWR acts as a safe environment in the agricultural matrix or as an ecological trap for aquatic fauna, focusing on amphibians and aquatic invertebrates. To address this issue, through a spatiotemporal, multi-taxon, multi-level, and multi-response biomonitoring approach, we aimed to:

(i) identify periods at risk for the amphibian community through the study of synchronisms between agrochemical fluxes and periods of vulnerability of the community (cf. Chap. I),

(ii) assess the effects of pesticides on enzymatic and morphological traits on two native amphibian species, the common toad (*Bufo bufo*) and the green frog (*Pelophylax sp.*), using a non-invasive method, namely, buccal swabbing (cf. Chap. II),

(iii) assess the effects of a pesticide mixture representative of the average CWR agrochemical pressure on enzymatic and behavioral traits in an aquatic invertebrate species, *Gammarus fossarum*, in mesocosms (cf. Chap. III),

(iv) assess the effects of agrochemical fluxes on the community structure of native benthic macroinvertebrates, and on an associated ecosystem function, the leaf-litter breakdown (cf. Chap. IV) (Fig. 7).

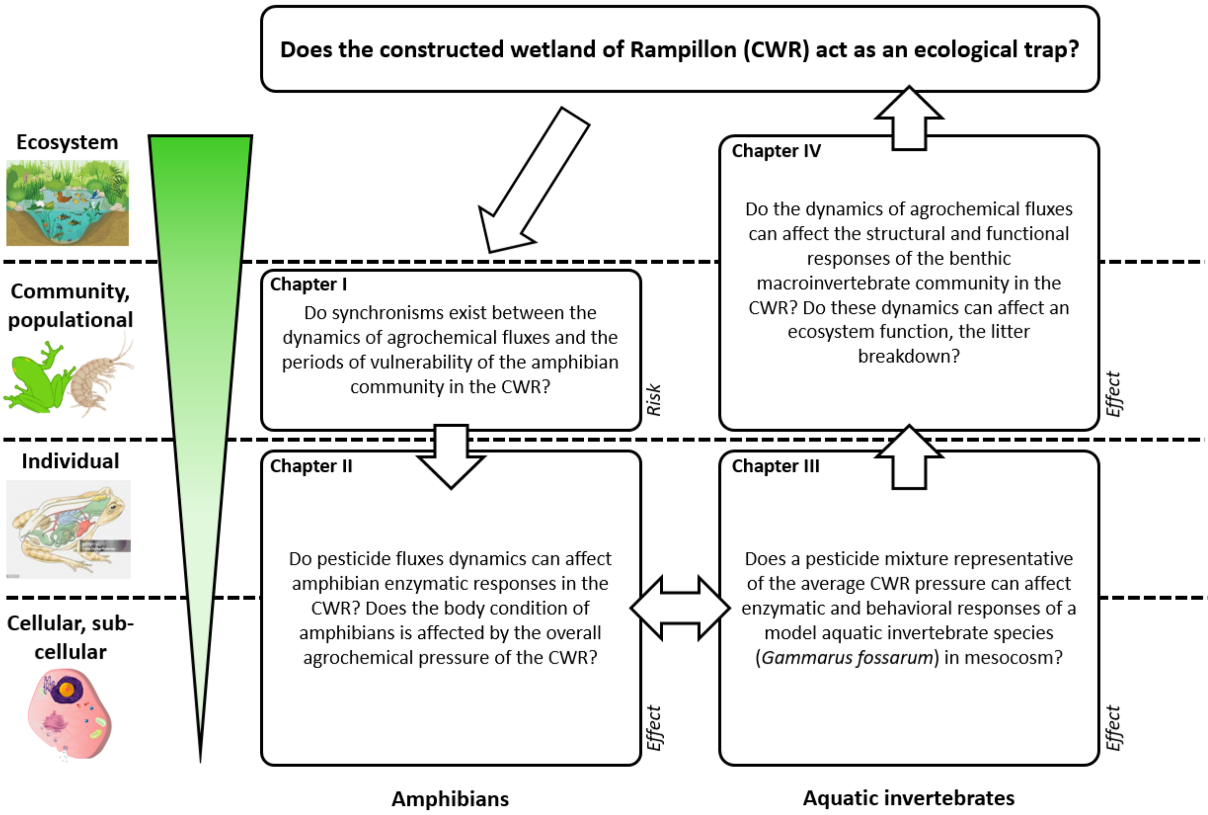


Fig. 7 Diagram presenting the conceptual framework of the thesis with the central, general question, and the various research specific questions addressed in chapters I, II, III, and IV of the "Results" section.

The general results expected are the validation of CWR's status as an ecological trap for aquatic fauna, with the existence of a notable risk for the amphibian community at the height of the breeding season, and the existence of pesticide and nitrate effects on enzymatic traits in amphibians and aquatic invertebrates, on individual traits, such as body condition on amphibians, and the behavior of *G. fossarum*, and effects on CWR benthic macroinvertebrate community structure and ecological functioning.

5. Appendix of the general introduction

Appendix 1 Effects of agrochemicals on amphibians with references.

	Controlled (laboratory / mesocosm / field)	Uncontrolled (field)
Lethal	(N. J. Baker et al., 2013; Berrill et al., 1994; Cottam & Higgins, 1946; Goodrum et al., 1949; **Hecnar, 1995**; **Karaoglu, 2022**; Lajmanovich et al., 2003; **Marco et al., 1999**; **Ortiz et al., 2004**; Relyea, 2009b)	(Carey & Bryant, 1995)
Biochemical	(Acquaroni et al., 2022; Attademo et al., 2021; Colombo et al., 2005; David et al., 2012; Ezemonye & Tongo, 2010; Greulich & Pflugmacher, 2004; Güngördü, 2013; Moutinho et al., 2020; Peltzer et al., 2013; **Wang et al., 2023**)	(Attademo et al., 2007)
Genetical	(Cabagna et al., 2006; Campana et al., 2003; Clements et al., 1997; Feng et al., 2004; Gonçalves et al., 2017; Herek et al., 2021; Jenkins et al., 2021; Lajmanovich et al., 2005; Li et al., 2010; Lopes et al., 2021; Mouchet et al., 2006; Ruiz de Arcaute et al., 2014; Silva et al., 2020; Soloneski et al., 2016; Sparling et al., 2015; **Xie et al., 2024**; Yadav et al., 2013; Yin et al., 2009; Yu et al., 2015)	(Borges, Santos, Benvindo-Souza, et al., 2019; Gonçalves et al., 2019; Gonçalves, Gambale, et al., 2017; Josende et al., 2015; Peluso et al., 2020; Sparling et al., 2015)
Endocrine, reproductive, developmental	(Adams, Leeb, & Brühl, 2021; Arukwe & Jenssen, 2005; Bach et al., 2016; Behrends et al., 2010; Brande-Lavridsen et al., 2008; Brunelli et al., 2009; Christensen et al., 2005; **Earl & Whiteman, 2009**; Edge et al., 2014; Fort et al., 2004; Haselman et al., 2018; Helbing et al., 1992; Hoffmann & Kloas, 2010; Hu et al., 2006; **Karaoglu, 2022**; Karlsson et al., 2021; Langlois et al., 2010; Larson et al., 1998; M. Li et al., 2016; Mortensen et al., 2006; **Ortiz et al., 2004**; **Ortiz-Santaliestra et al., 2012**; Pašková et al., 2011; Sullivan & Spence, 2003; Venturino et al., 2003; F.-X. Yang et al., 2005)	(Mosconi et al., 2005; Sanchez et al., 2014; Theodorakis et al., 2006)
Morpho-anatomical	(Bacchetta et al., 2008; Bonfanti et al., 2004; Brooks, 1981; Cooke, 1972; David et al., 2012; Fenoglio et al., 2009; Garcês et al., 2020; Ghodageri & Pancharatna, 2011; **Hecnar, 1995**; **Karaoglu, 2022**; Lajmanovich et al., 2003; **Ortiz et al., 2004**; Pašková et al., 2011; Pavan et al., 2021, 2021; Pawar & Katdare, 1984; Robles-Mendoza et al., 2009; Rohr et al., 2003; Seleem, 2019; Shojaei et al., 2021; Venturino et al., 2003)	(Agostini et al., 2013; Borges, Santos, Assis, et al., 2019; Christin et al., 2013; Haas et al., 2018; Ouellet et al., 1997; Peltzer et al., 2011)
Immunological	(Albert et al., 2007; Christin et al., 2003; Gilbertson et al., 2003; Hopkins & Hoverman, 2024; Kiesecker, 2002; Pochini & Hoverman, 2017; Rohr et al., 2017; Rumschlag et al., 2019; Silva et al., 2020; Taylor et al., 1999; **Wang et al., 2023**)	(Christin et al., 2003, 2013; McMurry et al., 2009)
Neurological, behavioral	(Agostini et al., 2020; Berrill et al., 1994; Brunelli et al., 2009; David et al., 2012; Denoël et al., 2012, 2013; **Dimitrova & Lukanov, 2024**; Kerby et al., 2012; Macagnan et al., 2017; McClelland et al., 2018; **Ortiz-Santaliestra et al., 2010**; Pavan et al., 2021; Peltzer et al., 2013; Shuman-Goodier & Propper, 2016; Sievers et al., 2019; **Wang et al., 2023**)	
Ecological	(Boone & James, 2003; Boone & Semlitsch, 2001; Buck et al., 2012; Groner & Relyea, 2011; Hua & Relyea, 2014; **Ortiz-Santaliestra et al., 2012**; Relyea, 2009; Relyea et al., 2005)	(**Bishop et al., 1999**; C. Davidson, 2004; **Hamer et al., 2004**)

In **bold**, References on the effects of nitrate. In addition, see Sánchez-Bayo & Mann (2011).

Appendix 2 Effects of agrochemicals on aquatic invertebrates with references.

	Controlled (laboratory / mesocosm / field)	Uncontrolled (field)
Lethal	(Bartlett et al., 2018; Goodrum et al., 1949; Kreutzweiser, 1997; Navis et al., 2013; Sandland & Carmosini, 2006; **Soucek & Dickinson, 2012**)	
Biochemical	(Bianco et al., 2013; Cossi et al., 2015; Duchet et al., 2011; Herbert et al., 2021; JanakiDevi et al., 2013; Kristoff et al., 2010; Muñiz-González et al., 2021; Serdar, 2019; Xuereb et al., 2007, 2009)	
Genetical	(JanakiDevi et al., 2013; Knapik & Ramsdorf, 2020; Muñiz-González et al., 2021)	
Endocrine, repro-ductive, develop-mental	(Cold & Forbes, 2004; Duchet et al., 2011; Favret & Lynn, 2010; LeBlanc et al., 2000; **A. P. Moore & Bringolf, 2018**; Muñiz-González et al., 2021; Navis et al., 2013; Nice et al., 2003; Omran & Salama, 2016; Palma et al., 2009; Pašková et al., 2011; Wirth et al., 2001)	
Morpho-anatomi-cal	(Bhide et al., 2004; Navis et al., 2013; Palma et al., 2009; Pašková et al., 2011; Pennati et al., 2006)	
Immunological	(Coors et al., 2008; Latanville & Stone, 2013; Muñiz-González et al., 2021; M. Ray et al., 2013; S. Ray et al., 2015; Sandland & Carmosini, 2006)	
Neurological, be-havioral	(Chmist et al., 2019; Cossi et al., 2015; Herbert et al., 2021; **A. P. Moore & Bringolf, 2018**; Rodrigues et al., 2016; Siregar et al., 2021; Xuereb et al., 2009)	
Ecological	(Auber et al., 2011; Buck et al., 2012; Bundschuh et al., 2012; Cornejo et al., 2021; Del Arco et al., 2015; Flores et al., 2014; Foit et al., 2012; Groner & Relyea, 2011; Hua & Relyea, 2014; Relyea, 2009)	(Berenzen et al., 2005; Brosed et al., 2016; Chiu et al., 2016; Fernández et al., 2015; Leonard et al., 2000; Liess & Ohe, 2005; **T. P. Moore et al., 2021**; Münze et al., 2015; **Nessel et al., 2021**; Reiber et al., 2020; Schäfer et al., 2007; Schepker et al., 2020; Simpson & Roger, 1995)

In **bold**, References on the effects of nitrate. In addition, see Sánchez-Bayo & Mann (2011).

II. General methods and materials

II. General methods and materials

This section is a brief description and general overview of the methods and materials used in this thesis. All methods and materials are described in detail in each manuscript project through the 4 chapters that will follow this section (see the section “III. Results”).

1. The agricultural Constructed Wetland of Rampillon (CWR)

The present thesis focuses on the agricultural Constructed Wetland of Rampillon (CWR) located in the Seine-et-Marne department in France, about 60 km southeast of Paris (48°32′19.5″N; 3°03′46.7″E) (Fig. 1). The 5,600 m^2 CWR, built in 2010, and situated in derivation from the stream “le ru des gouffres” (i.e., off-stream interception), collects runoff and drainage water from a 355-hectare agricultural catchment area subject to intensive crop rotation, mainly wheat, corn, and beets (85% of average crop rotation).

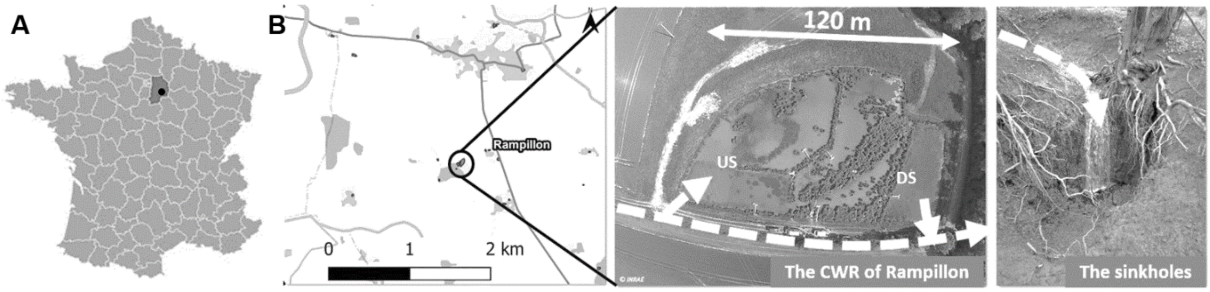


Fig. 1 Location of the CWR (A) in the Seine-et-Marne department in France, (B) location in the town of Rampillon, aerial photography of the CWR, and photography of the sinkholes. The dotted arrow corresponds to the “ru des gouffres”.

The CWR mitigates pesticides and nitrogen pollution before direct infiltration into sinkholes of the Champigny limestone aquifer that supplies 1.5 million Île-de-France inhabitants with water (Tournebize et al., 2012) (Fig. 1). The CWR intercepts an average of 40% of the mean runoffs, namely, 800,000 m^3 per year. The CWR has been equipped since 2012 to monitor agrochemical fluxes using three hydrological measurement stations (HMSs) situated upstream and downstream of the pond (but still within the CWR), and at the outlet of the CWR and catchment basin. Each HMS includes a flow rate measurement (Doppler probe) coupled with a composite sampling system controlled by the water volume that enables measurement of the incoming and outgoing fluxes of pesticides and nitrate. The strategy of flow-weight sampling gives access to biweekly flux concentration adapted to calculate incoming and exporting pollutant fluxes (but not the instantaneous variability of concentration). There is a contamination gradient from upstream to downstream due to the purification action of the pond (Lebrun et al., 2019; Letournel, Chaumont, et al., 2021). A 10-year period of continuous monitoring (2012–2022) and analysis of 531 pesticides and their metabolites showed that the CWR intercepts 40–

700 g of pesticides per year, depending on the year, and on the hydrological conditions (Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Letournel, Chaumont, et al., 2021).

The average pesticide concentration of incoming water is about 1 µg/L from January to December, with peak concentrations of approximately 10 µg/L observed in May and June, following the application of herbicides and fungicides that takes place from April to June (Fig. 2) (these are concentrations from composite water samples, based on multiple sampling over periods of 1 to 3 weeks, depending on hydrology, so they are average concentrations, rather than peak values). Over the year, nitrate concentrations are above the threshold of 30.1 mg/L, corresponding to a bad ecological quality class (Ministère de la transition écologique et solidaire, 2019) (Fig. 2) (C. Chaumont, unpublished). The average decrease in concentrations due to the purification abilities of the CWR is about 37% depending on the properties of the different molecules in pesticides (50% are herbicides), and about 11 mg/L for nitrate concentration (Letournel, Chaumont, et al., 2021). The major pesticides retrieved in the pond are the herbicides metamitron, quinmerac, mesotrione, metolachlor, ethofumesate, terbuthylazine, bentazone, isoproturon, nicosulfuron, imazamox, glyphosate and its metabolite AMPA as well as the insecticide thiamethoxam, the fungicide tebuconazole, and the molluscicide metaldehyde (partly justifying the choice of pesticides for the mixture studied in Chap. III).

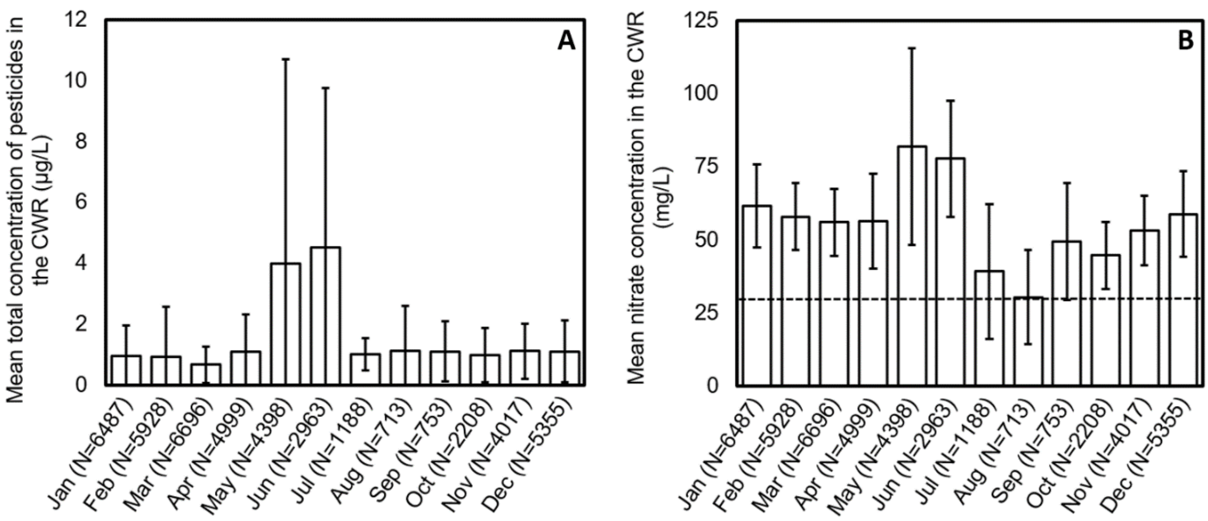


Fig. 2 (A) Pesticide concentration (µg/L) (531 molecules analyzed) (mean ± SD) and (B) Nitrate (NO_3^-) concentration (mg/L) (mean ± SD) per month at the inlet of the CWR based on continuous sampling from 2012 to 2020. The dotted line represents the threshold of nitrate concentration for the poor–bad water quality class (≥ 30.1 mg/L) according to the "Guide to Assessing the State of Continental Surface Waters" (rivers, canals, bodies of water) (Ministère de la transition écologique et solidaire, 2019).

The CWR has been extensively studied in previous works to assess its potential for reconcile the dual issues of water quality and biodiversity conservation in agricultural landscapes (Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Lebrun et al., 2019; Letournel, Chaumont, et al., 2021; Letournel, Pages, et al., 2021; Mander et al., 2021; Tournebize et al., 2012, 2017). The CWR is subdivided into three sub-basins: 1-meter depth sedimentation basin, 30 centimeters intermediate living basin colonized by reeds (*Phragmites australis*), rushes (Juncaceae), and true sedges or carex (*Carex sp.*), and an around 1-meter depth final basin. Previous faunistic inventories and opportunistic observations have shown that the CWR had the potential to support a noticeable level of biodiversity (Letournel, Pages, et al., 2021) (Fig. 3).

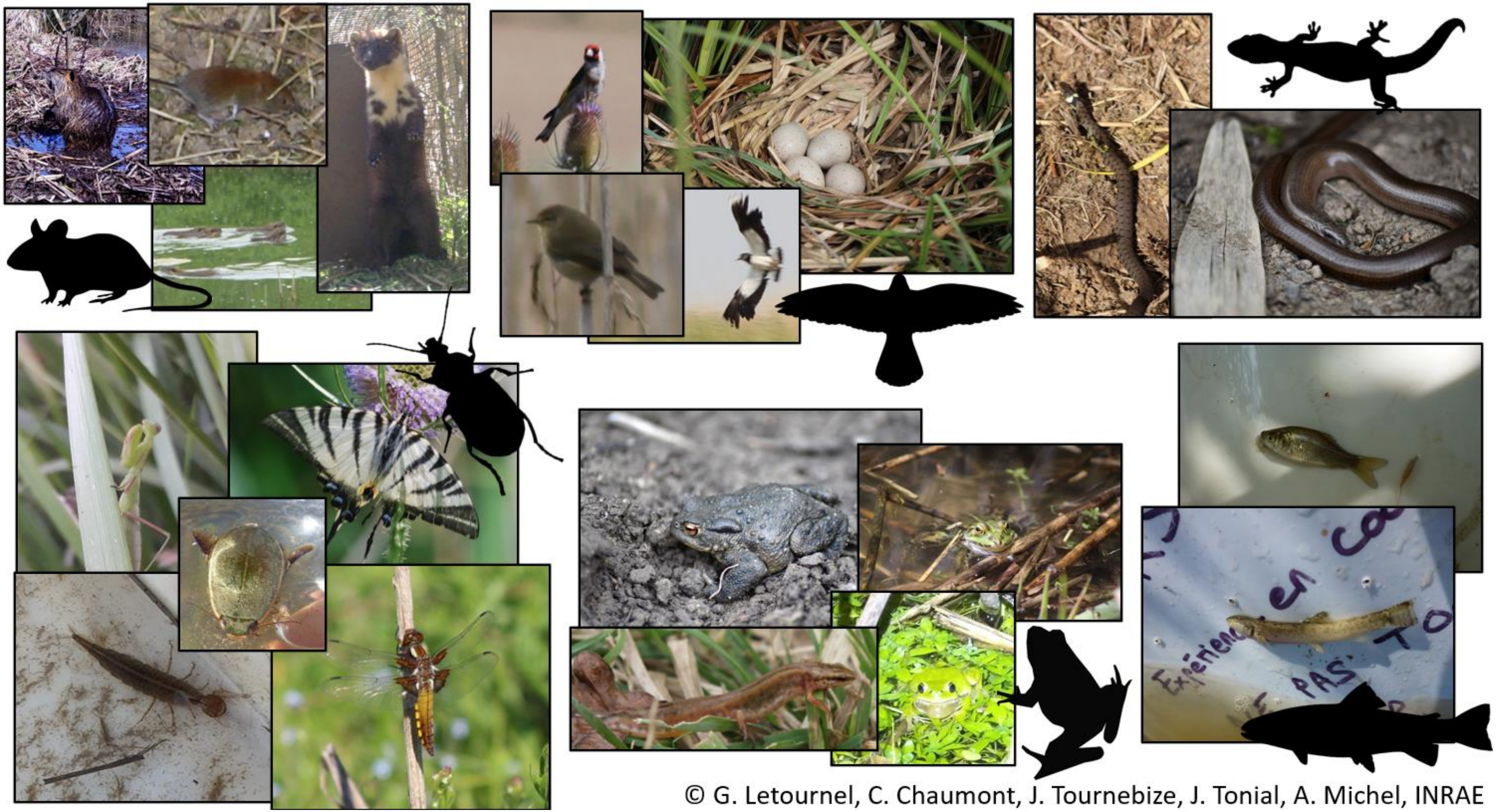


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Fig. 3 Visual sample of the CWR's faunal diversity.

2. Biomonitoring surveys general design

As stated in the introduction, in order to determine the CWR's role as an ecological shelter or trap for aquatic fauna, we conducted 4 main axes of study. We will present the general experimental design of each of these main axes of study here, but each will be explained and presented in detail in the section "III. Results".

Firstly, to study the synchronisms between amphibian community dynamics, and thus the periods of vulnerability of the amphibian community, and agrochemical dynamics, as well as the diversity of this community, we carried out faunistic inventories in the CWR and in comparison ponds, between 2021 and 2022. We also used naturalist references (i.e., herpetology guidebooks) to reconstruct the phenology of species present in the CWR, and continuous agrochemical fluxes data to study specifically the synchronisms between the amphibian community and agrochemicals.

Secondly, to study the effects of pesticides on the enzymatic activities and body condition of the common toad (*B. bufo*) and the green frog (*Pelophylax sp.*), we used a non-invasive approach, buccal swabbing, to collect saliva from the species studied, and carried out morphometric measurements, including total length and weight measurements. For this study, the method used for statistical analysis was Generalized Additive Models (GAM) to assess the relationships between the biological traits studied and pesticide pressures, due to the expected non-linear relationships between these two groups of variables.

Thirdly, to study the effects of the pesticide mixture (aclonifen, bentazone, chloridazon, S-metolachlor, glyphosate, chlorotoluron, metazachlor, boscalid, epoxiconazole, and tebuconazole, total of 64.5 µg/L), formed in the laboratory, on *G. fossarum*, we used outdoor mesocosms located directly within the CWR, to study biological responses of *G. fossarum* in semi-realistic conditions (i.e., by integrating climatic conditions into the understanding of the responses studied, in particular). In mesocosms, the mixture was added, only once, and its toxicity was monitored over time through a methodology of active biomonitoring. We carried out 4 exposure series, for a week each in the same water system, so the gammarids were caged for a week in microcosms, with population renewal after each week of exposure at different levels of pesticides concentration. We took advantage of an existing experiment to study the fate of pesticides in *in situ* mesocosms (ANR Pestipond). We studied their enzymatic responses, and their behavioral responses, including survival rate, locomotor and reproductive activity, as well as feeding rate. We used simple linear regressions to study the relationships between the biological traits measured in *G. fossarum* (enzymatic activities, behavioral traits), and pesticide pressures, and also between biological traits and natural confounding factors (temperature, nitrite, etc.).

Fourthly and lastly, to study the effects of agrochemical fluxes on the benthic macroinvertebrate community structure and functioning, a specific survey was developed to follow the community structural and functional responses, such as several diversity and sensitivity indices, and the leaf-litter breakdown. We used litterbags to carry out faunistic inventories and determine the rate of leaf-litter breakdown as a function of agrochemical exposure levels, over 4 months of measurements spaced 15 days apart. The method used for statistical analysis was Generalized Linear Models (GLM) to study, mainly, the effects of pesticide pressures on benthic macroinvertebrate community and functioning responses.

3. The comparison ponds

In this thesis, a total of 10 ponds were selected and studied, including the CWR, and 9 comparison ponds, all located in the Ile-de-France region (Fig. 4). The study of these ponds was intended to provide some hindsight on the various results obtained for the CWR. They were selected on the basis of consultation of naturalist data, and on the basis of field prospecting, to ensure the occurrence of amphibians notably. The selection criteria and characteristics of the comparison ponds are detailed in the section “III. Results”. Fig. 4 gives an overview of all the ponds studied, and of the different surveys carried out in each of them.

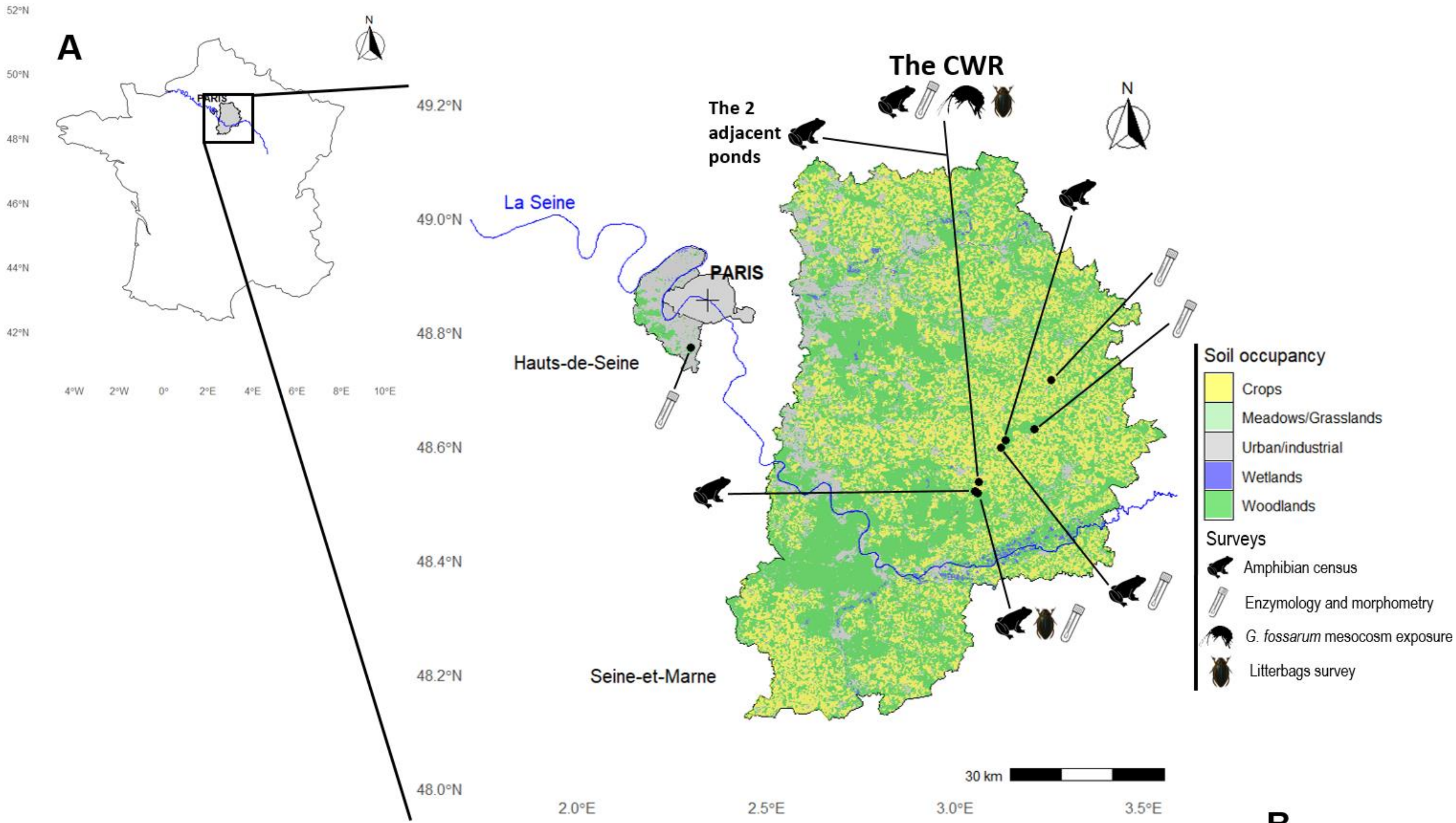


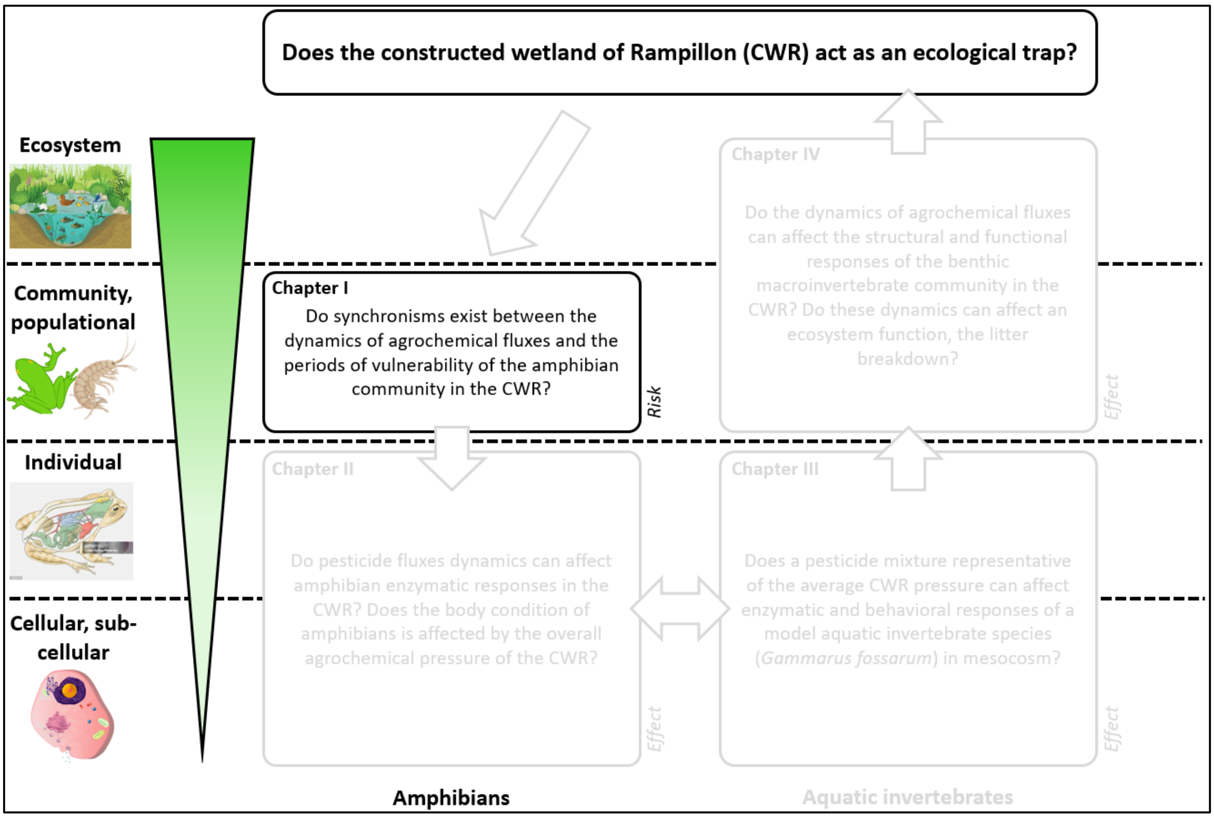
Fig. 4 Map of the study area (A) within France, and (B) location of the 10 ponds within the Ile-de-France region. The map was made thanks to R software (version 4.3.3, R Core Team 2024). The different surveys carried out in the ponds are specified for each pond.

III. Results

Chapter I

May an agricultural constructed wetland be an ecological trap? Exploring synchronisms between agrochemical fluxes and amphibian dynamics

Project of short communication / forum paper (in preparation for Oikos)



III. Results

Chapter I: May an agricultural constructed wetland be an ecological trap? Exploring synchronisms between agrochemical fluxes and amphibian ecological dynamics

Head of Chapter I

The results obtained within the framework of the present thesis will be presented through 4 chapters. The first chapter, entitled "*May an agricultural constructed wetland be an ecological trap? Exploring synchronisms between agrochemical fluxes and amphibian dynamics*", focused on the study of synchronisms between agrochemical flux dynamics and periods of vulnerability of the CWR amphibian community. This is therefore primarily a prospective ecological risk study. A given environment can be qualified as an ecological trap if, indeed, it allows the existence of such synchronisms between an environmental pressure, of an agrochemical type in the case of this thesis, and aquatic fauna. In this chapter, in addition to studying these synchronisms, the idea will be to take a more global interest in the level of the specific amphibian richness that characterizes the CWR, in relation to the selected comparison ponds, and we will attempt to address the issues relating to this richness in the context of the CWR's potential to act as an ecological trap for amphibians. Then, the research question of Chapter I is the following: *Do synchronisms exist between the dynamics of agrochemical fluxes and the periods of vulnerability of the amphibian community of the CWR?*

Title

May an agricultural constructed wetland be an ecological trap? Exploring synchronisms between agrochemical fluxes and amphibian ecological dynamics

(Short communication / forum paper in preparation for *Oikos*)

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Abstract

With the aim of mitigating water pollution coming from some agricultural practices, agricultural constructed wetlands are conceived to help purify water thanks to natural intrinsic biotic and abiotic processes. Incidentally, these constructed wetlands often harbor a noticeable biodiversity, including amphibians, which identify them as potential shelters for amphibian species.

However, precisely their primary role with regard to agrochemicals raises the question of the potential negative effects these contaminants may have on amphibians living in these environments, and therefore their actual status as ecological shelters or traps. Based on field observations in the agricultural constructed wetland of Rampillon (CWR) and a literature review, we studied the synchronisms between agrochemical fluxes dynamics and the ecological dynamics of amphibian community in the CWR to try to shed some light on its status. We carried out amphibian surveys in the CWR in 2021 and 2022, as well as in several comparison ponds in 2022. We also compared amphibian community vulnerability periods, i.e., periods when a high number of species use the pond simultaneously, with the dynamics of agrochemical fluxes in the CWR. We showed that CWR was relatively rich in amphibians (7 species, since 2017), but that synchronisms existed between amphibian community vulnerability periods and agrochemical fluxes dynamics. Although we cannot conclude on the status of the CWR at present, given the absence of data on the fitness of individuals, the existence of synchronisms between, respectively, amphibians and agrochemicals dynamics highlighted by our study, underlines the importance of addressing the ecotoxic risk faced by aquatic fauna within agricultural constructed wetlands, and thus, their potential for acting as ecological traps.

Keywords

Constructed wetland - Amphibians - Agricultural environment - Pesticides - Nitrate - Synchronisms

Statements and Declarations

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Alexandre Michel, Alienor Jeliazkov, Jérémie D. Lebrun, Cédric Chaumont, Mathieu Girondin and Julien Tournebize. The first draft of the manuscript was written by Alexandre Michel and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data Availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

Acknowledgments

We thank all the landowners for their support and all the colleagues who helped with the fieldwork.

Introduction

Pond ecosystems play a crucial role in supporting biodiversity in agricultural landscapes, including plants (Della Bella et al., 2007; Williams et al., 2004), and aquatic invertebrates (Ruggiero et al., 2008; Williams et al., 2004). Despite decades of conservation efforts (Boothby, 1999; Nicolet et al., 2007), ponds are still threatened by several pressures including agriculture intensification, pollution, and climate change (Heath & Whitehead, 1992; Indermuehle et al., 2008; Oertli et al., 2008; Wood et al., 2003). In this context, amphibians are currently experiencing high rates of species extinction (Luedtke et al., 2023; Wake & Vredenburg, 2008) mainly due to anthropogenic activities, including species over-exploitation, pollution, or else habitat modification (Collins & Storfer, 2003; Luedtke et al., 2023). Although habitat loss due to agriculture expansion is largely responsible for their decline (Curado et al., 2011; Luedtke et al., 2023), amphibians may find some shelter in agricultural ponds (Hartel & Von Wehrden, 2013; Knutson et al., 2004), in particular for reproduction.

Constructed agricultural ponds or wetlands (CWs) were first developed in the end of 20th century in Europe and North America, to purify water by promoting biological, physical, and chemical processes, such as microbial biodegradation, sediment sequestration, and photolysis (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Gregoire et al., 2009; Shutes, 2001). In addition to their primary role of water purification, some of these CWs showed some ability to support amphibian populations (Rannap et al., 2020; Strand & Weisner, 2013). Nevertheless, constructed wetlands remain systems at risk for aquatic and semi-aquatic organisms due to the potential toxicity of the contaminated water circulating within them (Stillway et al., 2019). As a result, certain CWs have the potential to act as ecological traps for animals (Zhang et al., 2020). Ecological traps are defined by Hale & Swearer (2016) as "mistakenly preferred habitats by animals, where their fitness is lower than in other available habitats". For instance, stormwater wetlands were shown to act as ecological traps for amphibians due to urban pollution, mainly metallic (Sievers et al., 2018). However, empirical evidence is missing on the potential for agricultural CWs to act as ecological traps. Yet, amphibians are known to be sensitive to pesticides, with genotoxic (Borges, Santos, Benvindo-Souza, et al., 2019; Gonçalves et al., 2019; Lowcock et al., 1997), immunotoxic (Christin et al., 2013), endocrino-, and reprotoxic effects (Bókony et al., 2018; McDaniel et al., 2008; Sanchez et al., 2014), observed even within wild populations. Amphibians are therefore likely to see their fitness affected in agricultural CWs. Therefore, the risk posed by these environments must be addressed to better envision compromises between water depollution and biodiversity conservation in agricultural landscapes.

Due to the temporality of agricultural practices and the specificities of the hydrology of each watershed, contaminant fluxes in general, and pesticides and nitrates in particular, will fluctuate over the course of the year. The potential effects on amphibians will depend on the chemical dynamics of the agrochemicals and the ecological dynamics of the amphibian population or community in question. In California, spatiotemporal overlaps were highlighted between the quantities of pesticides used and potential amphibian species richness on a large scale (i.e., on the scale of California), particularly in spring and early summer (Larsen et al., 2020). At a finest spatial scale (i.e., a few hundred hectares), temporal coincidence, i.e., synchronisms, between amphibian terrestrial migration and pesticide applications during spring has been shown in agricultural areas from Germany (Lenhardt et al., 2015). More generally, in amphibian breeding ponds, the pesticide exposome will vary in nature and intensity during spring (Goessens et al., 2022). Amphibians are likely to bioaccumulate certain pesticides, particularly at the time of the year when they are present in the water (Swanson et al., 2018). Thus, the identification of synchronisms between agrochemical fluxes dynamics and amphibian ecological dynamics is an important indicator of the level of exposure risk and, therefore, the occurrence of potential effects that amphibians may be exposed to. However, the risk to which amphibian communities may be exposed in agricultural constructed wetlands through time and phenological dynamics appears to be largely understudied. This can be approached through the study of the synchronisms between chemical and ecological dynamics.

Within the scope of the present study, we proposed to define synchronisms as the temporal concordance between notable agrochemical fluxes and amphibian community vulnerability periods, i.e., periods when the amphibian community is considered vulnerable, corresponding, in this study, to periods when most of amphibian species of a given community, are present in the water, regardless of the life stage considered, and thus potentially exposed to agrochemicals. We proposed to define periods at risk for the amphibian community, as the periods during which synchronisms are identified between notable agrochemical fluxes, and amphibian community high vulnerability. Indeed, we assumed that a higher number of amphibian species present in the water simultaneously would indicate an increased ecological risk for the amphibian community, due to the agrochemical contamination, as the probability of at least one species being affected by agrochemical pressure increases with the number of species present in the water. We are specifically interested in identifying synchronisms between agrochemical fluxes and periods when amphibians are present in the water, as we consider that toxic effects of agrochemicals in water are likely to occur in amphibians at the very time when they are exposed to agrochemicals in water. The existence of such synchronisms can be favored in agricultural constructed wetlands, making them potential ecological traps for amphibians.

In this study, through an indirect, prospective ecological risk assessment, we aimed to identify periods of synchronism between the dynamics of agrochemical fluxes and the ecological dynamics of an amphibian community. We focus on the agricultural constructed wetland of Rampillon (CWR) built in 2010 in the Seine-et-Marne region (north of France), at the downstream of a catchment basin subject to intensive agricultural practices (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Tournebize et al., 2012, 2017). Despite its well-documented levels of contamination, the CWR hosts a noticeable aquatic fauna, including a relatively high diversity of amphibians (7-8 species out of a regional diversity of 15) (Letournel, Pages, et al., 2021; Renault, 2012). However, whether this CWR acts as a shelter or an ecological trap remains an open question (Letournel, Chaumont, et al., 2021), mainly because the links between the CWR environmental conditions and its diversity are still unexplored. To address this question, our study directly confronts temporal dynamics of observation data, from amphibian surveys and environmental monitoring. In particular, we first aimed to compare the amphibian community richness of the CWR with those of comparison ponds. We expected the CWR to be characterized by a high level of amphibian richness compared to comparison ponds, making it a potential ecological trap for amphibians, allowing them to reproduce there but reducing their fitness. Second, we aimed to determine periods at risk for amphibians regarding agrochemicals exposure within the CWR. To do so, we studied the synchronisms between agrochemical fluxes and periods when amphibians are present in the water, i.e., during periods of vulnerability of the amphibian community. We expected these synchronisms to occur mainly from spring to early summer, when agrochemical fluxes are important in the CWR, and when most of amphibians are breeding.

Material and methods

This study is based on a compilation of amphibian monitoring data collected over 2 years in the CWR, spanning a period from early April 2021 to late June 2022. The survey that permitted to achieve the first objective - i.e., determine the potential for the CWR to be attractive for amphibians, so eligible to act as a potential ecological trap - was conducted in 2022 in the CWR and in 6 comparison ponds, to compare the amphibian richness in the CWR versus the amphibian richness of the 6 comparison ponds located in the same territory, through 10 sampling sessions every fortnight from February to June, 2022. The survey that permitted to achieve the second objective, i.e., identify synchronisms between agrochemical fluxes and vulnerability periods of the amphibian community in the CWR, was conducted in 2021 and 2022. We used the data collected during the 2021 and 2022 surveys in the CWR to build amphibian phenological dynamics in the CWR, with the aim to define the periods of vulnerability of the amphibian community, corresponding, in this study, to the periods when amphibians are present in the water.

Selection of the study ponds

To compare our assessment of amphibian richness of the CWR to other ponds, we proceeded to a selection of ponds to study. Those ponds, including the CWR are described just below.

The Constructed Wetland of Rampillon (CWR) is located in the Seine-et-Marne region in France, about 60 km southeast of Paris (48°32′19.5″N; 3°03′46.7″E). Located in a 355-hectare agricultural catchment area subject to intensive crop rotation, the role of the CWR is to limit the transfer of agrochemicals into the Champigny limestone aquifer that supplies 1.5 million Île-de-France inhabitants with water (Tournebize et al., 2012). The 5,600 m^2 CWR is subdivided in several sub-basins and characterized by an important habitat diversity. The CWR has been equipped since 2012 to monitor agrochemical fluxes using hydrological measurement stations situated upstream and downstream of the pond and at the outlet of the catchment basin. Based on a 10 years continuous monitoring of the water quality, characterized by continuous composite sampling controlled by flow rate, and analyses of 531 molecules including metabolites, the self-purification ability of the CWR has been estimated to be about 37% for total pesticides and about 11 mg/L for nitrate concentration (Letournel, Chaumont, et al., 2021).

In order to be able to compare the amphibian community living in the CWR, we had to select several comparison ponds in the same territory as the CWR. First, we looked for naturalist data on the French database GeoNat'îdF (https://geonature.arb-idf.fr/) to pre-select ponds where amphibians had already been observed in the last 10 years. When naturalist data were scarce and uncertain, we requested information about the presence of amphibians from landowners and, when possible, we prospected ponds to identify the presence of amphibians. In addition, we visualized soil occupancy surrounding the ponds thanks to cartography software such as Geoportail (https://www.geoportail.gouv.fr/) or Google Earth (https://www.google.com/intl/fr_fr/earth/) in order to find ponds as close as possible to the CWR and to avoid major landscape differences between ponds (see also the section "Land cover and characteristics of the ponds"). We also looked for evidence that might indicate that the crops surrounding the ponds were drained, which might indicate the risk of the pond receiving water contaminated with agrochemicals, with the initial aim of gathering ponds with contrasted levels of agrochemical contamination.

In total, we retained 6 comparison ponds. They were called P2, P3, P4, P5, P6, and P7, and were ordered according to a decreasing agrochemical contamination gradient (see the section "Land cover and characteristics of the ponds"). All ponds, including the CWR, were located in the region of Seine-et-Marne, within a 7 km radius buffer (Fig. 1). The CWR, P3, and P4 were very close to each other (ca 50 m).

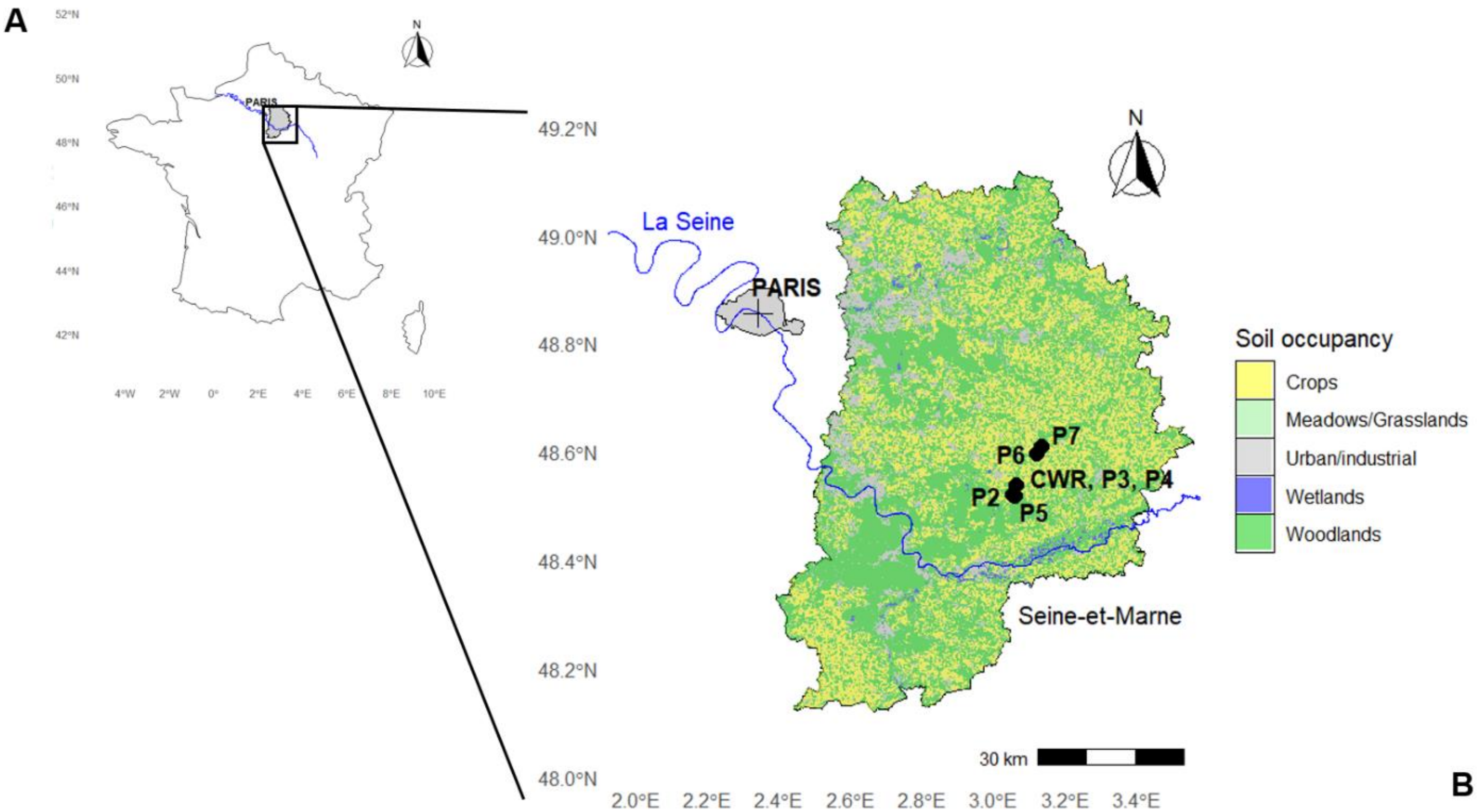


Fig. 1 Map of the study area (A) within France, and (B) location of the 7 ponds, including the Constructed Wetland of Rampillon (CWR), within the Seine-et-Marne region. The 6 comparison ponds were studied during the 2022 survey. The map was made thanks to R software (version 4.3.3, R Core Team 2024).

Land cover and characteristics of the ponds

The proportion of land cover types surrounding each pond (i.e., woodlands, meadows/grasslands, crops, urban/industrial lands) was determined in a 500-m radius buffer using QGIS 3.24.0-Tisler with the data package Theia OSO Land Cover Map 2021 (Thierion et al., 2022) (Fig.1, Table 1). Landscape connectivity, including pond connectivity, is crucial for amphibians, both urodeles (Joly et al., 2001; R. Ribeiro et al., 2011), and anurans (R. Ribeiro et al., 2011). The number of pond adjacent to the study ponds within a 1 km radius buffer was thus determined thanks to QGIS as a proxy of connectivity. Surface of ponds (m^2) and presence/absence of fish was also determined (Table 1). Ponds varied in surface area from 225 to 5,600 m^2, with the biggest ones sheltering fish (Table 1).

We also proceeded to physical and chemical measurements in every pond during the 10 amphibian sampling sessions, namely, every fortnight from February 22, 2022, to June 29, 2022 (see the section “Results: Amphibian richness in the CWR compared to other ponds”). We used multiparametric probes to measure temperature (°C), pH (no unit), conductivity (µS/cm), and oxygen saturation (%). We also measured nitrate and pesticide contamination level via water sampling. All water samples followed a similar protocol by using 500-mL amber flasks, preserved at −20°C until pesticide analyses, and 15-mL Falcon tubes for nitrogen measures preserved at 4°C. Pesticides were analyzed by an independent laboratory (Inovalys). For each aquatic sampling session and each pond, a total of 531 pesticides and their metabolites have been quantified. Taking all ponds and sampling sessions together, 80 molecules were actually detected in the water. Mean nitrate and pesticide concentrations for each pond are summarized in Table 1.

Table 1 Environmental characteristics of the 7 ponds (CWR, P2, P3, P4, P5, P6, and P7) during the 2022 amphibian survey. Ponds are in descending order of agrochemical contamination level. For physical and chemical conditions: mean ± SD. The physical and chemical measurements were taken from February 22 to June 29, 2022. Overall, 531 pesticides and their metabolites were analyzed. The number of ponds in a buffer of 1 km radius for ponds P6 and P7 is certainly biased due to the lack of data (photointerpretation difficulties linked with the forest cover).

		CWR	P2	P3	P4	P5	P6	P7
Land occupancy (500m radius) and connectivity	Woodlands (%)	7.1	14.8	6.1	6.2	55.3	45.5	54.1
	Meadows/grasslands (%)	29.5	0.5	5.8	5.6	10.8	2.4	1.9
	Crops (%)	62.0	82.8	87.0	87.2	33.9	51.9	43.8
	Urban/industrial (%)	1.0	1.9	1.0	1.0	0.0	0.0	0.1
	Number of adjacent ponds within 1 km radius	10	3	10	10	8	2	4
Pond characteristics	Surface (m^2)	5,600	2,400	225	100	1,000	1,000	300
	Fish	Presence	Presence	Absence	Absence	Absence	Presence	Absence
Water physical and chemical conditions	Temperature (°C)	17.3 ± 8.5	15.1 ± 6.6	11.8 ± 4.4	12.1 ± 4.6	12.2 ± 5.3	9.0 ± 3.5	12.1 ± 4.7
	pH (no unit)	8.1 ± 0.2	8.1 ± 0.5	7.3 ± 0.7	7.5 ± 0.2	7.0 ± 0.3	7.6 ± 0.3	6.8 ± 0.2
	Conductivity (µS/cm)	545.4 ± 132.5	461.5 ± 117.2	532.4 ± 15.8	543.8 ± 20.5	154.3 ± 15.8	284.6 ± 78.3	146.2 ± 20.0
	Oxygen saturation (%)	142.2 ± 42.6	120.1 ± 36.2	25.9 ± 18.0	48.0 ± 56.3	31.0 ± 17.5	62.2 ± 23.8	34.8 ± 23.6
Water quality	Nitrate (NO_3^-) (mg/L)	29.72 ± 19.20	7.28 ± 8.06	11.45 ± 10.73	8.20 ± 9.01	1.85 ± 1.63	3.01 ± 1.55	1.96 ± 1.44
	Total pesticides (µg/L)	14.26 ± 19.74	4.46 ± 1.49	1.26 ± 0.16	1.34 ± 0.29	0.34 ± 0.08	0.14 ± 0.03	0.09 ± 0.07
	Herbicides (µg/L)	10.62 ± 19.60	0.62 ± 1.03	0.22 ± 0.10	0.24 ± 0.16	0.10 ± 0.06	0.06 ± 0.01	0.08 ± 0.06
	Fongicides (µg/L)	0.12 ± 0.11	0.24 ± 0.13	0.09 ± 0.04	0.09 ± 0.05	0.02 ± 0.03	0.03 ± 0.01	0.01 ± 0.01
	Insecticides (µg/L)	0.12 ± 0.22	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Metabolites (µg/L)	3.39 ± 2.76	3.58 ± 1.24	0.92 ± 0.19	0.97 ± 0.29	0.21 ± 0.03	0.00 ± 0.00	0.01 ± 0.01

Amphibian surveys and diversity assessment

For the 2022 survey, amphibians were monitored in each pond (i.e., CWR, P2, P3, P4, P5, P6, and P7) every fortnight, from February 22, 2022, to June 29, 2022 (N = 10). Amphibian surveys were carried out at dusk in every pond according to the protocol described by Jeliazkov *et al.* (2014). For each pond, we proceeded to a 5-minute listening point to cense, and especially, identify species based on the identification of anuran males calling. Then, we visually censused individuals regardless of their stage using lamps in five equidistant and equidistributed plots. Finally, we dip-netted once per plot to census individuals or tadpoles/larvae underwater. In addition, Amphicapt-type traps (see Barrioz & Miaud, 2016) were used to increase the detectability of urodeles, tadpoles, and larvae. Along these three steps, we determined the taxonomical identity of the individuals at the species level (except for the group *Pelophylax sp.* for which we were limited to the genus level).

The 2021 amphibian survey was based on the same protocol, but concerned only the CWR, and the ponds P3 and P4. The 2021 survey extended from April to June (N = 5). The 2021 survey data were used for the study on the synchronisms between agrochemicals fluxes and amphibian community vulnerability periods (see the next section: "Synchronisms between agrochemicals fluxes and amphibian community vulnerability periods").

Our amphibian surveys were declared to the Regional and Interdepartmental Directorate for the Environment, Planning and Transport of Île-de-France (Direction Régionale et Interdépartementale de l'Environnement, de l'Aménagement et des Transports d'Île-de-France, DRIEAT) and authorized by prefectoral decree.

Synchronisms between agrochemicals fluxes and amphibian community vulnerability periods

The objective was to study the synchronisms between agrochemicals fluxes and amphibian community vulnerability periods within the CWR. To determine the agrochemical flux dynamics in the CWR, we used water quality data obtained from an 8 years continuous monitoring of nitrate and pesticides fluxes (i.e., data from 2012 to 2020). The CWR is indeed instrumented since 2012 with hydrological measurement stations, at the inlet and the outlet of the CWR, that permit continuous bi-monthly flow-weight samples for water quality monitoring of 531 pesticides, including their metabolites (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Bahi, Sauvage, Payraudeau, & Tournebize, 2023). We calculated means of nitrate and total pesticide concentrations quantified in the CWR for each 12 months based on this 8 years continuous monitoring.

Then, we determined the periods of vulnerability of the amphibian community over a typical year in the CWR by identifying the periods when most of amphibian species were present in the water, regardless of their life stage, corresponding thus to periods when most of amphibian species were potentially exposed to agrochemicals in the water. In addition, we built a phenological calendar for the CWR amphibian community, based on direct field observations in 2021 and 2022, by simply determining the species and respective stages observed during a given month. To do this, we considered a species to be present during a given month if we had observed at least one spawn or one individual of its species in the CWR in 2021 or 2022, regardless of its development stage (juvenile, pre-metamorph, metamorph, immature, adult), during this given month. To check the potential differences in the sampling effort across sessions, we calculated and reported the number of survey sessions carried out for each month. To compare our data with those of the literature and to position our field observations in a broader-scale phenological calendar, we used phenology information from several herpetology guidebooks (Duguet & Melki, 2003; Miaud & Muratet, 2018; Speybroeck et al., 2018). From these herpetology guidebooks, three types of phenological periods were considered, namely (i) a period of primary aquatic activity, during which reproduction, fertilization, egg-laying, juvenile development and metamorphosis can take place, (ii) a period of secondary aquatic activity, during which overwintering/hibernation of juveniles or adults can occur, and (iii) a period of terrestrial activity (in line with (Duguet & Melki, 2003; Miaud & Muratet, 2018; Speybroeck et al., 2018)). We plotted the phenology data for the species censed in the CWR in 2021 and 2022, for each month, by representing the presence/absence data for each species, and compared these with the data gathered from herpetology guidebooks. Thus, based on the combination of phenological data gathered directly in the field on the one hand, and obtained from guidebooks on the other hand, we assigned a level of vulnerability of the amphibian community to each month, by determining the number of amphibian species actually (direct observations in the field) or likely (guidebooks) present in the CWR for each month. Based on the maximal amphibian species richness of the CWR (i.e., 7 species), we defined three levels of amphibian community vulnerability, for each month, as follows: (i) high level, when $\geq 6/7$ amphibian species censed in the CWR were present simultaneously, (ii) intermediate level, when 5/7 amphibian species censed in the CWR were present simultaneously, (iii) low level, when $\leq 4/7$ amphibian species censed in the CWR were present simultaneously.

We then superimposed graphically agrochemical flux dynamics data in the CWR, phenological data for native amphibian species, and amphibian community vulnerability periods. To identify these periods of risk, we graphically identified the periods of synchronisms, when both agrochemical fluxes and amphibian community vulnerability were high, i.e., when the highest number of amphibian species were present simultaneously in the water in the CWR, and subject to notable agrochemical fluxes.

Results

Amphibian richness in the CWR compared to other ponds

Results of the 2022 survey, i.e., community composition of the ponds, and the total number of species censed (i.e., species richness), were compiled in Table 2. Overall, 10 species were observed in the study ponds in 2022, namely, 5 anurans, the green frog (*Pelophylax sp.*) (Fitzinger, 1843), the common toad (*Bufo bufo*) (Linnaeus, 1758), the agile frog (*Rana dalmatina*) (Fitzinger in Bonaparte, 1838), the common frog (*Rana temporaria*) (Linnaeus, 1758), and the European tree frog (*Hyla arborea*) (Linnaeus, 1758), and 5 urodeles, the palmate newt (*Lissotriton helveticus*) (Razoumowsky, 1789), the smooth newt (*Lissotriton vulgaris*) (Linnaeus, 1758), the northern crested newt (*Triturus cristatus*) (Laurenti, 1768), the alpine newt (*Ichthyosaura alpestris*) (Laurenti, 1768), and the fire salamander (*Salamandra salamandra*) (Linnaeus, 1758). Community composition and species richness varied across ponds, with a highest species richness in the CWR and P7, and a lowest species richness in P2 (Table 2). The green frog (*Pelophylax sp.*) and the palmate newt (*Lissotriton helveticus*) were the most common across the ponds, while the common frog (*Rana temporaria*) and the alpine newt (*Ichthyosaura alpestris*) were the rarest, present in the CWR and P7 respectively.

Table 2 Amphibian species observed in the different ponds (CWR, P2, P3, P4, P5, P6, and P7) during the 2022 survey

	CWR	P2	P3	P4	P5	P6	P7
Green frogs (*Pelophylax sp.*)	x	x		x	x	x	x
Palmate newt (*Lissotriton helveticus*)	x		x	x	x	x	x
Common toad (*Bufo bufo*)	x		x	x		x	x
Agile frog (*Rana dalmatina*)	x		x		x	x	x
Smooth newt (*Lissotriton vulgaris*)	x				x		x
Northern crested newt (*Triturus cristatus*)			x	x			
European tree frog (*Hyla arborea*)	x				x		
Fire salamander (*Salamandra salamandra*)						x	x
Common frog (*Rana temporaria*)	x						
Alpine newt (*Ichthyosaura alpestris*)							x
Species richness (February - June 2022)	7	1	4	4	5	5	7

Synchronisms between agrochemicals fluxes and amphibian community vulnerability periods

On the basis of the 8 years water quality continuous monitoring in the CWR, the average pesticide concentration of incoming water is about 1 µg/L from January until December, with peak concentrations of approximately 10 µg/L observed in May and June following the spreading of herbicides and fungicides that takes place from April to June (Fig. 2c). Nitrate concentrations following almost the same patterns with peaks in May and June.

The period when most amphibians are in the CWR at the same time - considered as vulnerable - extends from February to July, i.e., potential for 6-7 species to be in the water within the same period (Fig. 2a, b). The period from October to January is a potentially less critical period in terms of species richness (with 4 species). Theoretically, though, this is also a period when the common frog and the green frog can mostly be in water, or in sediment, for hivernate or hibernate, as tadpoles or adults (Fig. 2b). There are matches - considered as synchronisms - between the amphibian community most vulnerable periods and periods when concentration of agrochemicals are the highest in the CWR, i.e., in May and June specifically, when juveniles of most species of the CWR are still developing (Fig. 2).

a

Nb. of sp. (CWR)	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2021	NA	NA	NA	4	4	5	NA	NA	NA	NA	NA	NA
2022	NA	1	4	5	6	5	NA	NA	NA	NA	NA	NA
Mean	**NA**	**1**	**4**	**4.5**	**5**	**5**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**
Samp. effort	0	1	1	5	4	3	0	0	0	0	0	0

b

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Palmate newt (*Lissotriton helveticus*)												
Miaud & Muratet (2018)	-	X	X	X	X	X	X	X	X	-	-	-
Speybroeck et al. (2018)		X	X	X	X	X	X	X				
Duguet & Melki (2003)	-	X	X	X	X	X	X	X	X	X	-	-
Smooth newt (*Lissotriton vulgaris*)												
Miaud & Muratet (2018)	-	X	X	X	X	X	X	X	X	X	-	-
Speybroeck et al. (2018)	-	-	X	X	X	X	X	X	-	-	-	-
Duguet & Melki (2003)	-	X	X	X	X	X	X	X	X	X	-	-
Green frog (*Pelophylax sp.*)												
Miaud & Muratet (2018)	X	X	X	X	X	X	X	X	X	X	X	X
Speybroeck et al. (2018)	-	-	X	X	X	X	X	X	X	X	-	-
Duguet & Melki (2003)	-	-	-	X	X	X	X	X	X	-	-	-
Common frog (*Rana temporaria*)												
Miaud & Muratet (2018)		X	X	X	X	X	X	X	X	X		
Speybroeck et al. (2018)	-	X	X	X	X	X	X	-	-	-	-	-
Duguet & Melki (2003)	X	X	X	X	X	X	X	X	X	X	X	X
Agile frog (*Rana dalmatina*)												
Miaud & Muratet (2018)		X	X	X	X	X	X					
Speybroeck et al. (2018)		X	X	X	X	X	X					
Duguet & Melki (2003)		X	X	X	X	X	X					
European tree frog (*Hyla arborea*)												
Miaud & Muratet (2018)				X	X	X	X	X	X			
Speybroeck et al. (2018)			X	X	X	X	X	X				
Duguet & Melki (2003)			X	X	X	X	X	X	X			
Common toad (*Bufo bufo*)												
Miaud & Muratet (2018)		X	X	X	X	X	X					
Speybroeck et al. (2018)			X	X								
Duguet & Melki (2003)		X	X	X	X	X						

Stages observed in the CWR:
= spawn
= juvenile
= metamorph / immature
= adult

c

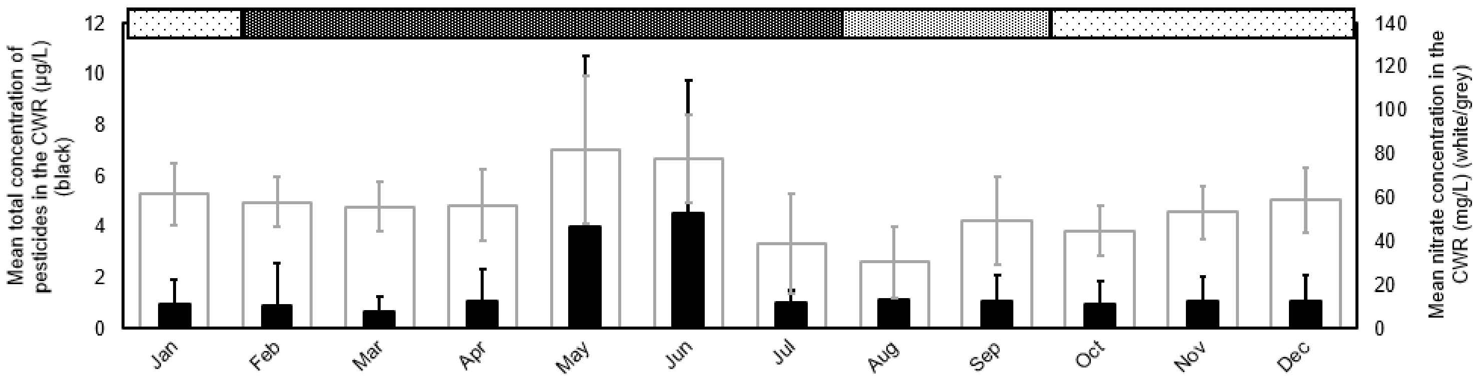


Fig. 2 Synchronisms between amphibian community vulnerability periods and the dynamics of agrochemical fluxes in the CWR over a year. (a) Table showing the number of amphibian species censed in the CWR, for each month, for the 2021 and 2022 surveys. Sampling effort (Samp. effort) corresponded to the number of survey sessions carried out for each month for the years 2021 and 2022. (b) Table showing the phenology data for the 7 amphibian species surveyed in the CWR in 2021 and 2022, determined directly in the field (grey hatching), or obtained from herpetology guidebooks (Duguet & Melki, 2003; Miaud & Muratet, 2018; Speybroeck et al., 2018) (symbols: X / - / blank). X = primary aquatic activity (presence, reproduction, fecundation, spawning, juvenile development, metamorphosis), - = secondary aquatic activity (adult / juvenile hivernation / hibernation), blank = no presence in the water, grey hatching = presence of the species considered according to direct field observation in the CWR during the 2021-2022 surveys. The various life stages observed in the field, in the CWR, are indicated by pictograms. The maximum sampling period in the CWR, for all years combined, is delimited by black vertical dotted lines (February-June). (c) Histograms represent pesticide concentration (µg/L) (531 molecules analyzed) (mean ± SD) (in black) and nitrate (NO_3^-) concentration (mg/L) (mean ± SD) (in white and grey) per month at the inlet of the CWR based on continuous sampling from 2012 to 2020. The frieze on the top of the plot, with differential fill levels, corresponds to the dynamics of the amphibian community vulnerability level over the year, according to the number of amphibian species actually or potentially present in the water during the same month: clearest filling level = low vulnerability (≤ 4/7 species present simultaneously in the water), intermediate filling level = intermediate vulnerability level (5/7 species present simultaneously in the water), highest filling level = high vulnerability level (≥ 6/7 species present simultaneously in the water).

Discussion

Overall, our study first shows the ability of the constructed wetland of Rampillon to support a relatively high diversity of amphibian species, i.e., 7 species, ex æquo with the woodland pond P7 as an uncontaminated comparison pond, compared to the studied ponds (from 1 to 7 species), and the regional diversity (15 species (Renault, 2012)). Moreover, the relatively high diversity of the CWR seems to maintain since 5 years, when referring to oldest surveys in the CWR in 2017 (Letournel, Pages, et al., 2021). In fact, we observed that the composition of the community of amphibians was almost the same in both 2017 and 2022, suggesting a certain stability of the community over the years (Letournel, Pages, et al., 2021), may be linked with a relatively stable environment, or with the potential high connectivity of the CWR within a pond network (Werner, Yurewicz, et al., 2007). Only *T. cristatus*, which was censed in 2017 in the CWR, was not anymore in 2021 or 2022. This finding may be linked to the fact that the species inhabits the ponds P3 and P4 that are located just 50 m next to the CWR, so the observation of *T. cristatus* individual in 2017 was possibly due to an anecdotal observation. Finally, the presence of *H. arborea* in the CWR constitutes an important result knowing that this species has been proposed as a focal species by the European Food Safety Authority (EFSA) based on its vulnerability towards pesticides (EFSA, 2018). Overall, this suggests, in the first instance, that the CWR would act as a shelter for amphibians, providing habitats, resources and therefore a favorable site for reproduction, stable over time. Paradoxically, however, the noticeable level of amphibian richness characterizing the CWR could make this environment an ecological trap for amphibians. Indeed, the CWR exhibits signals favorable to amphibian settlement, notably through the diversity of habitats it can offer (i.e., deep and shallow water zones, diversity of macrophyte populations, gentle slopes, etc.). Through its ecological attractiveness, CWR can therefore promote the use of this agrochemical-receiving environment by amphibians, and thus reduce their fitness due to the ecotoxic effects generated by these contaminants. Thus, a noticeable amphibian richness may also mean that other synecological phenomena could be masking the negative effects of the CWR. For instance, regular flows of individuals to the CWR, favored by the vicinity of more suitable occupied habitats (source-sink effects; see Harrison (1991)), may mask local declines in species abundance due to water toxicity linked with agrochemicals. Alternatively, effects on populations are not yet visible but may occur soon if the system we observe is not at the equilibrium and due to potential time delays in biological responses, in particular at the population and community levels, ranging from a few days to several years (Daskalova et al., 2020; Essl et al., 2015; Sánchez-Bayo & Mann, 2011). Thus, although the CWR appears to be favorable for amphibians, given the noticeable amphibian richness, the actual metacommunity dynamics into which CWR amphibians fit are not well known and may nuance this conclusion. To investigate this further, other studies could analyze the connectivity of the CWR with potential sources to assess the "sink" nature of the CWR in the context of potential source-sink dynamics, and thus assess the potential for the CWR to act as an ecological trap for amphibians.

In addition, we also highlight potentially deleterious synchronisms between agrochemical fluxes and amphibian community vulnerability periods in the CWR, in particular in May and June. Indeed, the period of the year during which the community vulnerability periods are occurring, i.e., the periods when the number of amphibian species of the community present at the same time in the water, was the highest, corresponded also to the period during which intense agrochemical fluxes occurred, making the CWR a potential ecological trap for amphibians. Amphibian community vulnerability periods were indeed synchronous with the highest nitrate and pesticide fluxes within the CWR, suggesting that significant toxic effects could occur on individuals during May and June, on a wide range of species, with potential long-term community consequences. Moreover, during these two months, the juveniles of most CWR species are in the process of developing, making this time of year more critical for the amphibian community (linked to potential developmental effects of pesticides on young stages, for example). Thus, in our study, the periods at risk for the amphibian community correspond to periods of pesticide and nitrate spraying, linked to agricultural practices in the drained watershed. The fungicide Fluxapyroxad, for example, is regularly detected in CWR during this high-risk period. As an SDHI fungicide (i.e., Succinate DesHydrogenase Inhibitors), Fluxapyroxad is able of disrupting carbohydrate and lipid metabolism in amphibians (Zhao et al., 2023), with potential repercussions on the health of individuals, and therefore populations. Nonetheless, chronic toxicity of pesticides (Jayawardena et al., 2011; Wrubleswski et al., 2018) and nitrate (Gomez Isaza et al., 2020) are also influent in amphibians. Thus, amphibians in the CWR are indeed subject to acute toxicity peaks, but the repeated chronic exposure even to lower concentrations of pesticides and nitrate during the rest of the year (e.g., amphibians hibernating in the water or sediment) is also to be taken into account, to limit risk underestimation.

There are some important limitations to the results obtained in this study. The complexity of ecosystems, of community and metacommunity dynamics, of the link between agrochemical contamination and impact on the major levels of ecological organization in the natural environment, as well as the differences in ponds' environmental characteristics, complicate the interpretability of our results. For example, the structure and dynamics of amphibian communities remain highly multifactorial, and the environmental differences between ponds complicates the interpretability of the results, as the presence of fish and connectivity, for example, are important factors influencing amphibian community composition (Cortwright & Nelson, 1990; Jeliazkov et al., 2019; Jumeau et al., 2020; Sredl & Collins, 1992; Venne et al., 2012; Werner, Skelly, et al., 2007; Werner, Yurewicz, et al., 2007). Moreover, although the higher risk is likely to occur when agrochemical fluxes are synchronous with amphibian community vulnerability periods, as we assumed in this paper, effects on individuals in field conditions are possibly unpredictable, given the vulnerability of amphibians to pesticides equally depends on both abiotic and biotic factors (Boone & James, 2003; Boone & Semlitsch, 2001, 2002; Relyea et al., 2005), and the complexity of mixture effects (Weisner et al., 2021). Thus, the risk periods determined for the CWR amphibian community may be more extended, or shifted, in reality, due to the actual complexity of the environment. In addition, the thresholds for the number of species chosen to classify the level of vulnerability of the amphibian community remain questionable. Furthermore, the phenological data in the literature is not specific to the biogeographical zone studied, adding uncertainty, especially to the extreme limits of the phenological calendar. Although this bias limits the precision of the definition of the real periods of vulnerability of the amphibian community in the CWR, it does allow us to be more protective. However, the phenological limits specific to local conditions based on field observations are clearly indicated in the calendar and still make it possible to maintain a realistic risk assessment approach for high-risk periods (i.e., May and June). Still is that we would need data on individual health, for example, to test the link between agrochemical fluxes and risk on amphibian populations effectively, to conclude on the ecological trap nature of the CWR. Nonetheless, the finding of synchronisms between amphibian community dynamics and agrochemical fluxes within the CWR itself remains an interesting result that, although it could benefit from further refinement, suggest that the CWR, despite its shelter-appearance, is a high-risk environment and a potential ecological trap for amphibians.

Conclusion

We carried out amphibian surveys in the CWR and other comparison ponds to discuss the CWR's status as an ecological shelter or trap. We found that the CWR was relatively rich in amphibians, compared with the comparison ponds, making the CWR a significant attractive environment for amphibians, and thus a potential ecological trap. We also highlighted synchronisms between amphibian community vulnerability periods, i.e., periods during which most amphibian species of the CWR are exposed to agrochemicals simultaneously in the water, and periods when agrochemical fluxes in the CWR were the highest, i.e., during May and June, specifically during which juveniles of most species are developing. These synchronisms are likely to make the CWR an ecological trap for amphibians, but further investigations are needed to test this hypothesis empirically and to assess the possible long-term risks undergone by amphibian populations in agricultural constructed wetlands.

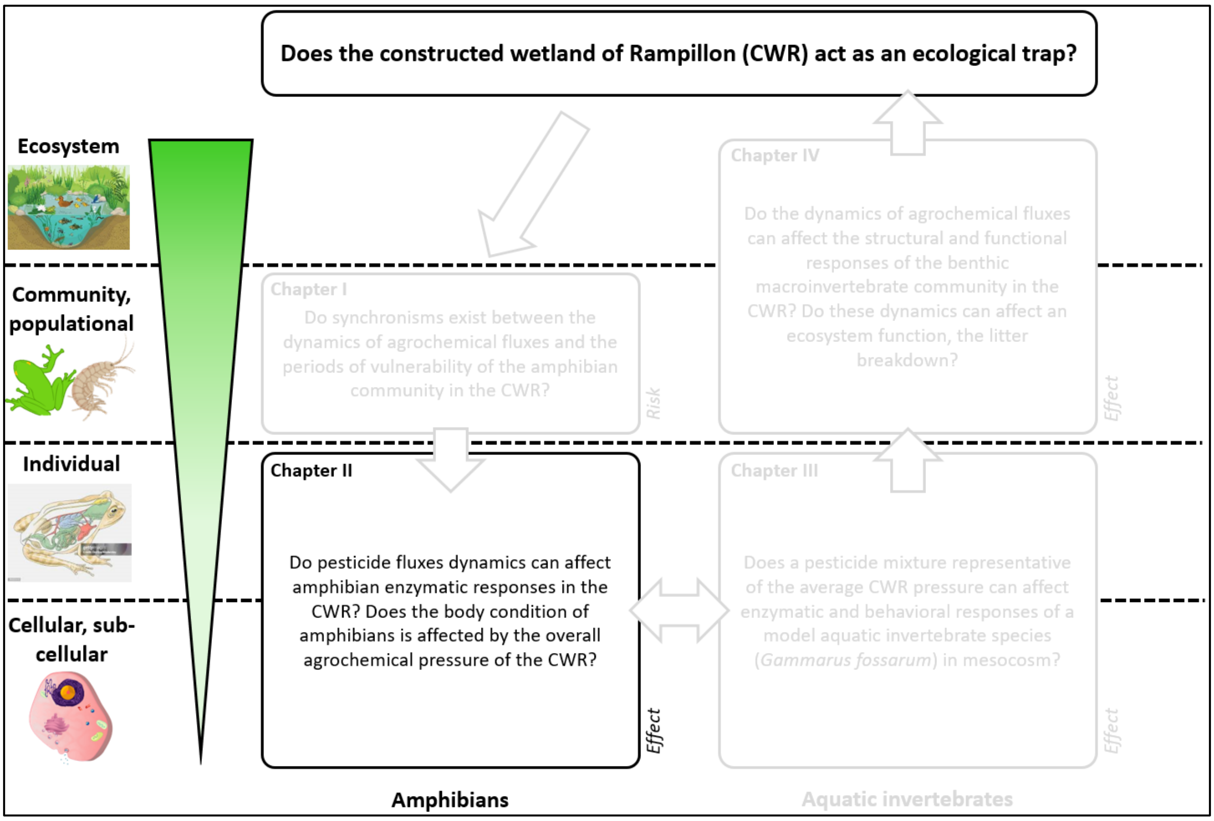
Epilog of Chapter I

The study of synchronisms between the dynamics of agrochemical fluxes and the periods of vulnerability of the amphibian community highlighted the potential of the CWR to act as an ecological trap for amphibians, in relation to the risk posed by high agrochemical fluxes in May and June, for the entire amphibian community, corresponding to a period of intense breeding and activity. Although this is a prospective ecological risk study, it does not provide any information on the effects these intense fluxes may have on individuals. The second chapter focuses at the potential effects of these fluxes on certain individual and sub-cellular traits in two native amphibian species, the common toad (*Bufo bufo*) and the green frog (*Pelophylax sp.*).

Chapter II

First *in situ* application of a non-invasive sampling approach to assess pesticide effects on amphibian enzymatic activities

Project of research article (in preparation for Environmental Chemistry and Ecotoxicology)



Chapter II: First *in situ* application of a non-invasive sampling approach to assess pesticide effects on amphibian enzymatic activities

Head of Chapter II

Chapter II, entitled "*First in situ application of a non-invasive sampling approach to assess pesticide effects on amphibian enzymatic activities*", will present the results of ecotoxicological monitoring of the enzymatic and morphological responses of *Bufo bufo* and *Pelophylax sp.* to agrochemical flux dynamics within the CWR. As in the study presented in Chapter I, the study presented in this chapter was also the subject of an inter-site comparison, in an attempt to highlight better pesticide-induced effects on this taxon, with potential vulnerability in the reproductive period as presented in Chapter I. The strength of this study lies in the method used to answer the research question. In collaboration with the French National Museum of Natural History (MNHN), with support from the Fédération Île-de-France de Recherche sur l'Environnement (FIRE), we used buccal swabbing for the two amphibian species studied, a method that has the advantage of being non-invasive and inexpensive. The use of buccal swabbing is common in amphibians for genetic analysis, but the use of buccal swabs directly in the field, for the specific purpose of studying the effects of pesticides on amphibian enzymes, is original. This study is therefore an opportunity to encourage the democratization of this technique in amphibians studied in the field, as a complement to, or partial replacement for, invasive approaches. The research questions of Chapter II are the following: *Do pesticide fluxes dynamics can affect amphibian enzymatic responses in the CWR? Does the body condition of amphibians is affected by the overall agrochemical pressure of the CWR?*

Title

First *in situ* application of a non-invasive sampling approach to assess pesticide effects on amphibian enzymatic activities

(Research article in preparation for *Environmental Chemistry and Ecotoxicology*)

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Abstract

Amphibians are particularly vulnerable to pesticide exposures according to numerous laboratory and field studies. Enzymatic activities in biological tissues and body condition are usually proposed as relevant biomarkers for studying the effects of pesticides on these organisms, especially in agricultural context. Nevertheless, measuring enzymatic activities on animals often requires invasive sampling methods, such as blood sampling or destruction of individuals in the case of juveniles in particular. Limiting the harmful effects of invasive methods and developing non-invasive approaches is crucial to the ethical principles that aim to minimize the impact on individuals. We aimed to test a non-invasive sampling approach, namely buccal swabbing, to investigate the effects of pesticides on the enzymatic activities of two native amphibian species, the common toad (*Bufo bufo*) and the green frog (*Pelophylax sp.*), in six ponds distributed along a pesticide contamination gradient. We also performed morphometric measurements to determine the body condition of the swabbed individuals. Our results show that buccal swabbing effectively allows quantifying the activities of 6 enzymes present in the saliva of wild amphibians and involved in neurological, non-specific immunity, and nutrition processes, supporting the relevance of this approach to assess their enzymatic responses *in situ*. Enzymatic levels of acetylcholinesterase, β-galactosidase, β-glucosidase, of cytotoxic biomarkers, such as glutathione S-transferase, and peroxidases, were either significantly correlated with pesticide concentrations, or responsive to synchronic-antagonistic effects of pesticide fluxes occurring in an agricultural constructed wetland, suggesting that buccal swabbing in amphibians is applicable in the field for this purpose, and that agricultural constructed wetland could have the potential to affect aquatic fauna. Body condition was similar between ponds regardless of the pesticide pressure level, probably because this variable reacts on longer time scales than enzymatic activities, or because of absence of effects, especially as this trait may be driven by other environmental factors. Our study highlights the relevance of using buccal swabbing to study the effects of pesticides on amphibians in the field while having limited impacts on animal welfare.

Keywords

Constructed wetland - Amphibians - Enzyme activity - Pesticides - Body condition - Non-invasive approaches - Ponds

Statements and Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Data availability

The data used in this study can be available upon request to the corresponding author.

Introduction

Amphibians are currently the most threatened vertebrates on Earth with 40.7% threatened species, with a decline mainly due to anthropogenic activities (Collins & Storfer, 2003; IPBES, 2019; Luedtke et al., 2023). In particular, intensive agriculture is one of the strongest factors of amphibian diversity decline, notably due to habitat loss and degradation (Collins & Storfer, 2003; Curado et al., 2011; Luedtke et al., 2023) and to the associated risk of pesticide pollution, which is of global concern (Leenhardt et al., 2022; Tang et al., 2021).

Pesticides are known to have deleterious effects on various biological functions and traits of amphibians under controlled conditions. These include toxic effects on biochemical, cellular, neurological, behavioral, endocrine, reproductive, metabolic, and morpho-anatomical traits (Acquaroni et al., 2022; Denoël et al., 2013; Fenoglio et al., 2009; Karlsson et al., 2021; Shenoy et al., 2009; Venturino et al., 2003; Venturino & Pechen de D'Angelo, 2005). Although effects are more complicated to demonstrate *in natura*, pesticides have been shown to be related with a variety of impairments in wild populations of amphibians monitored directly in the field. These include effects on genetic (Borges, Santos, Benvindo-Souza, et al., 2019; Gonçalves et al., 2019; Lowcock et al., 1997), morphological and anatomical (Borges, Santos, Assis, et al., 2019; Ouellet et al., 1997), immunological (Christin et al., 2013), endocrinological, and reproductive traits (Bókony et al., 2018; McDaniel et al., 2008; Sanchez et al., 2014). However, an accurate understanding of the relationship between pesticide exposures and amphibian responses remains a challenge (Mann et al., 2009), especially because biological responses in the field are multifactorial (Blaustein & Johnson, 2003; Boone & Semlitsch, 2001, 2002; da Rocha et al., 2020).

The ecological vulnerability of amphibians, particularly related to the high permeability of their skin to chemicals, including pesticides (Brühl et al., 2011; Kaufmann & Dohmen, 2016; Van Meter et al., 2015), makes amphibians sentinel organisms for pollution (Hazen et al., 2024). Because of the importance of amphibians to ecosystems through their role in food webs, energy flows, and ecosystem engineering, through physical habitat modification, by bioturbation or grazing notably (Hocking & Babbitt, 2014; Verburg et al., 2007; Whiles et al., 2006), pesticide-driven impairments in amphibians are likely to affect ecosystem functions (Whiles et al., 2006, 2013). Thus, the threat posed by agrochemicals to wild amphibians motivates further field investigations.

In particular, ponds and wetlands, on which most amphibians are extremely dependent for about half of their life cycle, are threatened by agriculture intensification, pollution, climate change, and human population growth (Czech & Parsons, 2022; Finlayson & Spiers, 1999; Heath & Whitehead, 1992; Indermuehle et al., 2008; Oertli et al., 2008; Wood et al., 2003), and the rate and intensity of their decline are alarming (N. C. Davidson, 2014; Fluet-Chouinard et al., 2023). Pesticide pollution of pond water is global (Chaumet et al., 2021; Frank et al., 1990; Miglioranza et al., 2002; Sarrazin et al., 2022; Uddin et al., 2013), and some waterbodies, notably agricultural constructed wetlands, are even designed to intercept agrochemicals (Shutes, 2001; Tournebize et al., 2013, 2017). Within agricultural landscapes, pesticides contaminating ponds and wetlands can therefore be the cause of disruptions to amphibian biological traits. Specifically in agricultural constructed wetlands, whose functioning can be closely linked to agricultural drainage, the potential effects of agrochemicals on amphibians will depend on the dynamics of the chemical fluxes circulating there, and therefore on the temporality of agricultural practices and catchment hydrology. Within the agricultural constructed wetland of Rampillon (Seine-et-Marne, France), for example, a demonstration site for intercepting and reducing diffuse pollution in the context of agricultural drainage, transfers of agrochemicals into the water will be particularly significant from spring to early summer, at the height of the breeding season for several amphibian species.

Enzymatic activities and morphological traits are often used to study the effects of pesticides on amphibians. At the enzymatic level, the effects of pesticides in detoxification processes of amphibians have been documented in controlled conditions (Ezemonye & Tongo, 2010; Ferrari et al., 2011; Güngördü et al., 2016), and suspected in the field (Attademo et al., 2007). However, enzymatic sampling often requires the sacrifice of individuals or the use of invasive methods that can cause stress or pain in the animals. Amphibian enzymes can be indeed extracted from whole organisms (e.g., tadpoles) (Güngördü et al., 2016; Rosenbaum et al., 2012), or from specific organs and tissues, including blood (Attademo et al., 2007; Ezemonye & Tongo, 2010; McDaniel et al., 2008). Nevertheless, promising non-lethal and non-invasive methods, namely the use of swabs, are increasingly being used. In fact, the use of skin swabbing in amphibians is relatively widespread (Barbi et al., 2023; Jiménez et al., 2021; Paetow et al., 2012), and continue to develop, with the recent use of dermal mucus swabs to study urodeles' stress response, for example (Van Meter et al., 2024). In parallel, the use of buccal swabs for enzymatic assays, have been developed recently to study the effects of pesticides in lizards (Chen et al., 2019; Mingo et al., 2016, 2017, 2019). Meanwhile, the use of buccal swabs in amphibians seems to remain confined to genetic studies (Broquet et al., 2007; Spear & Storfer, 2008), buccal swabbing, specifically for enzymatic assays, have also been developed recently in amphibians in controlled conditions (Cattin et al., 2022). However, to our knowledge, this method has never been used to assess the effects of pesticides on the enzymatic activities of amphibians in the field.

Several saliva enzymes involved in various biological functions appear as relevant to study the effect of pesticides on lizards (Chen et al., 2019; Mingo et al., 2016, 2017, 2019) and amphibians (Cattin et al., 2022). The acetylcholinesterase (AChE) is involved in neurotransmission, a biological process targeted by insecticides such as organophosphates or neonicotinoids (Jenkins et al., 2021; Venturino & Pechen de D'Angelo, 2005). The glutathione S-transferase (GST) acts in the second phase of detoxification of reactive oxygen species (ROS) (Sheehan et al., 2001). These ROS are linked to the free radicals present in cells under oxidative stress. Pesticides are known to induce this oxidative stress, and hence toxicity in animals (Abdollahi et al., 2004). Effects of pesticides on AChE and GST in amphibians have been well-studied in various tissues (Bassó et al., 2022; Ezemonye & Tongo, 2010; Güngördü, 2013; Lajmanovich et al., 2010; Rutkoski et al., 2020), but amphibian enzymatic activity assays have been carried out only one time in saliva by buccal swabbing, with no detection of AChE activity (Cattin et al., 2022).

Other enzymes, on which pesticide effects seem to be understudied in amphibians, are potentially relevant to study due to their involvement in various biological functions. These are for instance the β-galactosidase (GAL) and the β-glucosidase (GLU), involved in energy acquisition, the acid phosphatase (PAC), the alkaline phosphatase (PAL), and peroxidases (PEROX), involved in non-specific immunity and considered as cytotoxic markers. More specifically, GAL is a lysosomal hydrolase intervening in the hydrolysis of certain monosaccharides (Cañestro et al., 2001) and is used as an exposure biomarker in gammarids notably (Lebrun et al., 2017, 2020). GLU is an enzyme involved in the digestion of organic matter (Ketudat Cairns & Esen, 2010), whose activity can be inhibited by fungicides (Lebrun et al., 2021). PAC is a hydrolase present in lysosomes (Hourdry, 1974) used as a marker of cell lysis, since it is released in large quantities during the destruction of lysosomal membranes and cell autolysis (Lebrun et al., 2020) whose activity can be increased by insecticides in anurans (Renuka, 2007). PAL is a multifunctional and ubiquitous enzyme in vertebrate tissues (Renuka, 2007). Finally, PEROX are universal enzymes in living organisms, including microorganisms, plants, and animals, that catalyze the oxidation of numerous organic substrates in the presence of H_2O_2 (Hamid & Khalil-ur-Rehman, 2009; Lück, 1965; Pütter, 1974).

Alternatively, in field conditions, monitoring of body condition has been considered as a non-invasive and robust method (Cheron, 2021; Orton et al., 2014) to assess agriculture pressures on amphibians. Morphological abnormalities and low body condition have been more or less successfully, directly linked with pesticide or agriculture pressure (Babini et al., 2016; Borges, Santos, Assis, et al., 2019; Brodeur et al., 2011; Guerra & Aráoz, 2016). Thus, in combination with enzyme responses, these conditions are usually expected to provide complementary insights on amphibian response to pesticides.

The present study aimed to assess the pertinence of buccal swabbing for enzymatic activities measurements and body condition to detect *in situ* effects of pesticides in two autochthonous amphibian species, the common toad *Bufo bufo* (Linnaeus, 1758) (hereinafter "*B. bufo*"), and the green frog *Pelophylax sp.* (Fitzinger, 1843) (hereinafter "*Pelophylax sp.*"), in particular with a view to investigating the potential risk posed by pesticide fluxes dynamics in agricultural constructed wetlands. To do so, we studied six ponds characterized by contrasted pesticide levels, following a contamination gradient, and including the agricultural constructed wetland of Rampillon as an interceptor of pesticides, and monitored the responses of amphibian wild populations via body condition assessment and buccal swabbing for enzymatic analyses. We expected changes in enzymatic levels and in body condition for both amphibian species in the ponds with the highest toxicity levels, reflecting a highest stress in highest exposure conditions. Specifically we expected a negative relationship respectively between AChE, GAL, GLU, and pesticides, and a positive relationship respectively between GST, PAC, PAL, PEROX, and pesticides on the scale of the sampling session, reflecting a short-term response to pesticide exposure. Finally, we expected a negative relationship between body condition and pesticides on the scale of the pond including all sessions, as the temporal scale of response of the body condition, conditioned by growth time, life cycle, and environmental constraints, should be longer than the one of enzymatic responses.

Material and methods

Selection of the ponds and study species

We selected six ponds for the study following two primary criteria: (i) the ponds had to represent a contamination gradient, particularly concerning pesticide exposure, to increase the likelihood of detecting heterogeneous amphibian responses, and (ii) at least one of the studied species had to be potentially present in several ponds. To ensure the first criterion (i), on the base of an *a priori* selection, and on the base of the visualization of the soil occupancy surrounding the ponds, we used cartography software such as Geoportail (https://www.geoportail.gouv.fr/) or Google Earth (https://www.google.com/intl/fr_fr/earth/) to anticipate a potential gradient of agricultural pressure. We also looked for evidence that might indicates that the crops surrounding the ponds were drained, giving clues on the risk for the pond to receive water contaminated with agrochemicals. To ensure the second criterion (ii), building on previous studies, we were aware of the presence of the two species in some of the pre-selected ponds (in the CWR notably), but for the other, we used naturalist data on the French database GeoNat'îdF (https://geonature.arb-idf.fr/) to determine their potential presence in those ponds. When naturalist data were insufficient, we requested information from landowners and, when possible, we prospected ponds to identify the presence of the species.

As a result, six ponds were selected following a supposedly decreasing gradient of agricultural pressure, then confirmed with pesticide analyses (see details in the section “Materials and methods: Pesticide analysis and calculation of the sum of toxic units”): the agricultural constructed wetland of Rampillon and five other ponds (P2, P3, P4, P5, and P6), all located in the French Ile-de-France region. The agricultural constructed wetland of Rampillon (CWR) (Seine-et-Marne, France) has been constructed to mitigate nitrate and pesticide coming from drainage and runoff of agricultural area, while supporting aquatic biodiversity, including amphibians. This CWR has been extensively studied in previous works to assess its potential for reconcile the dual issues of water quality and biodiversity conservation in agricultural landscapes (Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Lebrun et al., 2019; Letournel, Chaumont, et al., 2021; Letournel, Pages, et al., 2021; Mander et al., 2021; Tournebize et al., 2012, 2017). The

CWR has been equipped since 2012 to monitor agrochemical fluxes using three hydrological measurement stations (HMSs) situated upstream and downstream of the pond and at the outlet of the catchment basin, permitting to monitor fluxes of 531 pesticides continuously (composite samples of about 15 days).

The six ponds harbored and allowed us to select the two focal species, namely the highly prevalent *Bufo bufo* (*B. bufo)* and *Pelophylax sp*. Because of the complexity of the *Pelophylax* genus due to its hybridogenetic nature and the difficulty in identifying species based on morphological criteria alone, we refrained from specifying the exact species present in the ponds. Both species are aquatic breeders but differ in their phenology, resulting in varying degrees of exposure to environmental pesticides. In temperate regions, including the studied area, *B. bufo* exhibits “explosive” breeding behavior (Sinsch, 1988), primarily in March, while *Pelophylax sp.* has a prolonged breeding season extending from spring through summer. As a result, we hypothesized that *B. bufo* would experience less pesticide exposure in water compared to *Pelophylax sp.*, which remains in aquatic habitats for longer periods, especially during the peak of agricultural application of agrochemicals, which coincides with high runoff of pesticides into hydrosystems.

Land cover and physical and chemical characteristics of the ponds

To check the proportion of land cover types surrounding each pond (i.e., woodlands, meadows/grasslands, crops, urban/industrial lands) we determined the land cover in a 500-m radius buffer using QGIS 3.24.0-Tisler with the data package Theia OSO Land Cover Map 2021 (Thierion et al., 2022). The ponds studied were different in terms of land cover, and therefore in terms of agricultural pressure, and then potentially different in terms of pesticide contamination levels, as desired (see selection criterion (i), section “Selection of the ponds and study species”, just above) (Fig.1, Table 1).

The CWR is a 5,600-m^2 agricultural pond that collects runoff and drainage water from a 355-hectare agricultural catchment area subject to intensive crop rotation, mainly wheat, corn, and beets (85% of average crop rotation). According to a dataset from a 10-year continuous monitoring of the water quality, the average pesticide concentration of incoming water in the CWR is about 1 µg/L from January to December, with peak concentrations of approximately 10 µg/L observed in May and June following the application of herbicides and fungicides that takes place from April to June. P2 is a 2,300-m^2 pond with a small amount of vegetation and is the outlet for the drainage system of the adjacent crops. P3 is an unused 600-m^2 village washhouse located between a sparsely trafficked road and a woodland. P4 is a 1,000-m^2 pond surrounded by woodland and located next to some crops but fed by rainwater. P5 is a 1,000-m^2 pond. P6 is an 800-m^2 pond located in the "Parc de Sceaux", a great urban park with a "Zero Phyto" policy (i.e., pesticide-free maintenance of outdoor spaces by local authorities, public and private managers) (Fig. 1). P6 was considered a control in this study, given the total absence of pesticides in the water (see the section: "Pesticide analysis and calculation of the sum of toxic units"), although certain limits to the inclusion of this pond in the analysis have to be discussed (i.e., urban location, see the Discussion section).

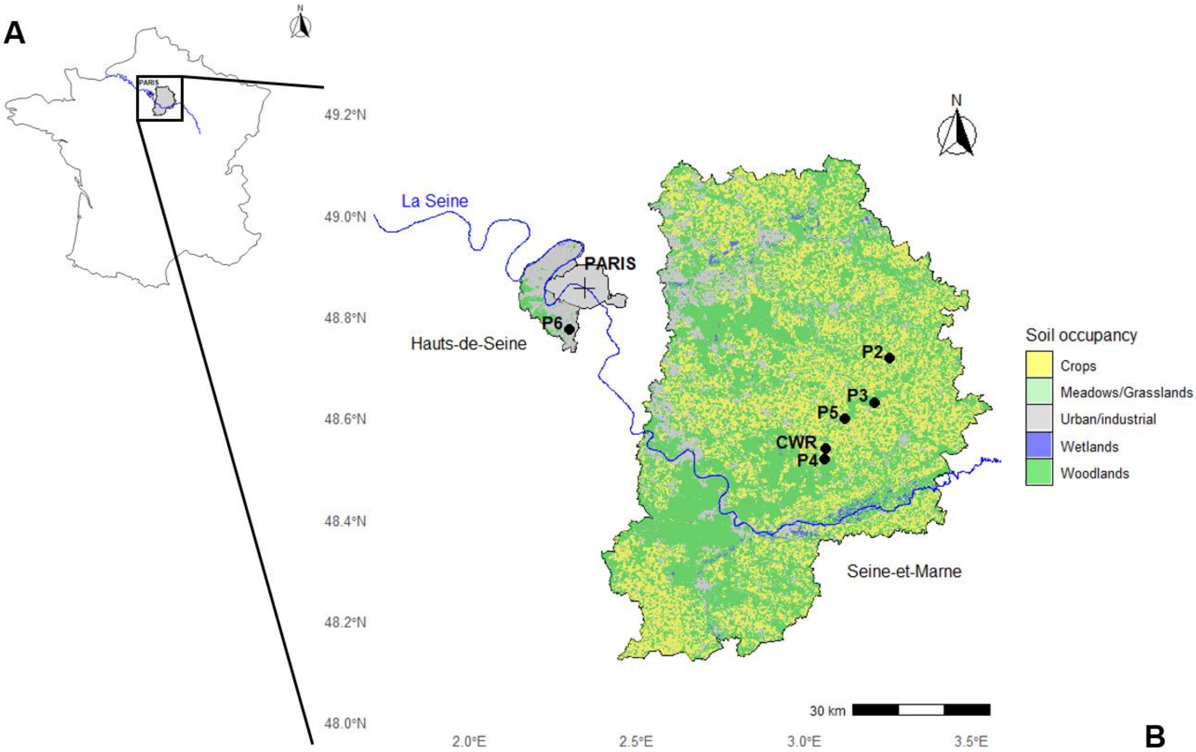


Fig. 1 Map of the study area (A) within France, and (B) location of the 6 ponds within the Ile-de-France region. The map was made thanks to R software (version 4.3.3, R Core Team 2024).

Table 1 Proportions of the different land cover surrounding the studied ponds in a 500-m radius buffer and their surfaces in 2023.

	CWR	P2	P3	P4	P5	P6
Woodlands (%)	7.1	41.6	43.4	56.3	48.7	48.2
Meadows/grasslands (%)	29.5	6.4	47.1	12.7	8.5	14.8
Crops (%)	62.0	52.0	5.3	30.9	42.8	1.8
Urban/industrial (%)	1.0	0.0	4.3	0.0	0.0	32.5
Surface (m^2)	5,600	2,300	600	1,000	1,000	1,100

We sampled water and carried out physical and chemical measurements (temperature, pH, conductivity, O_2 saturation) in all ponds using a multiparameter probe at different times (Table 2).

Table 2 Environmental characteristics of the six studied ponds according to the sampling sessions. Some measurement data are missing for some sessions and ponds (NA) due to technical problems in the field.

Pond	Date	Species	Session	Water temperature (°C)	pH (No unit)	Conductivity (µS/cm)	O_2 saturation (%)	Nitrate (mg/L)
CWR	March 13, 2023	*Bufo bufo*	CWR.A	NA	NA	NA	NA	NA
	May 31, 2023	*Pelophylax sp.*	CWR.A1	24.4	7.6	386.0	132.0	18.3
	June 28, 2023	*Pelophylax sp.*	CWR.A2	22.5	7.9	558.0	196.5	36.0
P2	March 13, 2023	*Bufo bufo*	P2.A	NA	NA	NA	NA	NA
P3	March 13, 2023	*Bufo bufo*	P3.A1	NA	NA	NA	NA	NA
	March 28, 2023	*Bufo bufo*	P3.A2	10.7	8.5	461.0	109.5	11.0
P4	May 31, 2023	*Pelophylax sp.*	P4.A1	16.5	7.3	250.0	NA	23.0
	June 28, 2023	*Pelophylax sp.*	P4.A2	18.9	6.9	189.0	0.0	NA
P5	March 14, 2023	*Bufo bufo*	P5.A	9.0	7.8	299.0	60.0	0.0
P6	March 16, 2023	*Bufo bufo*	P6.A	NA	NA	NA	NA	NA

Pesticide analysis and calculation of the sum of toxic units

Water sampling was realized for each session using 500 mL amber flasks for pesticide analysis at the same time of amphibian swabbing. Flasks were preserved at -20°C before analysis by an independent laboratory (Inovalys). For each aquatic sampling session and each pond, a total of 541 pesticides and their metabolites have been quantified. Overall, 43 molecules were detected and quantified in all water samples (Table 3; see details in Appendix 1). As expected and targeted by the sampling design, ponds were heterogeneous in terms of pesticide contamination and, within the same pond, total pesticide concentrations ($\Sigma[\text{pest}]_{\text{tot.}}$) were the same regardless of the sampling session. The least contaminated pond was P6 (0 µg/L) and the most contaminated pond was P2 (38.51 µg/L) (Table 3, Fig. 2). Figure 2 gives an overview of the total pesticide concentrations in each ponds, for the different sampling sessions in 2023, as well as specifying the mean total pesticide concentration values for the CWR based on the continuous monitoring data for 2023 (Fig. 2).

The total pesticide concentration of a given cocktail does not necessarily reflects its toxicity (Rizzati, 2016), so we also calculated the sum of toxic units (ΣTU). Since toxicity data for amphibians are still limited, and since we worked mainly on the aquatic phase of amphibians, we used ΣTU based on the relative toxicity of pesticides for fish as this group currently appears to be the best surrogate for predicting pesticide toxicity, at least for the aquatic stages of amphibians (EFSA Panel on Plant Protection Products and their Residues (PPR) et al., 2018; Ortiz-Santaliestra et al., 2018) (see Appendix 2). The ΣTU calculation method assumes additive toxic effects between pesticides. Although the behavior of pesticide mixtures may also involve complex interactions (Flores et al., 2014; Weisner et al., 2021), the additive approach is widely accepted and adapted to our purpose (Hela et al., 2005; C. S. Qu et al., 2011; Weber et al., 2018). The calculation was performed for each zone and each session as follows (1):

$$\sum_{i=1}^{n} \mathrm{TU} = \frac{\mathrm{C_i}}{\mathrm{TV_i}} \qquad \text{Sum of toxic units} \qquad (1)$$

where $\sum_{i=1}^{n}$ TU is the summed toxic unit for the pesticides quantified in our samples, for a given pond and session, n is the number of pesticides quantified for the given pond and session, C_i is the concentration of the pesticide i, TV is a toxicity value for fish (depending on the availability of the data and type of toxicity considered: Lethal concentration 50, LC_{50} / Effective concentration EC_{50}, or No Observed Effect Concentration / Lowest Observed Effect Concentration, NOEC/LOEC) of the pesticide i. Corresponding levels of ecological risk are as follows: ΣTU = 0.01 low risk, ΣTU = 0.1 medium risk, ΣTU = 1 high risk, and ΣTU > 1 = very high risk (Hela et al., 2005; C. S. Qu et al., 2011). Appendix 2 summarizes fish toxicity values of pesticides quantified in our samples.

However, since a certain level of uncertainty surrounded the use of ΣTU in our case, linked to the absence of certain toxicity data, to the complexity of the study framework, and linked to the uncertainty, all the same, of using fish toxicity data as surrogate for amphibians, we have focused our analyses on the use of total pesticide concentrations ($\Sigma[\text{pest}]_{\text{tot.}}$). Results based on the ΣTU approach will be presented in the Appendix section.

The different ponds and sessions were ordered according to a decreasing gradient of pesticide contamination level by comparing the mean of their $\Sigma[\text{pest}]_{\text{tot.}}$ (Table 3). In decreasing order of $\Sigma[\text{pest}]_{\text{tot.}}$, the pond-sessions were thus ordered as follows: P2.A > CWR.A > P3.A2 > P3.A1 > P5.A > P6.A for *B. bufo*, and CWR.A2 > CWR.A1 > P4.A2 > P4.A1 for *Pelophylax sp*.

Table 3 Pesticide concentrations (µg/L) with concentrations of the main families of pesticides including metabolites in the studied ponds for each sampling sessions, corresponding ΣTU (no unit). 43 molecules detected on 541 molecules quantified.

Pond	Date	Species	Session	[ ] Total pesticides	[ ] Herbicides	[ ] Fongicides	[ ] Insecticides	[ ] Molluscicides	[ ] Metabolites	ΣTU
CWR	March 13, 2023	*Bufo bufo*	CWR.A	1.95	0.07	0.06	0.00	0.00	1.81	0.00008
	May 31, 2023	*Pelophylax sp.*	CWR.A1	2.11	0.14	0.03	0.00	0.00	1.94	0.00010
	June 28, 2023	*Pelophylax sp.*	CWR.A2	3.30	0.31	0.05	0.00	0.06	2.88	0.00017
P2	March 13, 2023	*Bufo bufo*	P2.A	38.51	1.08	0.05	0.17	0.02	37.19	0.00078
P3	March 13, 2023	*Bufo bufo*	P3.A1	1.62	0.03	0.09	0.05	0.00	1.45	0.00013
	March 28, 2023	*Bufo bufo*	P3.A2	1.74	0.68	0.04	0.03	0.00	1.00	0.00086
P4	May 31, 2023	*Pelophylax sp.*	P4.A1	0.45	0.12	0.02	0.00	0.03	0.29	0.00011
	June 28, 2023	*Pelophylax sp.*	P4.A2	0.47	0.20	0.02	0.00	0.00	0.25	0.00024
P5	March 14, 2023	*Bufo bufo*	P5.A	0.16	0.10	0.01	0.00	0.05	0.01	0.00009
P6	March 16, 2023	*Bufo bufo*	P6.A	--	--	--	--	--	--	--

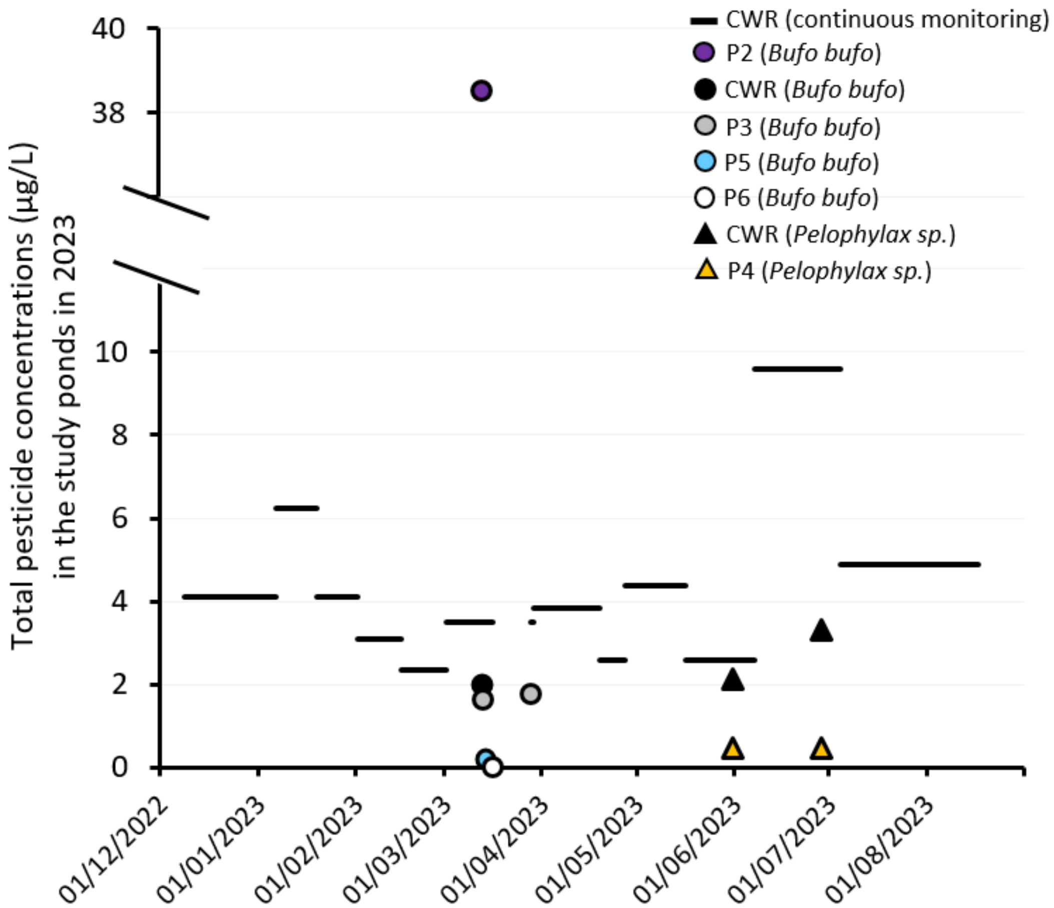


Fig. 2 Total pesticide concentrations (µg/L) in the study ponds in 2023 for each sampling session (CWR, P2, P3, P4, P5, P6), and in the CWR based on the continuous monitoring (2023). Horizontal lines correspond to the mean total pesticide concentrations in the CWR, based on the continuous monitoring (monitoring of 531 pesticides). Circles correspond to the single values of total pesticide concentration measured for each *Bufo bufo* sampling session (punctual sampling). Triangles correspond to the single values of total pesticide concentration measured for each *Pelophylax sp.* sampling session (punctual sampling).

Amphibian sampling: capture, buccal swabbing, morphometric measurements, and ethics and regulatory statement

Sampling sessions took place from March 9-28, 2023, and from May 31-June 28, 2023 and were nocturnal to maximize adult amphibian detectability. *B. bufo* was effectively present in the CWR, P2, P3, P5, and P6, and *Pelophylax sp.* was effectively present in the CWR and P4 (Appendix 3). For *B. bufo*, whose reproduction period, and therefore presence in the water, is relatively short, we paid close attention to the daily evolution of air temperature values in the region studied, since it is a determining factor in the timing of *B. bufo* breeding activity (Reading, 1998), and to certain naturalist references, so as not to miss its sampling. To try and get a reference baseline of enzymatic activities under potentially no or low exposure compared to aquatic phase (although the exposure of amphibians to pesticides in the terrestrial environment should not be overlooked; see Brühl et al. (2013); Leeb et al. (2020)), we sampled *B. bufo* during its terrestrial phase, enabled by the explosive nature of its reproductive migration. In total, we sampled 40 adults *B. bufo* in terrestrial phase, 107 in aquatic phase, and 57 adults *Pelophylax sp.* in aquatic phase (Appendix 3).

B. bufo and *Pelophylax sp.* were captured either by hand or with a net and placed in 16-L plastic buckets filled with a bottom of water and some pieces of vegetation. To account for potential sex differences in physiological variations, we sampled only males when possible (except for the pond P4, June 28 session, where we could capture almost exclusively females for *Pelophylax sp.* (6 out of 8 individuals)). We systematically wore protection gloves and used Virkon to sanitize equipment to limit diseases transmission risk (e.g., chytridiomycosis). Saliva sampling was done by using sterile plastic swabs with a viscose tip (COPAN 155C) and by rotating the swab head for a few seconds on the inside of the cheeks, the inside of the mandible and maxilla, and under the tongue (Fig. 3). Swabs, enclosed in their protection tube, were put directly on ice in a cool box. They were preserved at - 80°C the next day, after our return to the laboratory. We also carried out morphological measurements on each individual including their total length, i.e., from snout to cloaca, with a caliper and their weight with a digital scale, directly in the field, before releasing them at closest to where they had been captured.

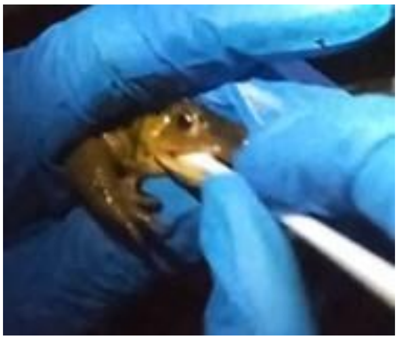


Fig. 3 Buccal swabbing of a common toad during the 2023 study.

Handlings of amphibians were declared to the Regional and Interdepartmental Directorate for the Environment, Planning and Transport of Île-de-France (Direction Régionale et Interdépartementale de l'Environnement, de l'Aménagement et des Transports d'Île-de-France, DRIEAT) and authorized by prefectoral decree (Reference: N°319). If necessary, the permission to enter private areas was given by the landowners.

Enzymatic assays

All the laboratory work performed in this study, i.e., protocols for sample preparation and analysis of amphibian enzyme activities, were based on existing works. For AChE and GST analyses, we relied on original studies (Ellman et al., 1961; Habig et al., 1974) and on studies focusing on the lizards *Podarcis muralis* (Laurenti, 1768) or *Eremias argus* (Peters, 1869) (Chen et al., 2019; Mingo et al., 2016, 2017, 2019). To explore the relevance of other potential exposure biomarkers, we also assessed enzymatic levels of GAL, GLU, PAC, PAL, and PEROX, by adapted enzymatic assays from Lebrun *et al.* (2017; 2020) on *Gammarus fossarum* (Koch, 1836).

Saliva from each swab was extracted in 500 µL of a lysis buffer consisting of 25 mM Tris-HCl solution and 0,1 % Triton X-100. Samples on the ice were homogenized (5 min, 1000 rpm, Shaker SI-100) and centrifuged (14,000 rcf, 4°C, 10 min). To inactivate enzymes, 180 µL of the supernatant from each sample was boiled for 5 minutes. This served as an optical control to ensure that the measurements reflected enzymatic activity rather than an abiotic reaction. The left 320 µL was used for enzymatic assays and protein quantitative analysis, via the Bradford method (Bradford, 1976), using bovine serum albumin for the calibration curve. All enzymatic assays were performed in microplates and monitored by spectrophotometry using a microplate reader (Berthold Technologies, Germany).

AChE activity was measured in a reaction medium that consisted of 180 µL citrate - potassium phosphate buffer, CPB (100 mM, pH 7,5), 5.5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (1 mM), 20 µL acetylthiocholine (76 mM) and 20 µL sample. The complexation of thiocholine with DTNB, revealed by a yellow coloration, was monitored at 405 nm, every minute for 10 min. GST activity was measured in a reaction medium that consisted of 150 µL CPB (100 mM, pH 6,6) and 0,1 % Triton X-100, 20 µL 1-chloro-2,4-dinitrobenzene (CDNB, 20 mM), 20 µL reduced glutathione (GSH) (200 mM), 20 µl sample. The conjugation of reduced glutathione to CDNB through the thiol group of the glutathione was monitored at 355 nm, every minute for 10 min. Hydrolases GAL, GLU, PAC, and PAL activity was measured in a reaction medium that consisted of 200 µL CPB (50 mM, pH 5) for GAL, GLU, and PAC, and 200 µL disodium phosphate (Na_2HPO_4) (pH 9,2) for PAL, and 25 mM of their respective substrates of conjugated p-nitrophenyl, 20 µL sample for GLU, PAC, PAL, and 30 µL sample for GAL. The reaction was stopped by adding 100 µL sodium hydroxide (NaOH) (1M) after 45 and 60 min of incubation for GAL, PAC, and PAL, and after 30 min for GLU. The liberation of p-nitrophenol (PNP) by enzymatic hydrolysis of the substrate was determined at 405 nm just after the end of incubation. PEROX activity was measured in a reaction medium that consisted of 200 µL CPB (50 mM, pH 5), 2,2′azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid (10 mM), and H_2O_2 (170 µl of 30% solution in 50 ml of water), 20 µL sample. Optical density was monitored kinetically at 405 nm, for 15 min, just after the extracts were submitted (readings at 1, 2, 4, 6, 8, 10, 12, and

15 min). Enzymatic activity was expressed in µmol/min/g of protein, equivalent to U/g of protein, with U corresponding to the quantity of enzyme needed to process one micromole of substrate in one minute, applying a molar extinction coefficient respectively of 0.0136 $\mu M^{-1}.cm^{-1}$ for AChE, and 0.0096 $\mu M^{-1}.cm^{-1}$ for GST. To determine the respective enzymatic activity of GAL, GLU, PAC, and PAL, we used a calibration curve of PNP.

Statistical analyses of the effects of pesticides on enzymatic activities

We investigated the statistical link between the enzymatic biomarkers and pesticide pressure expressed as total pesticide concentrations ($\Sigma[pest]_{tot.}$). To assess the statistical links between pesticides and the enzymatic activities, we used two complementary approaches. Firstly, we grouped the enzymatic activity data by pond (CWR, P2, P3, P4, P5, P6), session (i.e., date or 1st / 2nd aquatic session for a given pond), phase (terrestrial or aquatic) and species (*B. bufo* or *Pelophylax sp.*), and represented the distribution of each enzymatic activity using boxplots, according to the pond-phase-session combinations, and to the species considered. We performed Wilcoxon pairwise comparisons between each pond-phase-session combination pairs, for each species respectively, using R software (version 4.3.3, R Core Team 2024, package {stats}). This first approach is well suited to the data, as it allows direct comparison of pond-phase-session combinations without *a priori*. However, this basic approach made it more difficult to demonstrate a general link between enzyme activities and pesticide pressure levels.

Therefore, secondly, we further investigated the overall relation between the response variables (activity of AChE, GAL, GLU, GST, PAC, and PEROX, and protein quantity PROT), and the total pesticide concentrations. As we expected non-linear responses of some enzymatic activities to pesticide pressures, mainly due to hormesis (Erofeeva, 2022; Oliveira et al., 2018), we tested these links using generalized additive models (GAMs) (package {mgcv}) with a number of knots “k” limited to 3 to avoid over-parameterizing the models while allowing the detection of potential hump-shaped relationships.

First, to assess the general, overall effect of pesticides on enzymatic responses, we built a first group of GAMs as follows: Y ~ X, where Y was the response variable, and X was the total pesticide concentrations ($\Sigma[pest]_{tot.}$), for each species. Second, to discriminate certain family-specific pesticide effects (herbicides, fungicides, insecticides, molluscicides and metabolites), we separated as explanatory variables concentrations of herbicides, fungicides, insecticides, molluscicides, and metabolites. To limit collinearity issues between these pesticide variables, we performed 2 principal component analyses (PCA) (package {ade4}) on 2 datasets: (i) *B.*

bufo – Pesticide-family specific concentrations, and (ii) *Pelophylax sp.* – Pesticide-family specific concentrations. Each of the 2 PCA was therefore relative to the specific pesticide contamination data of the sampling sessions for each species. From each PCA, we extracted the coordinates of the pond-sessions along the first two principal axes that reflected different gradients of pesticide pressure. We used those values as predictors in the GAM model testing the effect of pesticide pressure on enzymatic activities. Thus, the second group of GAM was built as follows: Y ~ X1 + X2, where Y was the response variable, X1 corresponded to the first PCA gradient of pesticide pressure, and X2 corresponded to the second PCA gradient of pesticide pressure (see section "Results"). The shape of the relationships between the response variables and the pesticide variables were determined by graphically visualizing the GAM outputs of the significant relationships via the function *plot.gam* of the R package {mgcv}. We carried out the same work with the ΣTU approach (Results based on the ΣTU approach will be presented in the Appendix section).

Body condition calculation and statistical analyses

The body condition of each individual (BC_i) was determined using the method of Peig & Green (2009), for *B. bufo* and *Pelophylax sp.* respectively, as follows (2):

$$BC_i = M_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}} \qquad \text{Body condition} \qquad (2)$$

where L_0 corresponds to the arithmetic mean of the total length of all of the individuals of the study, M_i is the weight of the individual i, L_i is the total length of the individual i and b_{SMA} corresponds to the slope of the regression of the weight M on the length L.

After checking the normality of the data by performing Shapiro–Wilk test (package {stats}) and homoscedasticity of variance by performing Levene test (package {car}), we performed a non-parametric analysis of variance (ANOVA, Kruskal-Wallis test) of the body condition with the pond as an explanatory factor (package {stats}). Unlike the statistical treatment applied to enzymatic activity data, for BC_i we worked on a pond scale, rather than a session scale, given the temporal response scale of this index, that is expected to be of the order of several years compared to enzymatic traits.

Results

Session-specific links between enzymatic activities and pesticide pressure

Buccal swabbing sampling allowed us to detect enzymatic activities in both *B. bufo* and *Pelophylax sp.* effectively (Fig. 4). Only PAL activity was not detected at all for both species. For *B. bufo*, whose $\Sigma[\text{pest}]_{\text{tot.}}$ gradient was relatively strong (from 38.51 to 0 µg/L), some inter-pond variations were highlighted for GAL, GLU, GST, PAC, and PROT (Fig. 4B, C, D, E, G). Whatever the enzyme, activity values for the two *B. bufo* terrestrial phase sampling sessions were similar (Fig. 4), but variations were higher for GAL, GLU, and PEROX in the terrestrial phase compared to the aquatic phase in comparison with other enzymes (Fig. 4B, C, F VS. Fig. 4A, B, D, E, G). For *Pelophylax sp.*, whose $\Sigma[\text{pest}]_{\text{tot.}}$ gradient was less marked (from 3.30 to 0.45 µg/L), most of the responses presented at least one inter-pond significant difference, i.e., AChE, GAL, GLU, PEROX, and PROT (Fig. 4A, B, C, F, G). The mean salivary total protein quantity was twice as high in *Pelophylax sp.* (mean = 0.15 mg) as in *B. bufo* (mean = 0.07 mg) (Fig. 4G). The orders of magnitude of enzymatic activities in *B. bufo* and *Pelophylax sp.* were different for AChE and GST (Fig. 4A, D).

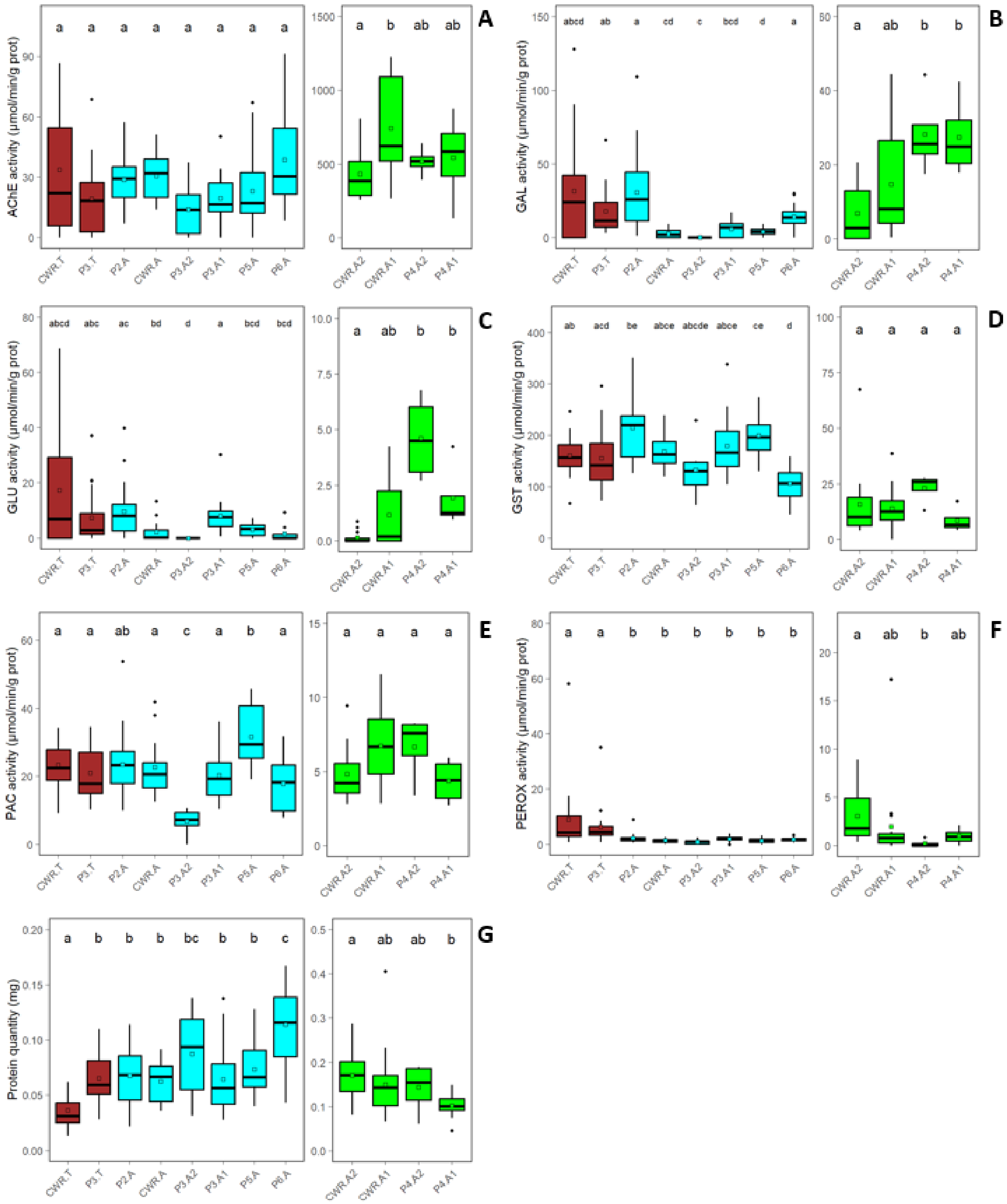


Fig. 4 Salivary enzymatic activities (µmol/min/g of protein) (A, B, C, D, E, F) or protein quantity (mg) (G) for *B. bufo* (brown and blue boxplots, box on left) and *Pelophylax sp.* (green boxplots, box on right). (A) AChE, (B) GAL, (C) GLU, (D) GST, (E) PAC, (F) PEROX. AChE = acetylcholinesterase, GAL = β-galactosidase, GLU = β-glucosidase, GST = glutathione S-transferase, PAC = acid phosphatase, PEROX = peroxidase. Ponds = CWR, P2, P3, P4, P5, P6. Phases: A = aquatic phase, T = terrestrial phase. Pond-phase-session combinations are in descending order according to the $\Sigma[\text{pest}]_{\text{tot.}}$. For *B. bufo*, according to the $\Sigma[\text{pest}]_{\text{tot.}}$ decreasing gradient (no values for terrestrial sessions): CWR.T = CWR terrestrial phase (March 9, 2023), P3.T = P3 terrestrial phase (March 9, 2023), P2.A = P2 aquatic phase (March 13, 2023), CWR.A = CWR aquatic phase (March 13, 2023), P3.A2 = P3 second aquatic phase (March 28, 2023), P3.A1 = P3 first aquatic phase (March 13, 2023), P5.A = P5 aquatic phase (March 14, 2023), P6.A = P6 aquatic phase (March 16, 2023). For *Pelophylax sp.*, according to the $\Sigma[\text{pest}]_{\text{tot.}}$ decreasing gradient: CWR.A2 = CWR second aquatic phase (June 28, 2023), CWR.A1 = CWR

first aquatic phase (May 31, 2023), P4.A2 = P4 second aquatic phase (June 28, 2023), P4.A1 = P4 first aquatic phase (May 31, 2023). Different letters indicate significant differences (pairwise Wilcoxon test, $P < 0.05$).

General relationships between enzymatic activities and pesticide pressure

The information provided by the pesticide variables $\Sigma[\text{pest}]_{\text{tot.}}$ and ΣTU was overall redundant, except for some significant relationships that were specific to an approach or the other (Table 4, Appendix 4-9). In *B. bufo*, GAL, GLU, GST, PEROX, and PROT were influenced by $\Sigma[\text{pest}]_{\text{tot.}}$, whereas in *Pelophylax sp.*, ACHE, GAL, GLU, and PROT were significantly associated with $\Sigma[\text{pest}]_{\text{tot.}}$ (Table 4, Appendix 6 and 8). In particular, for *B. bufo*, GAL, GLU, GST, and PEROX, responded positively to $\Sigma[\text{pest}]_{\text{tot.}}$, while the relationship between other response variables and $\Sigma[\text{pest}]_{\text{tot.}}$ was U-curve shaped. For *Pelophylax sp.*, AChE, GAL, GLU, tended to decrease with $\Sigma[\text{pest}]_{\text{tot.}}$ (Table 4, Appendix 8).

Table 4 GAM results concerning the relationship between response variables of *B. bufo* and *Pelophylax sp* and pesticide concentration variables. Response variables: ache = acetylcholinesterase (AChE), gal = β-galactosidase (GAL), glu = β-glucosidase (GLU), gst = glutathione S-transferase (GST), pac = acid phosphatase (PAC), perox = peroxidase (PEROX), prot = protein quantity (PROT). Explanatory variables: for (i) the first model group (i.e., global pesticide effects) = Total pesticides [ ] = $\Sigma[\text{pest}]_{\text{tot.}}$ = Total pesticides concentrations, for (ii) the second model group (i.e., family-specific pesticide effects) – *B. bufo* = ax1env_herb_inse_meta (variables contributing most to dimension 1 of the PCA: herbicides, insecticides, metabolites) and ax2env_fung_moll (variables contributing most to dimension 2 of the PCA: fungicides, molluscicides), for (ii) the second model group (i.e., family-specific pesticide effects) – *Pelophylax sp.* = ax1env_herb_fung _meta_moll (variables contributing most to dimension 1 of the PCA: herbicides, fungicides, metabolites, molluscicides) and ax2env_inse (variables contributing most to dimension 2 of the PCA: insecticides).

Species	Response variable	Explanatory variable	edf	F	relationship	p-value	R-squared
Bufo bufo	ache	**ax1env_herb_inse_meta**	**1.948**	**9.081**	∪	**<0.001**	0.135
		ax2env_fung_moll	1.000	3.155	/	0.079	
		Total pesticides [ ]	1.705	1.299	∪	0.305	0.014
	gal	**ax1env_herb_inse_meta**	**1.942**	**34.575**	∪	**<0.001**	0.386
		ax2env_fung_moll	1.000	0.948	/	0.333	
		Total pesticides [ ]	**1.857**	**34.620**	∪	**<0.001**	0.376
	glu	**ax1env_herb_inse_meta**	**1.407**	**12.671**	/	**<0.001**	0.204
		ax2env_fung_moll	**1.939**	**4.149**	∪	**0.019**	
		Total pesticides [ ]	**1.000**	**15.290**	/	**<0.001**	0.130
	gst	**ax1env_herb_inse_meta**	**1.000**	**50.500**	/	**<0.001**	0.364
		ax2env_fung_moll	**1.972**	**18.660**	∪	**<0.001**	
		Total pesticides [ ]	**1.406**	**8.625**	/	**<0.001**	0.129
	pac	**ax1env_herb_inse_meta**	**1.935**	**9.556**	∪	**<0.001**	0.322
		ax2env_fung_moll	**1.998**	**24.265**	∪	**<0.001**	
		Total pesticides [ ]	1.778	2.055	∪	0.159	0.026
	perox	**ax1env_herb_inse_meta**	**1.655**	**4.530**	∪	**0.036**	0.059
		ax2env_fung_moll	1.000	2.827	/	0.096	
		Total pesticides [ ]	**1.000**	**5.886**	/	**0.017**	0.044
	prot	**ax1env_herb_inse_meta**	**1.000**	**18.550**	\	**<0.001**	0.232
		ax2env_fung_moll	**1.960**	**13.830**	∩	**<0.001**	
		Total pesticides [ ]	**1.940**	**9.367**	∪	**<0.001**	0.148
Pelophylax sp.	ache	ax1env_herb_fung_meta_moll	1.013	0.076	—	0.806	0.178
		ax2env_inse	1.446	1.255	\	0.180	
		Total pesticides [ ]	**1.902**	**5.002**	∩	**0.010**	0.201
	gal	**ax1env_herb_fung_meta_moll**	**1.025**	**8.378**	\	**0.005**	0.279
		ax2env_inse	1.313	0.540	—	0.538	
		Total pesticides [ ]	**1.000**	**18.590**	\	**<0.001**	0.316
	glu	**ax1env_herb_fung_meta_moll**	**1.000**	**35.750**	\	**<0.001**	0.516
		ax2env_inse	**1.000**	**11.660**	/	**0.002**	
		Total pesticides [ ]	**1.000**	**26.240**	\	**<0.001**	0.399
	gst	ax1env_herb_fung_meta_moll	1.020	0.001	—	0.997	0.003
		ax2env_inse	1.114	0.902	/	0.285	
		Total pesticides [ ]	1.000	0.001	—	0.973	-0.027
	pac	ax1env_herb_fung_meta_moll	1.007	0.786	/	0.372	0.129
		ax2env_inse	1.795	2.001	∪	0.147	
		Total pesticides [ ]	1.808	2.252	∩	0.098	0.097
	perox	ax1env_herb_fung_meta_moll	1.000	3.159	/	0.084	0.031
		ax2env_inse	1.000	0.195	\	0.662	
		Total pesticides [ ]	1.000	3.182	/	0.083	0.054
	prot	**ax1env_herb_fung_meta_moll**	**1.020**	**4.465**	/	**0.033**	0.074
		ax2env_inse	1.628	1.413	∪	0.342	
		Total pesticides [ ]	**1.000**	**5.621**	/	**0.021**	0.076

Pesticide-family specific relationships between enzymatic activities and pesticide pressure

The first two axes of the PCA analyses of the pesticide variables allowed to explain from 88.6% up to 91.4% of the total pesticide data variation (Fig. 5). The gradients of pesticide variables revealed by the PCA are as follows: for the dataset (i) "*B. bufo* - Σ[pest]$_{tot.}$", the first gradient corresponded to herbicides - insecticides - metabolites (ax1env_herb_inse_meta), and the second gradient corresponded to fungicides - molluscicides (ax2env_fung_moll), for the dataset (ii) "*Pelophylax sp.* - Σ[pest]$_{tot.}$", the first gradient corresponded to herbicides - fungicides - metabolites - molluscicides (ax1env_herb_fung_meta_moll), and the second gradient corresponded to insecticides (ax2env_inse) (Fig. 5) (for the results of the PCA analyses based on the ΣTU approach, see Appendix 4).

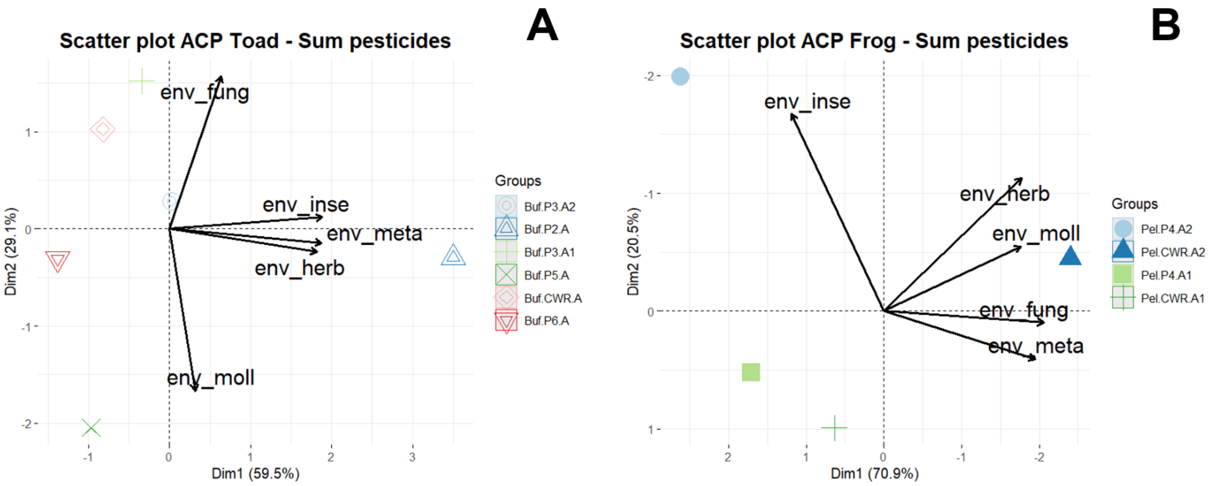


Fig. 5 PCA scatter plots for the datasets (A) *B. bufo* - $\Sigma[\text{pest}]_{\text{tot.}}$, (B) *Pelophylax sp.* - $\Sigma[\text{pest}]_{\text{tot.}}$. Variables: env_herb = herbicides concentration, env_fung = fungicides concentration, env_inse = insecticides concentration, env_moll = molluscicides concentration, env_meta = metabolites concentration. Groups correspond to the pond-phase-session combinations. The contribution in data variability of each dimension is indicated in brackets.

For *B. bufo*, family-specific effects were highlighted for GST and PROT. In particular, we observed a significant positive linear effect of the herbicides - insecticides - metabolites variables group on GST, but a U-shaped effect of the fungicides - molluscicides variable group. These relationships were strictly inversed for PROT, whose relationship with the herbicides - insecticides - metabolites variables group was negative linear, and whose relationship with the fungicides - molluscicides variables group was bell-shaped (Table 4, Appendix 6). For *Pelophylax sp.*, a family-specific effect was identified for GLU, with a linear negative effect of the herbicides - fungicides - metabolites - molluscicides variables group, and a linear positive effect of the insecticides variable (Table 4, Appendix 8).

Link between body condition and pond-level contamination

Body condition of both *B. bufo* and *Pelophylax sp.* was not significantly different among ponds (Fig. 6).

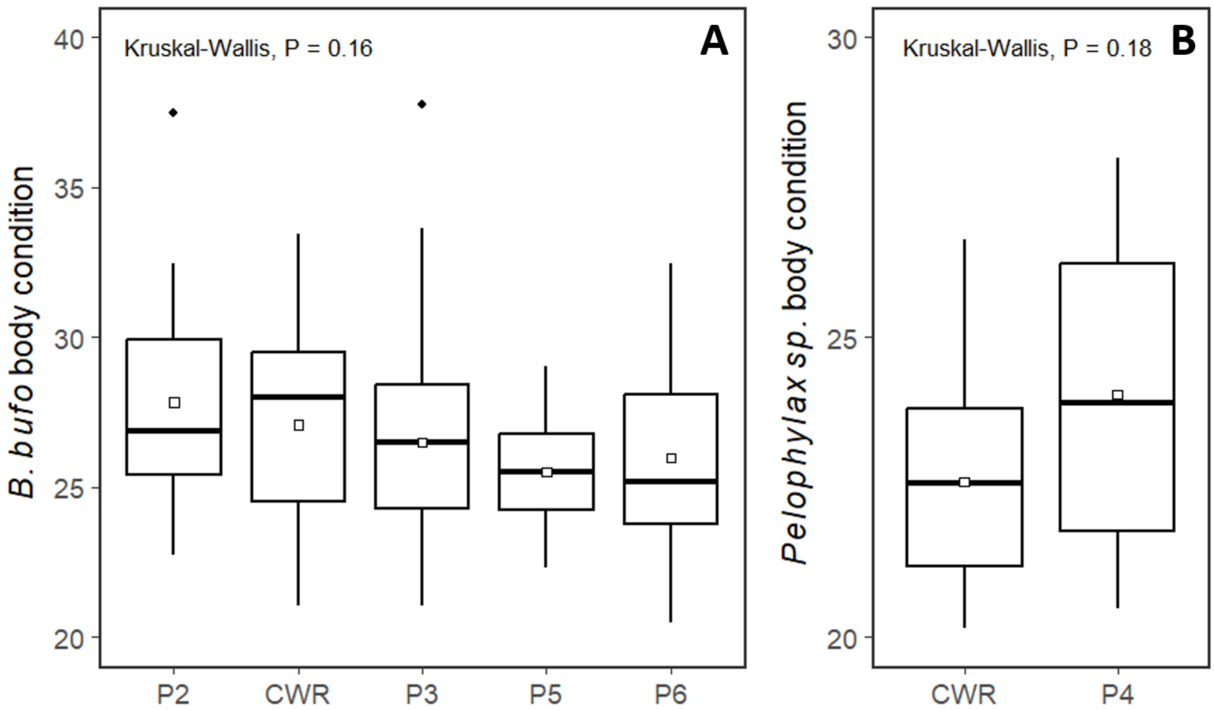


Fig. 6 Body condition of (A) *B. bufo* and (B) *Pelophylax sp*. across ponds (CWR, P2, P3, P4, P5, and P6) sorted in decreasing order of pesticide pressure according to the mean $\Sigma[\text{pest}]_{\text{tot.}}$. Kruskal-Wallis test significance shown on the top of the plot ($P < 0.05$).

Discussion

Relevance of buccal swabbing for measuring amphibian enzymatic activities in the field

First, we successfully detected enzyme activity using buccal swabbing in the field. Although we were unable to measure PAL activity in the saliva of the studied amphibians, likely because this enzyme is cytosolic or membrane-bound and therefore minimally excreted, leading to undetectably low levels, we were able to measure successfully the activity of the other enzymes. To our knowledge, our study is the first to use buccal swabs to study the effect of pesticides on amphibian salivary enzymes *in situ*, and the first to highlight the possibility of measuring the activity of GAL, GLU, PAC, and PEROX in amphibian saliva using buccal swabs. Commonly, the effects of pesticides on amphibian enzymatic traits are studied using various tissues, organs, or even the whole organism (Attademo et al., 2007; Güngördü et al., 2016; Leite et al., 2010). However, the recent work of Cattin et al. (2022) on the use of saliva swabbing to study enzymatic activities in *Xenopus laevis* (Daudin, 1803) under controlled conditions demonstrates the community's growing interest in the use and the development of non-invasive methods to develop biomarkers in amphibians.

Second, the comparison of our results with those obtained in other studies supports the idea that we succeeded to measure realistic enzymatic activities. The units used in the literature vary, but we have made an effort to standardize the discussion here by comparing these different units with the one used in this study (i.e., µmol/min/g protein = U/g). In particular, the levels of GST activity determined for *B. bufo* in our study corresponded to the same orders of magnitude obtained in Cattin et al. (2022) for the saliva of *X. laevis* (i.e., > 100 U/g). In other amphibian tissues, as serum, brain, liver, and lung tissues, or in whole organisms, in different species, values of GST activity range from a few dozen to a few hundred, up to ca. 600 U/g (ca. 600 nmol/min/mg protein in the original publication) (Ezemonye & Tongo, 2010; Gavrilović et al., 2021; Güngördü, 2013; Prokić et al., 2017; Rutkoski et al., 2020). Thus, *B. bufo* GST activity values obtained in our study seemed very realistic, meanwhile for *Pelophylax sp.*, values were quite low (ca. 20 U/g) maybe due to specific physiological reasons, as the inhibition of the GST activity, whether by an intrinsic or extrinsic specific factor.

In parallel, as for GST, at least in *B. bufo*, values measured for AChE activity were consistent. In various tissues and amphibian species, but not in saliva, AChE activity values range from a few units to a few dozen. Similarly, *B. bufo* AChE range activity (ca. 0-90 U/g) matched very well with values measured in various tissues and amphibian species of other studies (Bassó et al., 2022; Cattin et al., 2022; Foguth et al., 2020; Güngördü, 2013; Kroth et al., 2017; Lajmanovich et al., 2010; Rutkoski et al., 2020; Tongo et al., 2012). On the contrary, AChE activity was particularly high for *Pelophylax sp.* (ca. 500 U/g). Indeed, in other vertebrates, as the lizard *E. argus* or in human, acetylcholinesterase activity is higher in the brain (Chen et al., 2019), or in the serum (Omidpanah et al., 2018), compared to saliva where it does not reach such values as those measured in our study.

Although the enzymatic levels of GAL and GLU are undocumented in saliva of amphibians, and, thus, it is more difficult to assess the relevance of enzymatic levels obtained in our study, we achieved to detect and measure the activity of those two enzymes. Moreover, the overall activity values obtained for GLU in both *B. bufo* and *Pelophylax sp.* were close to those obtained in certain fish organs (e.g., *Hypostomus auroguttatus* (Kner, 1854) (Duarte et al., 2013), *Panaque nigrolineatus* (Peters, 1877), and *Hypostomus pyrineusi* (Miranda Ribeiro, 1920) (German & Bittong, 2009)). Although amphibian saliva and mouth glands are not directly involved in digestion (Clayton, 2005; Hedley, 2016), if GAL and GLU activity levels measured in amphibian saliva correlate with those of the digestive system, then these enzymes could be proposed as biomarkers, revealing potential pesticide effects on the feeding function of these organisms.

Finally, we noted differences in salivary protein levels between *B. bufo* and *Pelophylax sp.* that may be attributable to specific differences. For instance, during buccal swabbing, saliva appeared to be more abundant in *Pelophylax sp.*, perhaps due to the timing of the sampling in relation to the phenology of *B. bufo*, emerging from hibernation, or to the lower temperatures compared with those to which *Pelophylax sp.* was exposed (e.g., see Kurabuchi et al. (1995)), or to specific intrinsic differences (e.g., physio-anatomical particularities). However, enzymatic activities were systematically standardized by the protein quantity, so these differences did not interfere with our analyses nor the inter-specific comparisons. We have demonstrated that we could broaden the range of the enzymes studied in amphibians by using buccal swabs (e.g., GAL, GLU, PAC, PEROX), depending on the environmental issues raised.

Effects of pesticides on enzymatic activities and body condition

We found significant relationships between the enzymatic activities studied and the different types of pesticide pressure, highlighting potential effects of pesticide pollution on amphibians' health in retention wetland. In our study, the apparent stimulation of GST and PEROX in *B. bufo*, with potential family-specific pesticide effects highlighted for GST (i.e., in particular herbicides-insecticides-metabolites, and fungicides-molluscicides), was an important finding. These relationships suggest that pesticides may induce a cytotoxic stress, as evidenced by increased activity of the detoxifying enzymes GST and PEROX.

As we only had one measuring point specifically in the CWR for *B. bufo*, we were unable to show any temporal effects of the pesticides dynamics of the CWR on *B. bufo* enzymes, unlike *Pelophylax sp.* In fact, although this was less apparent from spot measurements of pesticides, the continuous monitoring of the water quality, on the other hand, enabled us to show that within the CWR, the difference in average exposure levels, to which *Pelophylax sp.* was exposed between the two sampling sessions, was very marked (i.e., greater exposure level late June, during CWR.A2, versus late May, during CWR.A1) (Fig. 2 and 4). Thus, the differences in enzymatic activities observed between these two sampling sessions could be linked to pesticide effects, possibly resulting in an inhibition of AChE, GAL, GLU, and a stimulation of PEROX in *Pelophylax sp*. Disruption of acetylcholinesterase by pesticides in amphibians can cause ultimately behavioral alterations (Peltzer et al., 2013), deleterious to individual survival. Moreover, the overall dynamics of pesticide fluxes in the CWR in 2023 are not exceptional in terms of intensity, with regard to the long-term water quality monitoring chronicles, and are therefore representative of the average chemical pressure characterizing the CWR, suggesting that such effects are indeed likely to occur under normal circumstances in native amphibians. Thus, in addition to measuring realistic activities, several significant relationships between pesticides and enzymatic activities were found in both species, and a potential temporal effect of the pesticide fluxes in the CWR have been detected in *Pelophylax sp.* This suggests that the use of buccal swabs in amphibians in the field could be implemented in a passive biomonitoring perspective of pesticide effects on amphibian enzymatic traits, notably in agricultural constructed wetlands context, where synchronic-antagonistic effects between agrochemical fluxes and aquatic fauna are likely to occur. Although inter-specific comparison was complicated due to sampling differences, we can suggest *Pelophylax sp.* as a species of choice to monitor effects of pesticide fluxes in the CWR because its phenology (and relatively long part of its life cycle spent within water) implies that it is subject to episodes of acute toxicity in this environment.

A negative link between pesticides and body condition or body size in the wild has been established in lizards (Mingo et al., 2017) and amphibians (Orton et al., 2014). Our study did not allow us to detect this link maybe due to environmental confounding factors, such as landscape and habitat characteristics that are key factors in amphibian body condition (Guillot et al., 2016). For instance, the body condition of *B. bufo* has been shown to be negatively associated with the level of landscape alteration (Janin et al., 2011). Similarly, for *Pelophylax sp.*, body condition has been found to be positively associated with the habitat suitability level (Breka et al., 2023). Given the absence of significant differences in amphibian body condition between the different ponds, despite their heterogeneity, we can deduce highly complex interlinked effects on this trait. A larger scale sampling would allow taking these potential other factors into account and better disentangling the effects of pesticides on body condition. Taking into account the effects of other factors in a larger sampling context would allow us to test the effects of pesticides on body condition in more detail.

Limitations of the study and perspectives

Overall, our study highlights that non-invasive swabbing is a promising method to assess the effects of pesticides on amphibians in the field. Although our results confirm the applicability of non-invasive swabbing to detect enzymatic response to pesticide pressure in amphibians, the interpretability of the results has shown to be complicated by the complexity of ecosystems, especially the number of environmental factors potentially influencing the relationships between pesticides and enzymatic responses in natural conditions. Notably, both abiotic and biotic factors can influence amphibian vulnerability to pesticides (Blaustein & Johnson, 2003; Boone & Semlitsch, 2001, 2002; da Rocha et al., 2020), including global change or infectious diseases (Collins, 2010; C. Davidson, 2004; Mann et al., 2009; Sparling et al., 2001) which makes it difficult to interpret the observations made in the field.

Moreover, in our case, the influence of chronic exposure of amphibians to pesticides, particularly from their earliest life stages, on the measured responses is unclear, as are the potential transgenerational effects that this chronic exposure may induce (Karlsson et al., 2021). Besides, despite our attempt to identify family-specific pesticide effects on the enzymatic responses studied, given the complexity of biological processes in the natural environment and the low statistical replication characterizing our experimental design, interpretation of the family-specific pesticide effects identified in our study remains highly complex and uncertain. In addition, although its nature of control is indisputable in view of the total absence of pesticides in the water, the inclusion of P6 in the ponds studied is questionable in the sense that this pond stands out from the others in terms of location and environment.

To overcome these difficulties in future investigations, we would need more spatio-temporal replications, including chronical data of pesticide exposure levels to assess chronic toxic effects, more balanced data of toxicity gradients for several species, and fine information on other environmental factors, such as water conditions and landscape. Developments are also needed with respect to the improvement of the toxic unit measure, to reduce their uncertainty and to adapt them to amphibians (EFSA, 2018). Further investigations will be needed to deepen the understanding of the relationships between enzymatic activities and pesticide pressure, and the environmental factors that may influence the sensitivity of amphibian biological responses.

Conclusion

We used buccal swabbing and body condition calculation in the common toad (*Bufo bufo*) and the green frog (*Pelophylax sp.*) to assess the impact of pesticides, in the natural environment, on these vulnerable organisms. We showed that buccal swabbing was relevant for quantifying the activity of certain enzymes in wild amphibians. We further highlighted potential effects of pesticides on certain enzymatic activities, including acetylcholinesterase, β-galactosidase, β-glucosidase, glutathione S-transferase, and peroxidases, respectively involved in the neuro-muscular function, energy acquisition, and detoxification processes, but not on body condition potentially due to confounding factors. In particular, we have potentially detected synchronic-antagonistic effects of pesticide fluxes in the agricultural constructed wetland on enzymatic activities in *Pelophylax sp.* Because this study is to our knowledge the first of its kind to test buccal swabbing on amphibians *in situ* in a context of pesticide exposure, it is mainly exploratory and opens avenues for further investigation to study the impacts of pesticides on amphibians more deeply. Nonetheless, we think that this study completes certain missing elements related to the scarcity of fieldwork studies and fits in with the logic of improving knowledge concerning both, the applicability of non-invasive approaches in amphibians, and the potential of agricultural constructed wetlands to affect aquatic fauna. The acquisition of additional data relating to the use of buccal swabbing in the field would allow refining the assessment of its relevance and robustness in the context of the study of the effects of pesticides on amphibians using non-invasive approaches.

Appendix

Appendix 1 The 43 quantified pesticides and their concentration (µg/L) depending on the pond and the session.

Class	Pesticide	CAS	*Bufo bufo*						*Pelophylax sp.*			
			CWR (March 13)	P2 (March 13)	P3 (March 13)	P3 (March 28)	P5 (March 14)	P6 (March 16)	CWR (May 31)	CWR (June 28)	P4 (May 31)	P4 (June 28)
Herbicide	Bentazone	25057-89-0	0.056	--	--	--	--	--	0.094	--	--	--
	Chloridazon	1698-60-8	--	--	--	--	--	--	0.011	0.018	--	--
	Chlorotoluron	15545-48-9	--	0.200	--	--	--	--	--	--	--	--
	Dimethenamid	87674-68-8	--	--	--	--	--	--	--	0.012	--	--
	Ethofumesate	26225-79-6	--	--	--	--	--	--	0.010	--	0.014	0.021
	Mesotrione	104206-82-8	--	--	--	--	--	--	--	0.021	--	--
	Metobromuron	3060-89-7	--	--	--	--	--	--	--	0.024	--	--
	Metolachlor	51218-45-2	--	--	--	--	--	--	0.028	0.170	0.017	0.010
	Nicosulfuron	111991-09-4	--	--	--	--	--	--	--	0.020	--	--
	Propyzamide	23950-58-5	0.017	0.490	0.018	0.017	0.036	--	--	--	0.035	0.017
	Prosulfocarb	52888-80-9	--	0.026	0.011	0.660	0.060	--	--	--	0.050	0.150
	Quinmerac	90717-03-6	--	0.360	--	--	--	--	--	0.043	--	--
Fungicide	2,4-Dinitrophenol	1326-82-5	0.043	0.034	0.042	--	--	--	--	--	--	--
	Azoxystrobin	131860-33-8	--	--	--	--	--	--	0.014	--	--	--
	Cyproconazole	94361-06-5	--	--	--	--	--	--	--	0.011	--	--
	Fluopyram	658066-35-4	--	0.008	0.043	0.035	0.007	--	--	--	0.016	0.016
	Fluxapyroxad	907204-31-3	0.021	0.011	--	--	--	--	0.016	0.034	--	--
Insecticide	Chlorantraniliprole	500008-45-7	--	0.170	0.048	0.028	--	--	--	--	--	--
	Hexachlorocyclohexane	608-73-1	--	--	--	--	--	--	--	--	--	0.001
Metabolite	1-(3-Chloro-4-methylphenyl)-3-methylurea	22175-22-0	--	0.094	--	--	--	--	--	--	0.052	0.035
	2-Hydroxyatrazine	2163-68-0	0.027	0.036	0.028	0.025	--	--	0.036	0.063	0.082	0.047
	4-Methoxy-6-(trifluoromethyl)-1,3,5-triazin-2-amine	5311-05-7	--	0.025	--	--	--	--	--	--	0.027	0.043
	Acetochlor ESA	187022-11-3	--	0.044	--	--	--	--	--	--	--	--
	Alachlor ESA	142363-53-9	0.054	--	--	--	--	--	--	--	--	--
	Alachlor OXA	171262-17-2	--	--	--	--	--	--	--	0.026	--	--
	AMPA	77521-29-0	0.017	--	0.016	--	0.011	--	--	--	0.041	0.072
	Deethylatrazine	6190-65-4	0.034	--	--	--	--	--	0.043	0.030	--	--
	Dimethachlor CGA 369873	1418095-08-5	--	0.620	--	--	--	--	--	--	--	--
	Dimethenamid OXA	380412-59-9	--	0.240	--	--	--	--	--	0.057	--	--
	Dimethenamide ESA	205939-58-8	0.020	0.990	--	--	--	--	--	0.082	--	--
	Flufenacet ESA	201668-32-8	0.042	0.380	0.028	0.024	--	--	--	0.022	0.066	0.024
	Flufenacet OXA	201668-31-7	--	0.200	--	--	--	--	--	--	--	--
	Metazachlor ESA	172960-62-2	0.053	21.000	--	--	--	--	0.026	0.034	--	--
	Metazachlor OXA	1231244-60-2	0.013	7.800	--	--	--	--	--	--	--	--
	Methyldesphenylchloridazon	17254-80-7	0.290	0.160	--	--	--	--	0.470	0.690	--	--
	Metolachlor ESA	171118-09-5	0.610	3.200	0.940	0.500	--	--	0.890	1.100	0.017	0.031
	Metolachlor OXA	152019-73-3	0.120	0.730	0.069	0.049	--	--	0.093	0.340	--	--
	Metolachlor-NOA 413173	1418095-19-8	0.400	1.000	0.340	0.250	--	--	0.320	0.330	--	--
	Prosulfocarb sulfoxide	51954-81-5	--	--	--	0.130	--	--	--	--	--	--
	S-Metolachlor CGA 357704	1217465-10-5	0.047	0.410	0.033	0.017	--	--	0.020	0.052	--	--
	S-Metolachlor CGA 368208	1173021-76-5	0.070	0.240	--	--	--	--	0.037	0.042	--	--
	Terbutylazine-2-hydroxy	66753-07-9	0.016	0.022	--	--	--	--	--	0.011	--	--
Molluscicide	Metaldehyde	9002-91-9	--	0.020	--	--	0.045	--	--	0.063	0.032	--

Appendix 2 The 43 quantified pesticides and corresponding acute and chronic toxicity data for fish (except for the Gekkota *Coleonyx variegatus*).

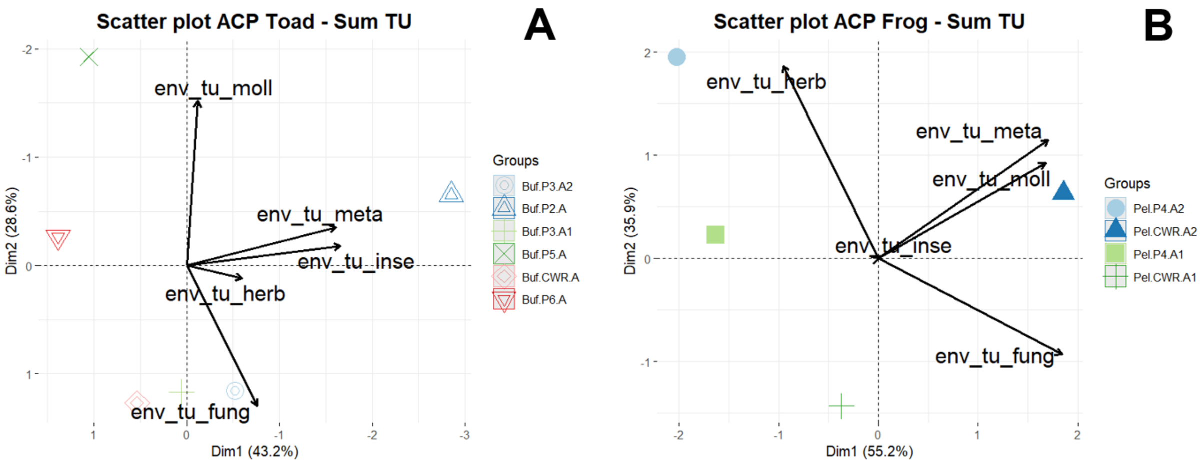
Pesticide	CAS	Class	Toxicity									
			Acute toxicity					Chronic toxicity				
			LC50/EC50	Unit	Model organism	Duration	Reference	NOEC/LOEC	Unit	Model organism	Duration	Reference
Bentazone	25057-89-0	Herbicide	> 100	mg/L	*Oncorhynchus mykiss*	96h	PPDB	48	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Chloridazon	1698-60-8	Herbicide	41.3	mg/L	*Oncorhynchus mykiss*	96h	PPDB	3.16	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Chlorotoluron	15545-48-9	Herbicide	7.7	mg/L	*Oncorhynchus mykiss*	96h	PPDB	0.4	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Dimethenamid	87674-68-8	Herbicide	2.6	mg/L	*Oncorhynchus mykiss*	96h	PPDB	2.5	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Ethofumesate	26225-79-6	Herbicide	10.92	mg/L	*Cyprinus carpio*	96h	PPDB	0.156	mg/L	*Danio rerio*	21d	PPDB
Mesotrione	104206-82-8	Herbicide	> 120	mg/L	*Lepomis macrochirus*	96h	PPDB	12.5	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Metobromuron	3060-89-7	Herbicide	43	mg/L	*Oncorhynchus mykiss*	96h	PPDB	0.25	mg/L	*Pimephales promelas*	21d	PPDB
Metolachlor	51218-45-2	Herbicide	> 3.9	mg/L	*Oncorhynchus mykiss*	96h	PPDB	1	mg/L	*Cyprinodon variegatus*	21d	PPDB
Nicosulfuron	111991-09-4	Herbicide	65.7	mg/L	*Oncorhynchus mykiss*	96h	PPDB	10	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Propyzamide	23950-58-5	Herbicide	> 4.7	mg/L	*Oncorhynchus mykiss*	96h	PPDB	0.94	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Prosulfocarb	52888-80-9	Herbicide	0.84	mg/L	*Oncorhynchus mykiss*	96h	PPDB	0.31	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Quinmerac	90717-03-6	Herbicide	86.8	mg/L	*Oncorhynchus mykiss*	96h	PPDB	3.16	mg/L	*Oncorhynchus mykiss*	21d	PPDB
2,4-Dinitrophenol	51-28-5	Fungicide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Azoxystrobin	131860-33-8	Fungicide	0.47	mg/L	*Oncorhynchus mykiss*	96h	PPDB	0.147	mg/L	*Pimephales promelas*	21d	PPDB
Cyproconazole	94361-06-5	Fungicide	19	mg/L	*Oncorhynchus mykiss*	96h	PPDB	0.65	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Fluopyram	658066-35-4	Fungicide	> 0.98	mg/L	*Coleonyx variegatus*	96h	PPDB	0.135	mg/L	*Pimephales promelas*	21d	PPDB
Fluxapyroxad	907204-31-3	Fungicide	0.466	mg/L	*Pimephales promelas*	96h	PPDB	0.036	mg/L	*Pimephales promelas*	21d	PPDB
Chlorantraniliprole	500008-45-7	Insecticide	> 1.09	mg/L	*Oncorhynchus mykiss*	96h	PPDB	0.11	mg/L	*Oncorhynchus mykiss*	21d	PPDB
Hexachlorocyclohexane	608-73-1	Insecticide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Metaldehyde	9002-91-9	Molluscicide	75	mg/L	*Oncorhynchus mykiss*	96h	PPDB	37.5	mg/L	*Oncorhynchus mykiss*	21d	PPDB
1-(3-Chloro-4-methylphenyl)-3-methylurea	22175-22-0	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2-Hydroxyatrazine	2163-68-0	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methoxy-6-(trifluoromethyl)-1,3,5-triazin-2-amine	5311-05-7	Metabolite	> 1.36	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Acetochlor ESA	187022-11-3	Metabolite	> 180	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Alachlor ESA	142363-53-9	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Alachlor OXA	171262-17-2	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
AMPA	77521-29-0	Metabolite	> 100	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Deethylatrazine	6190-65-4	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Dimethachlor CGA 369873	1418095-08-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Dimethenamid OXA	380412-59-9	Metabolite	> 87	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Dimethenamide ESA	205939-58-8	Metabolite	> 100	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Flufenacet ESA	201668-32-8	Metabolite	> 86.7	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Flufenacet OXA	201668-31-7	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Metazachlor ESA	172960-62-2	Metabolite	93.8	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Metazachlor OXA	1231244-60-2	Metabolite	100	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Methyldesphenylchloridazon	17254-80-7	Metabolite	100	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Metolachlor ESA	171118-09-5	Metabolite	43	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Metolachlor OXA	152019-73-3	Metabolite	> 100	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA
Metolachlor-NOA 413173	1418095-19-8	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Prosulfocarb sulfoxide	51954-81-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S-Metolachlor CGA 357704	1217465-10-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S-Metolachlor CGA 368208	1173021-76-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Terbutylazine-2-hydroxy	66753-07-9	Metabolite	> 2.5	mg/L	*Oncorhynchus mykiss*	96h	PPDB	NA	NA	NA	NA	NA

CAS refers to the CAS Registry Number of the substance considered. LC_{50} = Lethal Concentration 50, EC_{50} = Effective Concentration 50, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration. The model organism refers to the species or the taxon on which the toxicity test was performed and the duration refers to the duration of the toxicity test (h = hour, d = day). For the references: PPDB = Pesticide Properties DataBase website (https://sitem.herts.ac.uk/aeru/ppdb/), INERIS = Substances chimiques INERIS website (https://substances.ineris.fr/).

Appendix 3 Number of individuals sampled per pond, species, phase (A = aquatic, T = terrestrial, 1 = 1st session, 2 = 2nd session), session, during the study.

Pond	Date	Session	*Bufo bufo*		*Pelophylax sp.*	
			Terrestrial	Aquatic	Terrestrial	Aquatic
CWR	March 9, 2023	CWR.T	20	--	--	--
	March 13, 2023	CWR.A	--	20	--	--
	May 31, 2023	CWR.A1	--	--	--	21
	June 28, 2023	CWR.A2	--	--	--	20
P2	March 13, 2023	P2.A	--	20	--	--
P3	March 9, 2023	P3.T	20	--	--	--
	March 13, 2023	P3.A1	--	20	--	--
	March 28, 2023	P3.A2	--	7	--	--
P4	May 31, 2023	P4.A1	--	--	--	8
	June 28, 2023	P4.A2	--	--	--	8
P5	March 14, 2023	P5.A	--	20	--	--
P6	March 16, 2023	P6.A	--	20	--	--
		Total	40	107	--	57

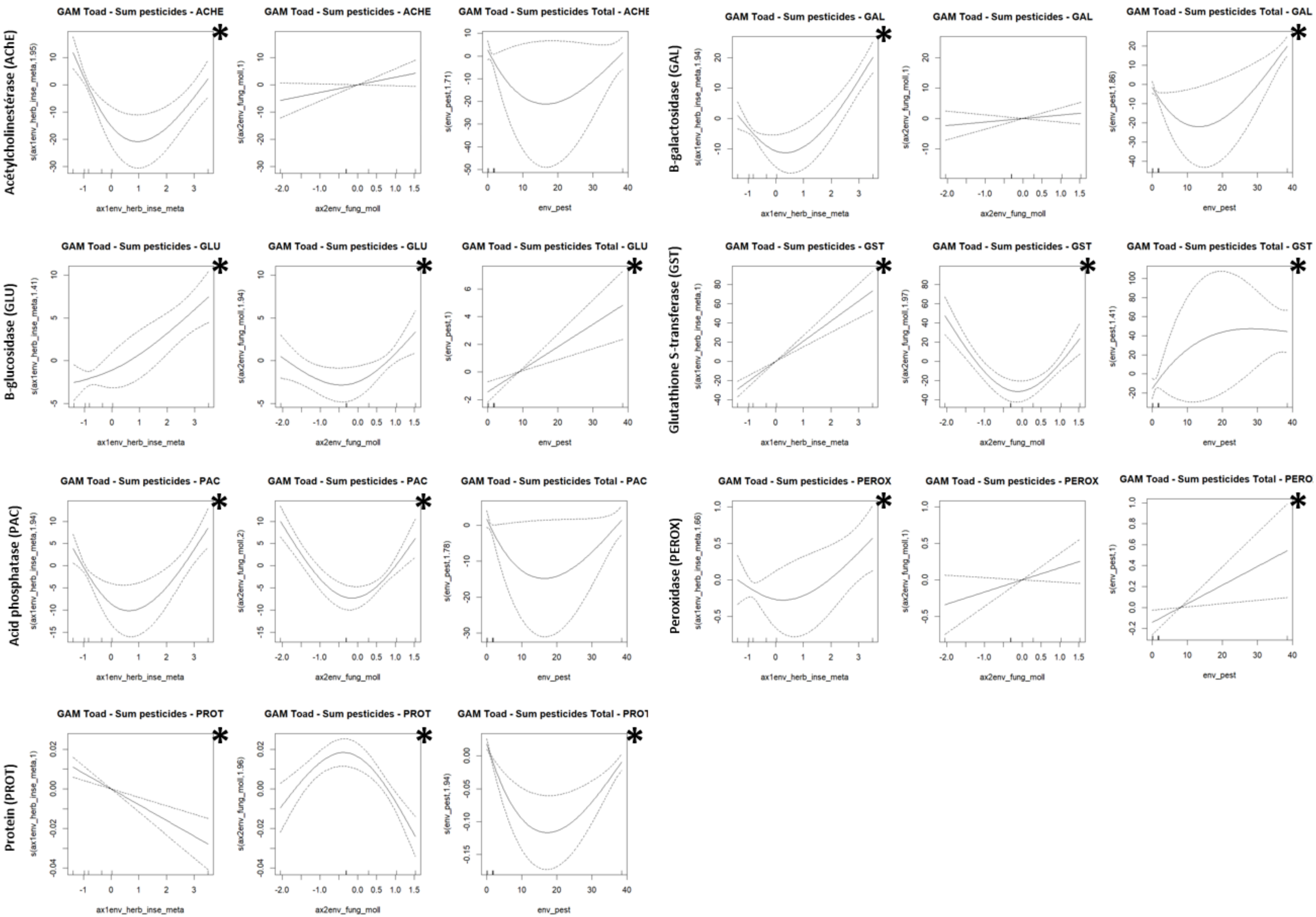
Appendix 4 PCA scatter plots for the datasets (A) *B. bufo* - ΣTU, (B) *Pelophylax sp.* - ΣTU. Variables: env_tu_herb = herbicides ΣTU, env_tu_fung = fungicides ΣTU, env_tu_inse = insecticides ΣTU, env_tu_molluscicides = molluscicides ΣTU, env_tu_meta = metabolites ΣTU. Groups correspond to the pond-phase-session combinations. The contribution in data variability of each dimension is indicated in brackets.



Appendix 5 GAM results concerning the relationship between response variables of *B. bufo* and *Pelophylax sp.* and ΣTU variables. Response variables: ache = acetylcholinesterase (AChE), gal = β-galactosidase (GAL), glu = β-glucosidase (GLU), gst = glutathione S-transferase (GST), pac = acid phosphatase (PAC), perox = peroxidase (PEROX), prot = protein quantity (PROT). Explanatory variables: for (i) the first model group (i.e., global pesticide effects) = Total ΣTU = Total sum of toxic units, for (ii) the second model group (i.e., family-specific pesticide effects) – *B. bufo* = ax1env_herb_inse_meta (variables contributing most to dimension 1 of the PCA: herbicides, insecticides, metabolites) and ax2env_fung_moll (variables contributing most to dimension 2 of the PCA: fungicides, molluscicides), for (ii) the second model group (i.e., family-specific pesticide effects) – *Pelophylax sp.* = ax1env_fung _meta_moll (variables contributing most to dimension 1 of the PCA: fungicides, metabolites, molluscicides) and ax2env_herb (variables contributing most to dimension 2 of the PCA: herbicides).

Species	Response variable	Explanatory variable	edf	F	relationship	p-value	R-squared
Bufo bufo	ache	**ax1env_herb_inse_meta**	**1.946**	**8.950**	∪	**<0.001**	0.135
		ax2env_fung_moll	**1.000**	**6.791**	\	**0.011**	
		Total ΣTU	**1.843**	**3.227**	∪	**0.034**	0.055
	gal	**ax1env_herb_inse_meta**	**1.390**	**12.738**	/	**0.008**	0.397
		ax2env_fung_moll	1.745	4.065	∩	0.060	
		Total ΣTU	**1.000**	**18.510**	/	**<0.001**	0.142
	glu	**ax1env_herb_inse_meta**	**1.000**	**16.562**	/	**<0.001**	0.132
		ax2env_fung_moll	1.000	0.022	—	0.882	
		Total ΣTU	**1.934**	**9.478**	∩	**<0.001**	0.164
	gst	**ax1env_herb_inse_meta**	**1.565**	**23.620**	/	**<0.001**	0.340
		ax2env_fung_moll	**2.000**	**16.700**	∪	**<0.001**	
		Total ΣTU	**1.974**	**23.700**	∩	**<0.001**	0.308
	pac	**ax1env_herb_inse_meta**	**1.908**	**5.389**	∪	**0.007**	0.279
		ax2env_fung_moll	**2.000**	**18.384**	∪	**<0.001**	
		Total ΣTU	**1.912**	**7.221**	∩	**0.002**	0.107
	perox	ax1env_herb_inse_meta	1.000	2.255	/	0.136	0.048
		ax2env_fung_moll	1.722	1.200	∩	0.284	
		Total ΣTU	1.000	0.817	/	0.368	-0.002
	prot	**ax1env_herb_inse_meta**	**1.814**	**13.431**	∩	**<0.001**	0.258
		ax2env_fung_moll	**2.000**	**9.541**	∩	**<0.001**	
		Total ΣTU	**1.967**	**17.310**	∪	**<0.001**	0.244
Pelophylax sp.	ache	ax1env_fung_meta_moll	1.000	2.061	\	0.160	0.189
		ax2env_herb	**1.000**	**7.407**	\	**0.010**	
		Total ΣTU	**1.746**	**5.534**	\	**0.019**	0.181
	gal	**ax1env_fung_meta_moll**	**1.000**	**17.440**	\	**<0.001**	0.292
		ax2env_herb	1.000	1.246	/	0.272	
		Total ΣTU	**1.902**	**4.547**	∪	**0.017**	0.178
	glu	**ax1env_fung_meta_moll**	**1.004**	**13.915**	\	**<0.001**	0.505
		ax2env_herb	**1.698**	**3.103**	/	**0.038**	
		Total ΣTU	**1.968**	**17.710**	∪	**<0.001**	0.479
	gst	ax1env_fung_meta_moll	1.007	0.152	—	0.692	0.003
		ax2env_herb	1.520	0.611	/	0.432	
		Total ΣTU	1.000	2.137	/	0.152	0.029
	pac	ax1env_fung_meta_moll	1.000	0.000	—	0.997	0.129
		ax2env_herb	1.765	2.925	∪	0.088	
		Total ΣTU	1.799	2.492	∪	0.121	0.087
	perox	ax1env_fung_meta_moll	1.000	3.204	/	0.082	0.031
		ax2env_herb	1.000	0.153	\	0.698	
		Total ΣTU	1.618	0.716	∩	0.470	0.016
	prot	**ax1env_fung_meta_moll**	**1.008**	**6.073**	/	**0.016**	0.074
		ax2env_herb	1.608	0.722	∪	0.473	
		Total ΣTU	1.435	0.958	∩	0.507	0.007

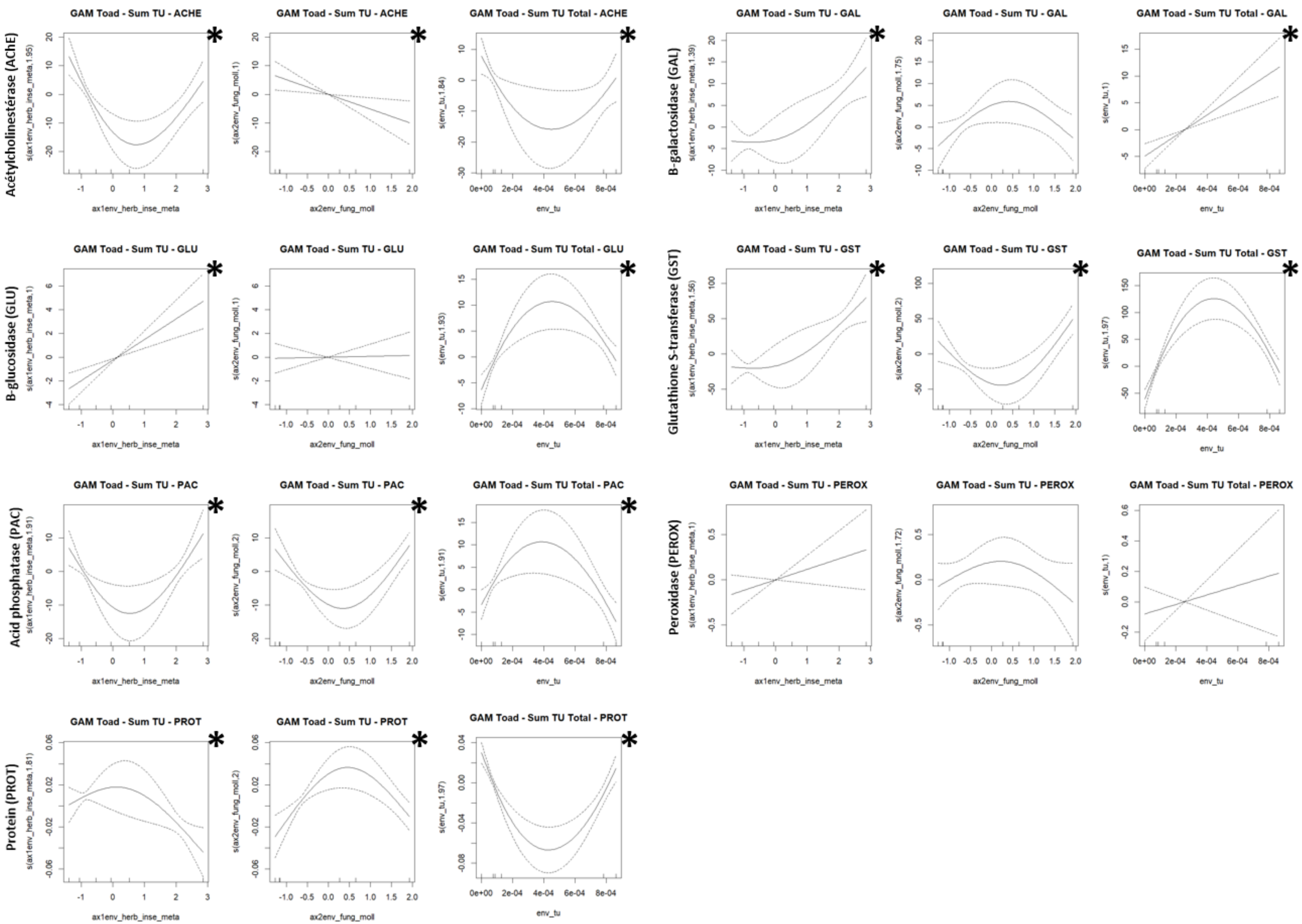
Appendix 6 Generalized additive model (GAM) partial dependence plots for the 56 models for the pesticide variable "Total pesticide concentrations" for *B. bufo*.



Acétylcholinestérase (AChE)
GAM Toad - Sum pesticides - ACHE
GAM Toad - Sum pesticides - ACHE
GAM Toad - Sum pesticides Total - ACHE
B-galactosidase (GAL)
GAM Toad - Sum pesticides - GAL
GAM Toad - Sum pesticides - GAL
GAM Toad - Sum pesticides Total - GAL
B-glucosidase (GLU)
GAM Toad - Sum pesticides - GLU
GAM Toad - Sum pesticides - GLU
GAM Toad - Sum pesticides Total - GLU
Glutathione S-transferase (GST)
GAM Toad - Sum pesticides - GST
GAM Toad - Sum pesticides - GST
GAM Toad - Sum pesticides Total - GST
Acid phosphatase (PAC)
GAM Toad - Sum pesticides - PAC
GAM Toad - Sum pesticides - PAC
GAM Toad - Sum pesticides Total - PAC
Peroxidase (PEROX)
GAM Toad - Sum pesticides - PEROX
GAM Toad - Sum pesticides - PEROX
GAM Toad - Sum pesticides Total - PERO
Protein (PROT)
GAM Toad - Sum pesticides - PROT
GAM Toad - Sum pesticides - PROT
GAM Toad - Sum pesticides Total - PROT
ax1env_herb_inse_meta
ax2env_fung_moll
env_pest

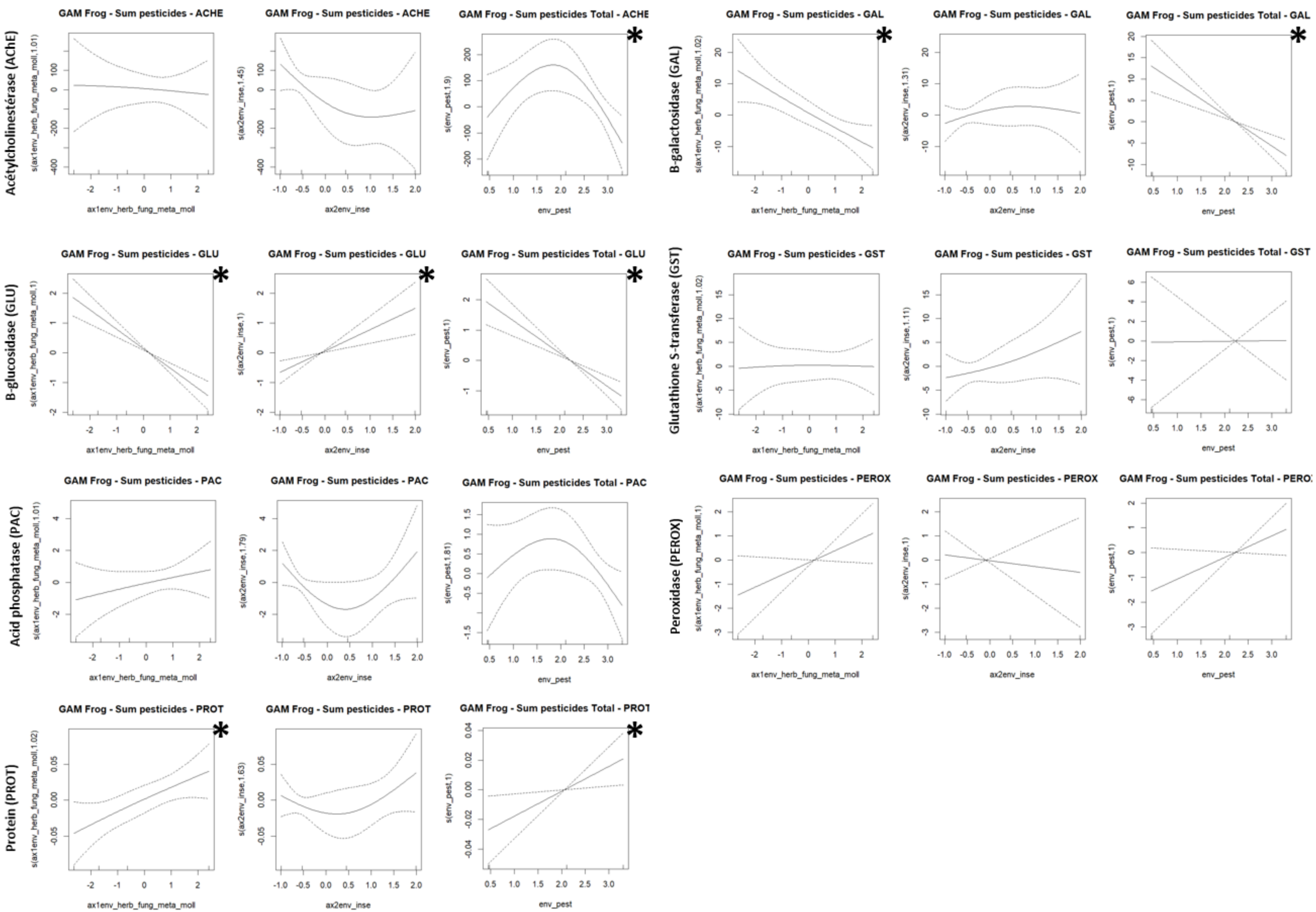
The black line corresponds to the best fit for the relationship between response variables and predictor variables. Upper and lower dashed lines correspond to 95% confidence interval. Each small dash on the inside of the x-axis corresponds to an observation. Two models were run for each response variable, a first model with pesticide data at the family level, then a second model with aggregated pesticide data, i.e., total pesticide concentrations. For each response variable, three plots are shown, from left to right: partial dependence plot for PCA dimension 1, partial dependence plot for PCA dimension 2 (first model), partial dependence plot for total pesticide concentrations (second model). Response variables: AChE = acetylcholinesterase, GAL = β-galactosidase, GLU = β-glucosidase, GST = glutathione S-transferase, PAC = acid phosphatase, PEROX = peroxidase. Explanatory variables: ax1env_herb_inse_meta = variables contributing most to dimension 1 of the PCA: herbicides, insecticides, metabolites, ax2env_fung_moll = variables contributing most to dimension 2 of the PCA: fungicides, molluscicides, env_pest = total pesticides concentrations. The black asterisk indicates whether the relationship was significant ($P < 0.05$).

Appendix 7 Generalized additive model (GAM) partial dependence plots for the 56 models for the pesticide variable "ΣTU" for *B. bufo*.


Acétylcholinestérase (AChE)
GAM Toad - Sum TU - ACHE
s(ax1env_herb_inse_meta,1.95)
ax1env_herb_inse_meta
GAM Toad - Sum TU - ACHE
s(ax2env_fung_moll,1)
ax2env_fung_moll
GAM Toad - Sum TU Total - ACHE
s(env_tu,1.84)
env_tu
B-galactosidase (GAL)
GAM Toad - Sum TU - GAL
s(ax1env_herb_inse_meta,1.39)
ax1env_herb_inse_meta
GAM Toad - Sum TU - GAL
s(ax2env_fung_moll,1.75)
ax2env_fung_moll
GAM Toad - Sum TU Total - GAL
s(env_tu,1)
env_tu
B-glucosidase (GLU)
GAM Toad - Sum TU - GLU
s(ax1env_herb_inse_meta,1)
ax1env_herb_inse_meta
GAM Toad - Sum TU - GLU
s(ax2env_fung_moll,1)
ax2env_fung_moll
GAM Toad - Sum TU Total - GLU
s(env_tu,1.93)
env_tu
Glutathione S-transferase (GST)
GAM Toad - Sum TU - GST
s(ax1env_herb_inse_meta,1.56)
ax1env_herb_inse_meta
GAM Toad - Sum TU - GST
s(ax2env_fung_moll,2)
ax2env_fung_moll
GAM Toad - Sum TU Total - GST
s(env_tu,1.97)
env_tu
Acid phosphatase (PAC)
GAM Toad - Sum TU - PAC
s(ax1env_herb_inse_meta,1.91)
ax1env_herb_inse_meta
GAM Toad - Sum TU - PAC
s(ax2env_fung_moll,2)
ax2env_fung_moll
GAM Toad - Sum TU Total - PAC
s(env_tu,1.91)
env_tu
Peroxidase (PEROX)
GAM Toad - Sum TU - PEROX
s(ax1env_herb_inse_meta,1)
ax1env_herb_inse_meta
GAM Toad - Sum TU - PEROX
s(ax2env_fung_moll,1.72)
ax2env_fung_moll
GAM Toad - Sum TU Total - PEROX
s(env_tu,1)
env_tu
Protein (PROT)
GAM Toad - Sum TU - PROT
s(ax1env_herb_inse_meta,1.81)
ax1env_herb_inse_meta
GAM Toad - Sum TU - PROT
s(ax2env_fung_moll,2)
ax2env_fung_moll
GAM Toad - Sum TU Total - PROT
s(env_tu,1.97)
env_tu

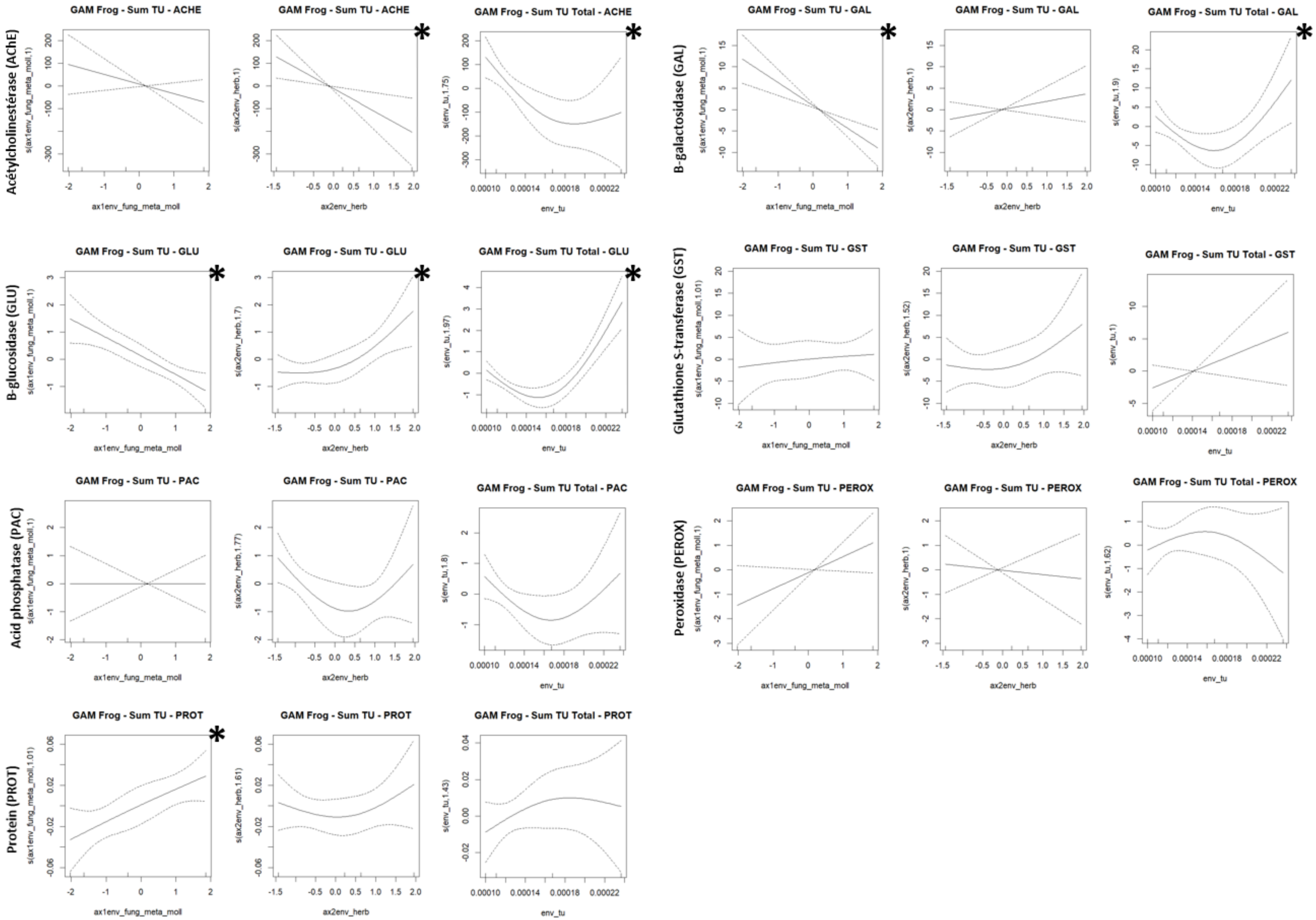
The black line corresponds to the best fit for the relationship between response variables and predictor variables. Upper and lower dashed lines correspond to 95% confidence interval. Each small dash on the inside of the x-axis corresponds to an observation. Two models were run for each response variable, a first model with pesticide data at the family level, then a second model with aggregated pesticide data, i.e., total ΣTU. For each response variable, three plots are shown, from left to right: partial dependence plot for PCA dimension 1, partial dependence plot for PCA dimension 2 (first model), partial dependence plot for total ΣTU (second model). Response variables: AChE = acetylcholinesterase, GAL = β-galactosidase, GLU = β-glucosidase, GST = glutathione S-transferase, PAC = acid phosphatase, PEROX = peroxidase. Explanatory variables: ax1env_herb_inse_meta = variables contributing most to dimension 1 of the PCA: herbicides, insecticides, metabolites, ax2env_fung_moll = variables contributing most to dimension 2 of the PCA: fungicides, molluscicides, env_tu = total ΣTU. The black asterisk indicates whether the relationship was significant ($P < 0.05$).

Appendix 8 Generalized additive model (GAM) partial dependence plots for the 56 models for the pesticide variable “Total pesticide concentrations” for *Pelophylax sp*.


Acétylcholinestérase (AChE)
GAM Frog - Sum pesticides - ACHE
GAM Frog - Sum pesticides - ACHE
GAM Frog - Sum pesticides Total - ACHE
B-galactosidase (GAL)
GAM Frog - Sum pesticides - GAL
GAM Frog - Sum pesticides - GAL
GAM Frog - Sum pesticides Total - GAL
B-glucosidase (GLU)
GAM Frog - Sum pesticides - GLU
GAM Frog - Sum pesticides - GLU
GAM Frog - Sum pesticides Total - GLU
Glutathione S-transferase (GST)
GAM Frog - Sum pesticides - GST
GAM Frog - Sum pesticides - GST
GAM Frog - Sum pesticides Total - GST
Acid phosphatase (PAC)
GAM Frog - Sum pesticides - PAC
GAM Frog - Sum pesticides - PAC
GAM Frog - Sum pesticides Total - PAC
Peroxidase (PEROX)
GAM Frog - Sum pesticides - PEROX
GAM Frog - Sum pesticides - PEROX
GAM Frog - Sum pesticides Total - PERO)
Protein (PROT)
GAM Frog - Sum pesticides - PROT
GAM Frog - Sum pesticides - PROT
GAM Frog - Sum pesticides Total - PROT
ax1env_herb_fung_meta_moll
ax2env_inse
env_pest

The black line corresponds to the best fit for the relationship between response variables and predictor variables. Upper and lower dashed lines correspond to 95% confidence interval. Each small dash on the inside of the x-axis corresponds to an observation. Two models were run for each response variable, a first model with pesticide data at the family level, then a second model with aggregated pesticide data, i.e., total pesticide concentrations. For each response variable, three plots are shown, from left to right: partial dependence plot for PCA dimension 1, partial dependence plot for PCA dimension 2 (first model), partial dependence plot for total pesticide concentrations (second model). Response variables: AChE = acetylcholinesterase, GAL = β-galactosidase, GLU = β-glucosidase, GST = glutathione S-transferase, PAC = acid phosphatase, PEROX = peroxidase. Explanatory variables: ax1env_herb_fung_meta_moll = variables contributing most to dimension 1 of the PCA: herbicides, fungicides, metabolites, molluscicides, ax2env_inse = variables contributing most to dimension 2 of the PCA: insecticides, env_pest = total pesticides concentrations. The black asterisk indicates whether the relationship was significant ($P < 0.05$).

Appendix 9 Generalized additive model (GAM) partial dependence plots for the 56 models for the pesticide variable "ΣTU" for *Pelophylax sp*.


Acétylcholinestérase (AChE)
GAM Frog - Sum TU - ACHE
s(ax1env_fung_meta_moll,1)
ax1env_fung_meta_moll
GAM Frog - Sum TU - ACHE
s(ax2env_herb,1)
ax2env_herb
GAM Frog - Sum TU Total - ACHE
s(env_tu,1.75)
env_tu
B-galactosidase (GAL)
GAM Frog - Sum TU - GAL
s(ax1env_fung_meta_moll,1)
ax1env_fung_meta_moll
GAM Frog - Sum TU - GAL
s(ax2env_herb,1)
ax2env_herb
GAM Frog - Sum TU Total - GAL
s(env_tu,1.9)
env_tu
B-glucosidase (GLU)
GAM Frog - Sum TU - GLU
s(ax1env_fung_meta_moll,1)
ax1env_fung_meta_moll
GAM Frog - Sum TU - GLU
s(ax2env_herb,1.7)
ax2env_herb
GAM Frog - Sum TU Total - GLU
s(env_tu,1.97)
env_tu
Glutathione S-transferase (GST)
GAM Frog - Sum TU - GST
s(ax1env_fung_meta_moll,1.01)
ax1env_fung_meta_moll
GAM Frog - Sum TU - GST
s(ax2env_herb,1.52)
ax2env_herb
GAM Frog - Sum TU Total - GST
s(env_tu,1)
env_tu
Acid phosphatase (PAC)
GAM Frog - Sum TU - PAC
s(ax1env_fung_meta_moll,1)
ax1env_fung_meta_moll
GAM Frog - Sum TU - PAC
s(ax2env_herb,1.77)
ax2env_herb
GAM Frog - Sum TU Total - PAC
s(env_tu,1.8)
env_tu
Peroxidase (PEROX)
GAM Frog - Sum TU - PEROX
s(ax1env_fung_meta_moll,1)
ax1env_fung_meta_moll
GAM Frog - Sum TU - PEROX
s(ax2env_herb,1)
ax2env_herb
GAM Frog - Sum TU Total - PEROX
s(env_tu,1.62)
env_tu
Protein (PROT)
GAM Frog - Sum TU - PROT
s(ax1env_fung_meta_moll,1.01)
ax1env_fung_meta_moll
GAM Frog - Sum TU - PROT
s(ax2env_herb,1.61)
ax2env_herb
GAM Frog - Sum TU Total - PROT
s(env_tu,1.43)
env_tu

The black line corresponds to the best fit for the relationship between response variables and predictor variables. Upper and lower dashed lines correspond to 95% confidence interval. Each small dash on the inside of the x-axis corresponds to an observation. Two models were run for each response variable, a first model with pesticide data at the family level, then a second model with aggregated pesticide data, i.e., total ΣTU. For each response variable, three plots are shown, from left to right: partial dependence plot for PCA dimension 1, partial dependence plot for PCA dimension 2 (first model), partial dependence plot for total ΣTU (second model). Response variables: AChE = acetylcholinesterase, GAL = β-galactosidase, GLU = β-glucosidase, GST = glutathione S-transferase, PAC = acid phosphatase, PEROX = peroxidase. Explanatory variables: ax1env_fung_meta_moll = variables contributing most to dimension 1 of the PCA: fungicides, metabolites, molluscicides, ax2env_herb = variables contributing most to dimension 2 of the PCA: herbicides, env_tu = total ΣTU. The black asterisk indicates whether the relationship was significant ($P < 0.05$).

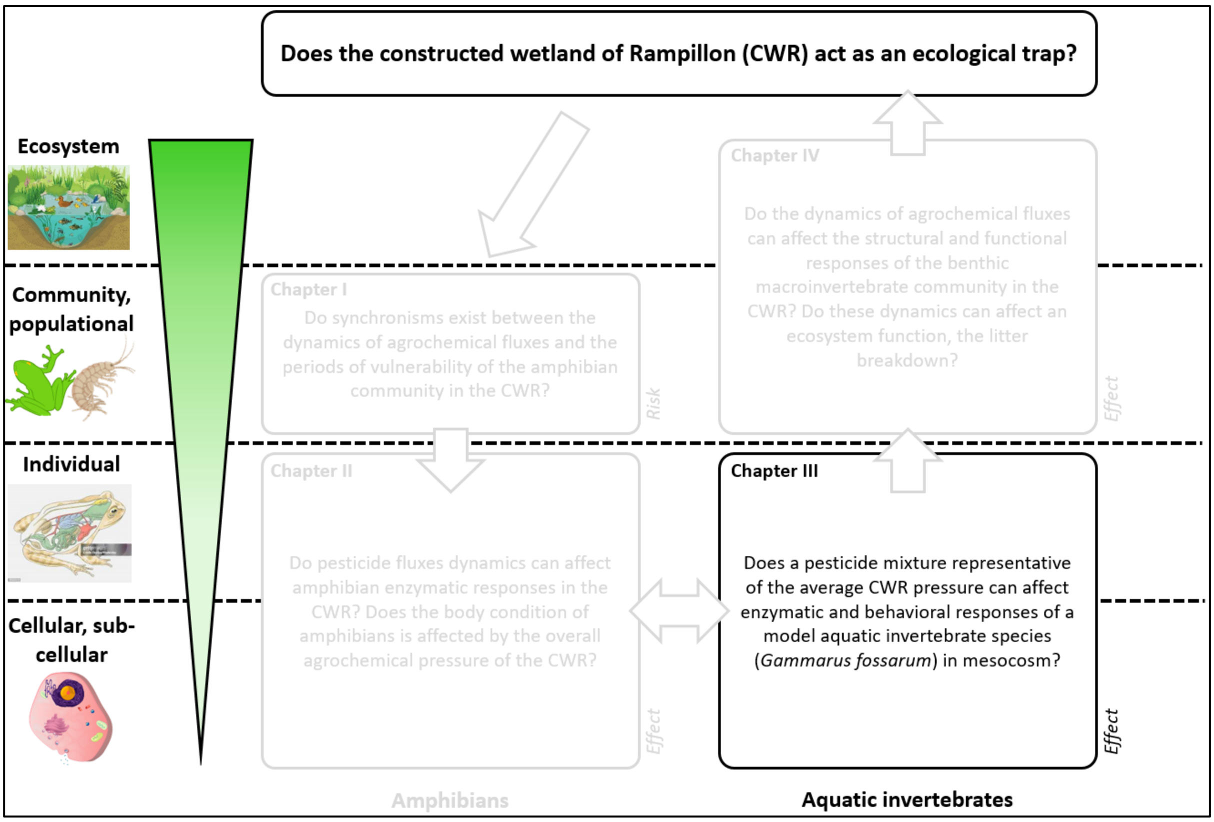
Epilog of Chapter II

In addition to the synchronisms between agrochemicals and amphibians at community level highlighted in Chapter I, Chapter II, on the same level, identified synchronisms coupled with antagonistic effects of pesticides on amphibian enzymatic activities. The results presented in Chapter II underline CWR's ability to act as an ecological trap for amphibians. No effect of pesticides on amphibian body condition could be identified, however, but future, more targeted studies could investigate this issue in more detail. Furthermore, the use of buccal swabbing in wild amphibian populations to study the effects of pesticides on their enzymatic activities seems promising, in the same way as methodologically similar studies on Lacertidae reptiles tended to highlight. In particular, levels of activity of acetylcholinesterase (neurotransmission), glutathione S-transferase (detoxification), and peroxidases (non-specific immunity), appeared to be modified by pesticide exposure. The development of non-invasive approaches, such as this, is an important element in improving the welfare of animals used in research into the adverse effects of pesticides. Although negative effects of pesticides on the enzymatic activities of amphibians were observed, the complexity of the environment and its influence on living organisms remains an important element to take into account, and can have a significant influence on these biological responses. This is what the design of the experiment presented in the next chapter can help to overcome. Chapter III presents the results of an experiment designed to study the effects of pesticides on aquatic invertebrates, this time under semi-controlled conditions in outdoor mesocosms.

Chapter III

Use of behavioral and biochemical biomarkers to assess the effects of a pesticide mixture on *Gammarus fossarum* in mesocosms implanted in an agricultural constructed wetland (Seine-et-Marne, France)

Project of research article (in preparation for Environmental Research)



Chapter III: Use of behavioral and biochemical biomarkers to assess the effects of a pesticide mixture on *Gammarus fossarum* in mesocosms implanted in an agricultural constructed wetland (Seine-et-Marne, France)

Head of Chapter III

As in the case of amphibians, Chapter III, entitled "*Use of behavioral and biochemical biomarkers to assess the effects of a pesticide mixture on* Gammarus fossarum *in mesocosms implanted in an agricultural constructed wetland (Seine-et-Marne, France)*", combines multi-level approaches to identify effects of pesticide on aquatic fauna. This study assessed the effects of a pesticide mixture at sub-cellular (enzyme) and individual (behavioral) levels in a model aquatic invertebrate species, *Gammarus fossarum*, under outdoor mesocosm conditions (*in situ*). The aim of the study is thus to determine the potential effects of a mixture of pesticides, representative of the overall CWR pressure, on aquatic fauna, to precise the role of the CWR as an ecological shelter or trap. The Crustacea Amphipoda genus *Gammarus spp.* is widely used in ecotoxicology studies, as a sentinel species for the quality of freshwater environments. Although we used non-native gammarids in this study, through an active biomonitoring approach (i.e., caging), Gammaridae are well represented in the CWR. The potential effects highlighted under mesocosm conditions could therefore reflect potential mismatches in certain native organisms. The research question of Chapter III is thus the following: *Does a pesticide mixture representative of the average CWR pressure can affect enzymatic and behavioral responses of a model aquatic invertebrate species (*Gammarus fossarum*) in mesocosm?*

Title

Use of behavioral and biochemical biomarkers to assess the effects of a pesticide mixture on *Gammarus fossarum* in mesocosms implanted in an agricultural constructed wetland (Seine-et-Marne, France)

(Research article in preparation for *Environmental Research*)

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Abstract

Pesticides, used in crops to optimize food production, can be transferred to the hydrosphere via runoff and drainage networks. Once in the aquatic environment, pesticides interact with biota and are likely to generate adverse effects on aquatic fauna and associated ecological functions.

Constructed wetlands (CWs), at the interface between the agricultural plot and the aquatic environment, help mitigate pollution induced by pesticides and nitrogenous pressures thanks to their natural purification properties. However, these infrastructures could in turn become significant sources of contaminants, with potential effects on native aquatic fauna. We aimed to assess the potential for an agricultural CW (northern France) to cause such effects on aquatic invertebrates. We exposed an aquatic invertebrate sentinel species, *Gammarus fossarum*, caged in outdoor mesocosms set up directly in the agricultural CW, to a mixture of pesticides representative of the overall chemical pressure circulating in the CW. We carried out several exposure series, each lasting one week, based on a systematic renewal of gammarids. At the end of each exposure week we studied individual responses (survival rate, amplexus rate, locomotor activity, ingestion rate), and biochemical responses of *G. fossarum* (enzymes involved in neurotransmission, energy acquisition, non-specific immunity, detoxification, molting). The results highlight effects of pesticides on the amplexus rate, locomotor activity, enzymatic levels of enzymes involved in neurotransmission, energy acquisition, non-specific immunity, and molting. This suggests that behavioral and sub-cellular disturbances may occur in aquatic fauna of agricultural CWs, with possible population-wide repercussions. The use of sentinel aquatic invertebrate species, such as *Gammarus spp.*, in semi-controlled outdoor mesocosms, may prove relevant for assessing the unintended effects of pesticides circulating in agricultural CWs on aquatic fauna.

Keywords

Constructed wetland - Aquatic invertebrates - Individual performances - Enzyme activity - Pesticides - Outdoor mesocosms - Ponds

Statements and Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data used in this study can be available upon request to the corresponding author.

Introduction

Although the intensification of agriculture plays a major role in the decline of biodiversity worldwide (Attwood et al., 2008; Dudley & Alexander, 2017; J. M. H. Green et al., 2019), agricultural landscapes tend to offer some shelters for aquatic fauna, due to the presence of agricultural water bodies. Animal taxa that appear to benefit from these habitats include amphibians (Knutson et al., 2004), and aquatic invertebrates (Cereghino et al., 2008; Ruggiero et al., 2008), with some ponds exhibiting noticeable species rarity values (Biggs et al., 2007).

However, nowadays, pesticides are used on a massive global scale. They reach the hydrosphere by spraying, flowing or via drainage systems (Meite et al., 2018; Tournebize et al., 2017), and they became ubiquitous in the environment (Leenhardt et al., 2022; Tang et al., 2021), sometimes at concentrations higher than quality standards not to exceed to conserve the integrity of the environment (Starner & Goh, 2012). Pesticides are contaminating ponds worldwide (Chaumet et al., 2021; Frank et al., 1990; Miglioranza et al., 2002; Sarrazin et al., 2022; Uddin et al., 2013), and are likely to generate deleterious effects on aquatic fauna, including aquatic invertebrates assemblages (Ito et al., 2020; Leenhardt et al., 2022; Rohr & Crumrine, 2005; Schepker et al., 2020). In fine, pesticides have the potential for disrupting freshwater ecosystem functions, especially litter breakdown, due to deleterious effects on aquatic invertebrate and ecosystem engineers (Brosed et al., 2016; Flores et al., 2014; McMahon et al., 2012).

Restoring ecological functionality can permit to mitigate pesticide transfers (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Tournebize et al., 2024). As an example of a nature-based solution, agricultural constructed wetlands, landscape elements at the interface between agricultural plots and the aquatic environment, are designed to intercept drainage water and naturally reduce pesticide and nitrate concentrations circulating in the hydrosphere, through biotic and abiotic natural processes (Gersberg et al., 1983, 1986; Gregoire et al., 2009; Imfeld et al., 2021; Tournebize et al., 2013, 2017). These semi-artificial hydroecosystems can become favorable shelters for numerous species, such as amphibians (Rannap et al., 2020; Strand & Weisner, 2013), and aquatic invertebrates (Becerra-Jurado et al., 2014; Huikkonen et al., 2020), helping to restore ecological continuity. Because of their retention function, however, agricultural constructed wetlands are potential interceptors of pesticides and nitrates, which, by acting as ecological traps, can have unintended effects on aquatic fauna they support (Stillway et al., 2019; Zhang et al., 2020).

Some invertebrates, such as gammarids, are commonly designated as sentinel species for environmental quality biomonitoring. Their sensitivity to pesticides, demonstrated at environmentally realistic concentrations (Cold & Forbes, 2004) make them relevant bioindicators. Sensitivity of *Gammarus sp.* to pesticides has already been studied in stream or pond mesocosms conditions (Böttger et al., 2013; Crane et al., 1999; Heckmann et al., 2005). Several biomarkers have been developed and studied in the genus *Gammarus spp.* as part of studies on the effects of pesticides under controlled laboratory conditions. These include biochemical (Demirci et al., 2018; Lebrun et al., 2020, 2021, 2023; Serdar, 2019), and genotoxic biomarkers (Lacaze et al., 2010), as early indicators of toxicity, and behavioral biomarkers (Lebrun et al., 2020, 2021, 2023), as indicators at the interface between organism physiology and population ecology. The use of these biomarkers makes it possible to link the effects of pesticides at the sub-cellular, cellular and individual levels, and potentially bridge the gap with repercussions of pesticides at population level. Biochemical traits, such as enzymatic activities, and certain behavioral traits therefore appear to be relevant for studying the ecotoxicity of pesticides *in situ*. In this context, the use of outdoor mesocosms, where conditions are intermediate between those in the laboratory and in the field, coupled with an active biomonitoring approach, enhances the realism of responses observed in aquatic invertebrates in relation to pesticide exposure (Gerhardt et al., 2012; Hasenbein et al., 2016), for accurate assessment of pesticide ecotoxicity.

The aim of this study was to investigate the potential risk to aquatic fauna posed by the agricultural constructed wetland of Rampillon (CWR) (Seine-et-Marne, France), constructed in 2010 to mitigate pesticide and nitrate pollution coming from drainage system of a catchment basin subject to intensive agricultural practices. To this end, in outdoor mesocosms directly implanted in the CWR, *G. fossarum* was exposed to a field realistic mixture of pesticides (7 herbicides and 3 fungicides) representative of the average pressure circulating in the CWR. *G. fossarum* was encaged in microcosms within mesocosms, through an active biomonitoring approach. We investigated several individual responses in *G. fossarum*, including survival and various behavioral responses, namely locomotor activity, amplexus rate and leaf ingestion rate. We also studied several sub-cellular responses including the activity of different enzymes involved in neurotransmission (acetylcholinesterase), nutrition function (β-galactosidase, β-glucosidase), growth (chitobiase), and non-specific immunity (acid and alkaline phosphatases, peroxidases). We expected a negative influence of the pesticide mixture on *G. fossarum*, with significant differences in responses between controls and treated mesocosms.

Material and methods

Study site

The present study focuses on the constructed wetland of Rampillon (48°32′19.5″N; 3°03′46.7″E), 70 km southeast of Paris, located in the Seine-et-Marne department in France. Constructed in 2010, the CWR is a pilot project started in 2005 that aims to reduce chemical pollutions from subsurface drained catchment upstream.

With an area of about 5,600 m^2, namely 0.15 % of the surface of its catchment basin, and a volume of 2,600 m^3, the CW is situated in derivation in relation to the stream “le Ru des Gouffres” which carries agrochemicals (nitrate, pesticides) received from runoff and from the drainage network of a 355 ha catchment basin located in the region of Brie as typical region under intensive agriculture. The stream “le Ru des Gouffres” partly flows into the CW by way of a sluice gate, and water spread through the different basins before run near the downstream part of the CWR and meet “le Ru des Gouffres” that flows directly into sinkholes that give access to the aquifer of Champigny supplying 1.5 millions of Île-de-France inhabitants. The CWR is subdivided in three sub-basins: one 1 meter depth sedimentation basin, one 30 centimeters intermediate living basin colonized by reeds (*Phragmites australis*), sedges (Juncaceae) and true sedges or carex (*Carex sp.*) and an around 1 meter depth final basin (Fig. 1).

The CWR is instrumented since 2012 using three hydrological measurement stations situated at its upstream and its downstream area and one situated at the outlet of the catchment basin. Each of the stations includes a flow rate measurement (Doppler probe) coupled with a composite sampling system controlled by the volume obtained in order to determine the incoming and outgoing flows of pesticides and a spectro-UV probe to characterize the dynamics of turbidity, TOC and NO3. The CWR reduces around 40% of pollutions. The CWR is colonized by a great diversity of taxa, including odonates and amphibians representing respectively 96% and 73% of the biodiversity identified in the Brie region (Letournel, Pages, et al., 2021).

Mesocosms

Six mesocosms were implanted in the intermediate basin of the CWR. Four mesocosms were referred to as “treated mesocosms” (TM) because they corresponded to the mesocosms in which the pesticide mixture under study was injected. The other two mesocosms corresponded to the “control mesocosms” (CM), in which the pesticide mixture was not injected.

The four treated mesocosms were 2 m long, 50 cm wide, 50 cm high, and they had a surface of 1 m^2 for a volume of 300-350 L. The two control mesocosms were twice as small due to technical constraints (Fig. 1) They all are made of polyvinyl chloride (PVC) with walls made of PVC glass. Certain pieces for structure covering are made of aluminum and a silicone joint complete the waterproofness of the angles. The first 15 cm of each mesocosms were buried in the sediment of the CWR and the water column in mesocosms measured consequently 35 cm. The content of each mesocosm was thus isolated from the ambient water of the CWR. On the six mesocosms, one CM and two TM were covered with a white geotextile protection of 6 mm thickness, initially planned to limit the degradation of pesticides by photolysis. In addition, one out of the covered treated mesocosm and one out of the uncovered one were equipped with a system permitting recirculation of water in closed circuit between the two extremities of the mesocosms to assess a potential effect of the convection in mesocosms and to re-homogenize the reactor water before the one-time automatic sampling.

Fig. 1 The constructed wetland of Rampillon (photography to the left), and mesocosms within the pond (photography to the right).

Hereafter, the abbreviations "UC" and "CC" will be used to designate respectively the "uncovered control mesocosm" (i.e., control without the geotextile protection), and the "covered control mesocosm" (i.e., control with the geotextile protection). The abbreviations "UT1", "UT2", "CT1", and "CT2", will be used to designate respectively the two "uncovered treated mesocosms", and the two "covered treated mesocosms".

Pesticide mixture

The mixture of pesticides was composed of 10 pesticides, including 7 herbicides and 3 fungicides, as follows: aclonifen (3 µg/L), bentazone (7.7 µg/L), chloridazon (8.46 µg/L), S-metolachlor (2.51 µg/L), glyphosate (9.5 µg/L), chlorotoluron (6.23 µg/L), metazachlor (7.75 µg/L), boscalid (1.69 µg/L), epoxiconazole (9.53 µg/L), and tebuconazole (7.64 µg/L). The mixture of pesticides was injected only once in the 4 treated mesocosms (UT1, UT2, CT1, and CT2) on March 25. Pesticide concentrations therefore declined naturally over time.

Environmental conditions and water quality parameters monitoring

During the 4 exposure series, 10 environmental variables and water quality parameters were monitored: temperature, oxygen saturation, conductivity, pH, cumulated ray, NO_3^-, NO_2^-, NH_4^+, sum of total pesticide concentrations, and sum of toxic units.

Overall, 40 pesticides and their metabolites, including the 10 pesticides tested, were quantified in the samples by a subcontractor laboratory with a typical limit of detection of 0.01 µg/L.

Despite high frequency monitoring, means and standard deviations of the various environmental variables were studied in the form of histograms.

Gammarus fossarum. collection and caging

A first lot of *Gammarus sp.* was collected with a net and by kick sampling in the morning of March 22, 2021, in the “Ru de Saint-Blandin” (Guérard, France), a forest stream as reference site, i.e., poorly contaminated in metals and pesticides (Lebrun et al., 2017, 2020). Gammarids with a size of 1 ± 0.2 cm were separated using a series of sieves with different sizes of mesh and transported into the lab in a cool box filled with water from the stream. At the sampling moment, the stream was characterized by a pH of 8.32, a temperature of 5.5°C, and a conductivity of 676 µS/cm. With exactly the same method, a second lot of *Gammarus sp.* was collected on the morning of April 21, 2021, in the stream characterized by a pH of 7.68, a temperature of 3.5°C, and a conductivity of 717 µS/cm. Back to the laboratory, individuals were stocked in 32 cm length × 23 cm width × 13 cm height plastic boxes filled with water of the “Ru de Saint-Blandin”. Boxes with gammarids were aerated through Tetra® oxygen pumps and disposed in a thermostated cupboard set at 10°C under a 12:12-h light:dark photoperiod. Alder leaves were generously distributed in boxes to feed individuals. The water of boxes was changed with clear unfiltered water of the stream once it get too charged in particles and nutriments (corresponding to 1/3 of the total volume changed every 3 days).

At the laboratory, thirty small cages (five cages per mesocosm) were made consisting in plastic cylindrical boxes of 10 cm height and 6 cm diameter, openable with a lid and modified by replace the mainly part of the bottom and of the lid by a circle cut net with a 500 µm nylon mesh. A wire was added at the back of the lid of each box to attach them into mesocosms. Alder leaves collected in autumn 2020 at Aulnoy (Seine-et-Marne, France) were gently washed with unfiltered tap water, left to dry at ambient temperature during three days and weighted dry. Two leaves were attributed per cage. Individuals stocked in the thermostated cupboard were sorted and 20 individuals were put in 24 cages according to the sex ratio 10 females:10 males with two leaves of alder per cage. For each condition, the firth cage contained alder leaves without individuals in order to assess the natural decomposition of the leaves. The thirty cages were placed in a cool box filled with water and transported to the CWR. Arrived at the CWR, cages were placed in the six mesocosms (five cages per mesocosms) by hanging them on a transversal metallic axe thanks to the wire. All cages were immersed in the water column without contact the sediment of the CWR. In all, four series of thirty cages (24 cages × 20 individuals = 480 ind. per series) were exposed in mesocosms from March 25, 2021, to April 22, 2021, for a period of one week each (about 168 h of exposure per series) at time 0, 7, 14, 21 days after pesticides mixture application (the mixture of pesticides was injected only once in the 4 treated mesocosms (UT1, UT2, CT1, and CT2) on March 25, followed by natural dissipation over time). The first series was exposed from March 25 to April 1st, the second from April 1st to April 8, the third from April 8 to April 15, and the last one from April 15 to April 22. Every week, after the exposure of a series during one week, individual responses of exposed individuals were assessed directly in field and then a new series of individuals was encaged and put in mesocosms.

G. fossarum individual responses monitoring

After each exposure series, individual responses, namely (i) survival rate, (ii) locomotor activity, (iii) amplexus rate, (iv) ingestion rate, of all exposed gammarids were assessed directly in the field on a laboratory bench located in the CWR. The contents of each cage, i.e., gammarids and leaves, were transferred into plastic beakers to perform additional measurements.

The survival rate of *G. fossarum* after 7 days of exposure was expressed in percentage and was determined as follows (1):

$$\text{Survival rate} = \frac{\text{Nb.survivors} \times 100}{\text{Nb.initial}} \qquad \text{(1) Survival rate}$$

where Nb. survivors is the number of individuals still alive at the end of the exposure series, Nb. initial is the number of individuals initially caged, i.e., N = 20.

Locomotor activity was calculated by measuring the number of individuals crossing a radial mark created in the cyclic bottom of each beaker was counted four times for periods of 30 seconds with intervals of 30 seconds. This response was expressed in number of passage per individual per minute, and was determined as follows:

$$\text{Locomotor activity} = \frac{\text{Total nb.pass.}}{\frac{\text{Nb.survivors} - \text{Nb.amp.}}{2}} \qquad \text{(2) Locomotor activity}$$

where Total nb. pass. is the total number of individual passages on the radial mark during the 2 minutes of monitoring, Nb. survivors is the number of individuals still alive at the end of the exposure series, Nb. amp. is the number of amplexus. We have subtracted the number of amplexus as we consider that only the male participates in the movement during the amplexus.

The amplexus rate, expressed in percentage, corresponded to the percentage of individuals in amplexus, i.e., reproductive individuals. Amplexus rate was determined as follows:

$$Amplexus\ rate = \frac{Nb.amp. \times 100 \times 2}{Nb.survivors} \qquad \text{(3) Amplexus rate}$$

where Nb. amp. is the number of amplexus, Nb. survivors is the number of individuals still alive at the end of the exposure series.

Once those measures were done, all individuals of each cage were dry on paper towel and were placed in Falcon® plastic tubes whose the net weight was determined in laboratory. Tubes were then placed in polystyrene cool boxes containing ice before stocking them at -20°C in a freezer. Tubes containing gammarids were weighted in the laboratory to determine the mass individuals gained in one exposure series. In parallel, leaves contained in cages were collected, dried up and weighted dry to determine the mass loss after one exposure series. Natural leaf degradation was determined to estimate the natural degradation of leaves, without the intervention of gammarids, by using a fifth cage per mesocosm. We determined a natural leaf degradation factor (NLF), expressed in percentage, that corresponded to the percentage of leaves dry weight that was not decomposed naturally and was estimated as follows:

$$\text{NLF} = \frac{\frac{W_t \times 100}{W_0}}{100} \qquad \text{(4) Natural leaf degradation factor}$$

where W_t is the remaining dry weight of leaves at the end of the exposure series in the fifth cage, without gammarids, W_0 is the initial dry weight of leaves at the beginning of the exposure series.

Then, the ingestion rate, expressed in mg of leaves consumed per mg of gammarid dry biomass per individual per day, determined as follows:

$$\text{Ingestion rate} = \frac{\frac{\frac{(W_0 - W_t) \times \text{NLF}}{\text{fresh gamm.biom.} \times 30\%}}{\text{Nb.survivors} \times \frac{\text{Nb.initial} + \text{Nb.survivors}}{2}}}{\text{caging duration}} \qquad \text{(5) Ingestion rate}$$

where W_0 is the initial dry weight of leaves at the beginning of the exposure series, W_t is the remaining dry weight of leaves at the end of the exposure series, NLF is the natural leaf degradation factor (see (4)), fresh gamm. biom. is the fresh gammarid biomass, i.e., the biomass of non-dried individuals still alive at the end of the exposure series, Nb. survivors is the number of individuals still alive at the end of the exposure series, Nb. initial is the number of individuals initially caged, caging duration is the number of days gammarids have been caged (i.e., 7 days per exposure session). 30% corresponded to the estimated dry weight for gammarids (factor determined in several previous in-house projects).

G. fossarum enzymatic responses monitoring

The activity of 7 enzymes was measured: acetylcholinesterase (AChE), β-galactosidase (GAL), β-glucosidase (GLU), chitobiase (NAG), acid phosphatase (PAC), alkaline phosphatase (PAL), peroxidases (PEROX). Protein quantity was also measured.

These analyses were carried out in males only, to avoid any sex-dependent variation (Charron et al., 2013; Rollin et al., 2023). At the end of each series of exposure, 3 male gammarids per replicate were collected and preserved at -20°C until enzymatic activities were analyzed. Each batch of 3 male gammarids was ground (Mixer Mill 400 - Retsch ® ; 2min ; 30Hz) using steel and glass beads in 1.5 ml cold citrate/phosphate buffer (CPB 0.05 M, pH5). After centrifugation (14,000g, 4°C, 20 min), the supernatant was collected and used for total protein quantification and color-metric analysis of the 7 enzyme activities in 96-well microplates (Lebrun et al., 2020). To inactivate enzymes, 250 µL of the supernatant from each sample was boiled for 5 minutes. This served as an optical control to ensure that the measurements reflected enzymatic activity rather than an abiotic reaction. The left 250 µL was used for protein quantitative analysis via the Bradford method (Bradford, 1976) and enzymatic assays. All enzymatic assays were based on spectrophotometry using a microplate reader (Berthold Technologies, Germany).

GAL, GLU, NAG, PAC, and PAL activity was measured in reaction medium that consisted of 200 µL citrate phosphate buffer (CPB) (pH 5) for GAL, GLU, PAC, and NAG, or 200 µL disodium phosphate (Na_2HPO_4) (pH 9,2) for PAL, and 25 mM of their respective substrates of conjugated p-nitrophenyl for GAL, GLU, PAC, and PAL, and 12.5 mM for NAG, 20 µL sample for GLU, NAG, PAC, PAL, and 50 µL sample for GAL. The reaction was stopped by adding 100 µL sodium carbonate (Na_2CO_3) (1M), after 3 and 5 min for GLU, after 10 and 15 min for GAL, and NAG, or by adding 100 µL sodium hydroxide (NaOH) (1M) after 10 and 20 min for PAC, and PAL. The liberation of p-nitrophenol by enzymatic hydrolysis of the substrate was determined at 405 nm just after the end of incubation. PEROX activity was measured in a reaction medium that consisted of 200 µL citrate phosphate buffer (50 mM, pH 5), 2,2′azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid (10 mM), and H_2O_2 (20 µL, 0.3 mM), 10 µL sample. Optical density (at 405 nm) was monitored just after the extracts were submitted, followed by the next readings spaced at 1, 2, and 3 min. Blanks were produced by substituting H_2O_2 substrate with ultrapure water for peroxidase. AChE activity was measured in a reaction medium that consisted of 180 µL potassium phosphate buffer (100 mM, pH 7.4), 5.5’-dithiobis-(2-nitrobenzoic acid) (DTNB) (1 mM), 20 µL acetylthiocholine (76 mM) and 20 µL sample. Optical density (at 405 nm) was monitored every minute for 10 min, using a molar extinction coefficient of 0.0136 $\mu M^{-1}.cm^{-1}$ for AChE, and 0.036 $\mu M^{-1}.cm^{-1}$ for PEROX.

For GAL, GLU, NAG, PAC, and PAL, enzymatic activity was expressed U/g of wet weight of gammarid. One unit of activity was defined as the amount of enzyme that catalysed 1 µmol substrate in 1 min, and was calculated as follows:

$$\text{Enzymatic activity} = \frac{1}{2}\sum_{i=1}^{2}\frac{\frac{\frac{\text{Abs.}_{t_i}}{\text{aPNP}}}{t_i}\times\frac{1}{\text{MM}_{\text{PNP}}}\times \text{V}_{\text{enz.}}}{\text{wet weight}}$$

where Abs._{ti} is the absorbance of the sample corrected by the absorbance of the control (i.e., the boiled sample) at the incubation time t_i (3 and 5 min for GLU, 10 and 15 min for GAL and NAG, 10 and 20 min for PAC and PAL), aPNP is the directrix of the PNP calibration line, MM_{PNP} is the molar mass of PNP, $V_{enz.}$ is the cytosolic volume, wet weight is the total wet weight or mass of the 3 male gammarids used for the enzymatic analyses.

For AChE and PEROX, enzymatic activity was expressed in U/g of gammarid wet weight, with U corresponding to the quantity of enzyme needed to process one micromole of substrate in one minute, and was calculated as follows:

$$\text{Enzymatic activity} = \frac{\frac{\frac{\text{a}_{\text{kinetics}}}{\varepsilon}}{\text{L}}\times \text{DF}\times \text{V}_{\text{enz.}}}{\text{wet weight}}$$

where $a_{kinetics}$ is the directrix coefficient of the line describing the temporal evolution of optical densities corrected by control (i.e., the boiled sample), ε the molar extinction coefficient, L is the optical path, DF is the dilution factor, $V_{enz.}$ is the cytosolic volume, wet weight is the total wet weight or mass of the 3 male gammarids used for the enzymatic analyses.

The protein content of the samples (PROT) was quantified using Bradford's reagent (Sigma-Aldrich ®) and bovine serum albumin for curve calibration.

Statistical analyses

All statistical analyses were performed using R software (version 4.3.3, R Core Team 2024) and using the total dataset (6 mesocosms × 4 cages × 4 exposure series = 96 observations). Firstly, in order to identify potential correlations between environmental and water quality variables, we performed principal component analyses (PCA) (package {ade4}) on the following variables: temperature, oxygen saturation, conductivity, pH, cumulated ray, NO_3^-, NO_2^-, and NH_4^+. We have taken the pesticide variable "sum of total pesticide concentrations" into account in one of the PCAs, and the pesticide variable "sum of toxic units" in the other.

The individual responses of *G. fossarum* (survival rate, locomotor activity, amplexus rate, and ingestion rate), and the enzymatic responses (acetylcholinesterase, β-galactosidase, β-glucosidase, chitobiase, alkaline phosphatase, acid phosphatase, and peroxidase activity, and protein quantity) were studied by calculating the means and standard deviations of each response for each exposure modality (UC, UT1, UT2, CC, CT1, CT2) and for each exposure series (S1, S2, S3, S4) and using a boxplot representation. For each response, we performed Wilcoxon-Mann-Whitney U-test ($P < 0.05$) (package {ggpubr}) between control modalities (UC or CC) and each corresponding treated mesocosm (UT1, UT2, CT1, or CT2), respectively, namely: UC VS. UT1, UC VS. UT2, CC VS. CT1, and CC VS. CT2.

In addition, we performed correlation plots (package {corrplot}) to determine the relationship between the response variables and pesticides, generally built as follows: Y ~ X where Y was the response variable (individual or enzymatic responses), and X was the explanatory variables reflecting pesticides pollution levels (sum of total pesticide concentrations or the sum of toxic units). Correlation plots also permitted to highlight the potential confounding effects of environmental conditions on the response variables.

Results

Environmental conditions and water quality parameters

Overall, environmental conditions and water quality parameters were heterogeneous according to the different exposure modalities and series (Fig. 2). Temperature was similar in all mesocosms (Fig. 2A), while differences between covered and uncovered mesocosms were observed for oxygen saturation (Fig. 2B), conductivity (Fig. 2C), pH (Fig. 2D), cumulated ray (Fig. 2E), ammonium (Fig. 2G), and nitrite concentration (Fig. 2H). Nitrate concentrations tended to decrease over the course of the exposure series (Fig. 2F), while ammonium concentrations increased (Fig. 2G). Total pesticide concentrations and ΣTU also decreased over time (Fig. 2I, J).

Nitrate concentrations were higher in control mesocosms (Fig. 2F), and the two pesticide variables were higher in the treated mesocosms but were different from zero in control mesocosms (Fig. 2I, J).

Correlations between variables were highlighted (Fig. 3). In particular, oxygen saturation, cumulated ray, and pH were correlated, as well as nitrite and ammonium concentrations were. Globally, oxygen saturation, cumulated ray, and pH were negatively correlated to conductivity, nitrite and ammonium concentrations. The total pesticide concentration was negatively correlated with nitrate concentration (Fig. 3A), but ΣTU was not and was positively correlated with temperature (Fig. 3B). Temperature-induced variability was relatively low (Fig. 3).

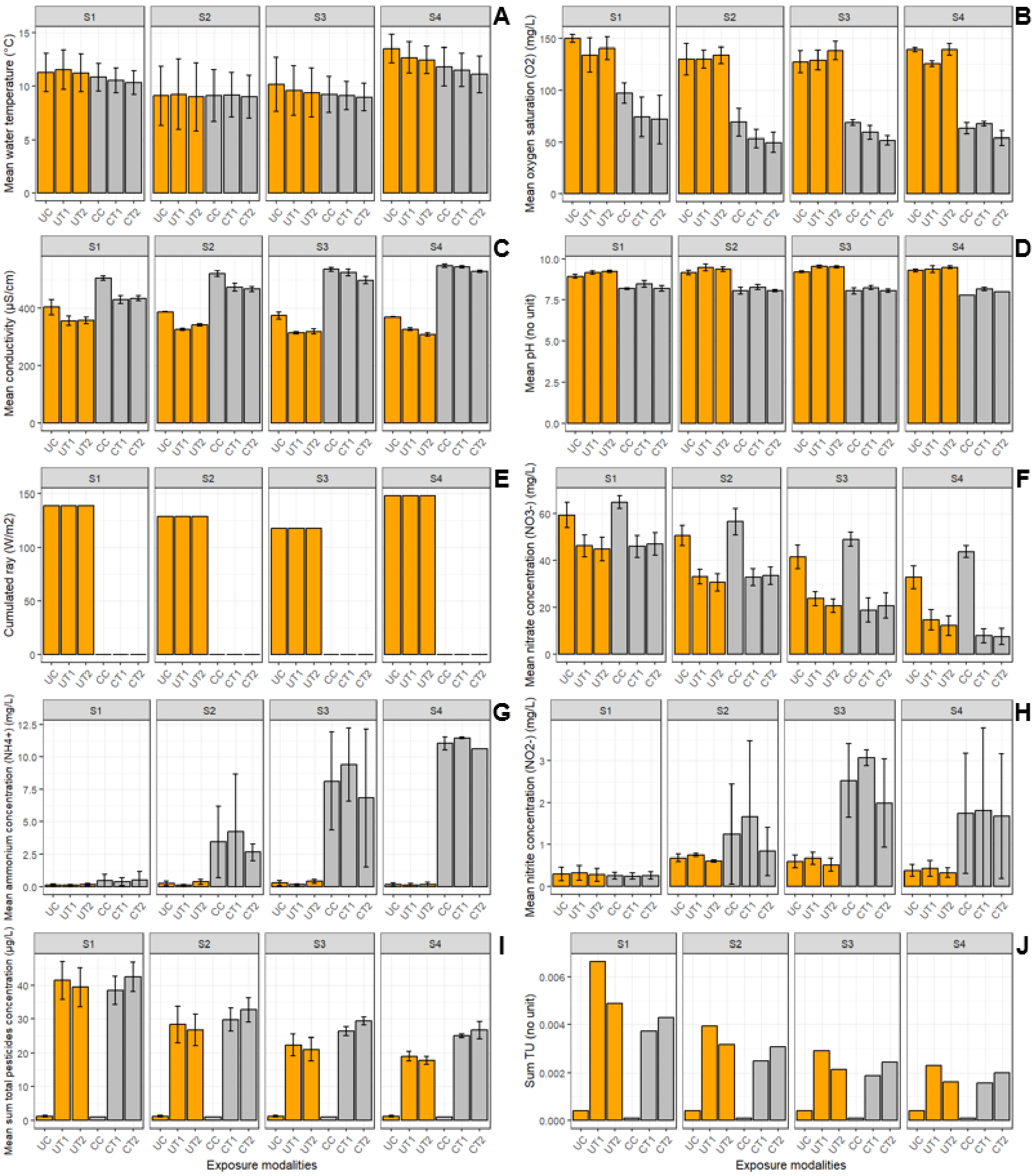


Fig. 2 Environmental conditions and water quality according to series and exposure modalities. (A) Mean water temperature (°C), (B) Mean oxygen saturation (mg/L), (C) Mean conductivity (µS/cm), (D) Mean pH (no unit), (E) Cumulated ray (W/m^2), (F) Mean nitrate concentration (mg/L), (G) Mean ammonium concentration (mg/L), (H) Mean nitrite concentration (mg/L), (I) Mean sum total pesticides concentration (µg/L), (J) Sum TU (no unit). Exposure modalities: UC = Uncovered Control mesocosm, UT1 / UT2 = Uncovered Treated mesocosms, CC = Covered Control mesocosm, CT1 / CT2 = Covered Treated mesocosms. Series: S1 = March 25-April 1st, S2 = April 1st-8, S3 = April 8-15, S4 = April 15-22.

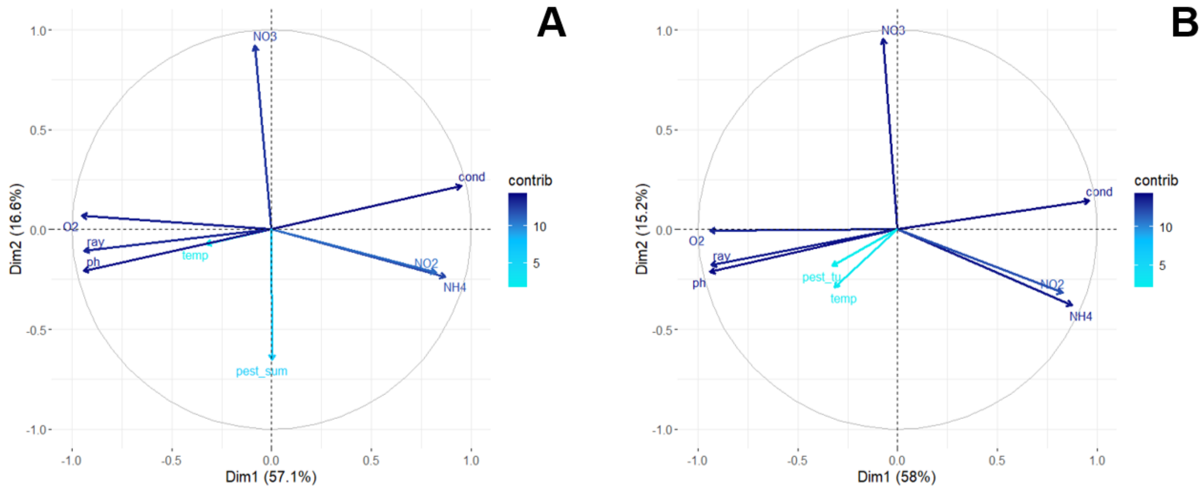


Fig. 3 Principal Component Analysis (PCA) graph highlighting relationships between the environmental conditions and water quality parameters. (A) PCA graph with the pesticide variable "Total pesticides concentration" (pest_sum) VS. (B) PCA graph with the pesticide variable "ΣTU" (pest_tu). Variables: cond = conductivity, NH4 = ammonium NH_4^+ concentration, N2 = nitrite NO_2^- concentration, NO3 = nitrate NO_3^- concentration, O_2 = oxygen saturation, ph = pH, ray = cumulated ray, pest_sum = total pesticides concentration, pest_tu = sum of TU (ΣTU).

Individual responses

No strong differences were found between control and treated mesocosms regardless of the individual response studied, except for the ingestion rate that was more responsive (Fig. 4). In particular, no clear patterns between control and treated mesocosms were observed for *G. fossarum* survival rate (Fig. 4A). However, significant heterogeneous differences were observed from S3, notably we observed a significant lower survival rate in UC in comparison with UT1 and UT2. For covered mesocosms, we observed a significant lower survival rate in CT1 compared to the control CC (Fig. 4A). Globally, amplexus rate was erratic (Fig. 4B). No clear pattern was found for the locomotor activity, but it tended to decrease over the series all modalities included (Fig. 4C). Almost none significant difference was found for the ingestion rate for the uncovered mesocosms. When a significant difference existed in the covered mesocosms, we observed systemically a higher ingestion rate in CC compared to at least one of the associated treated mesocosm (Fig. 4D). As observed for the locomotor activity, overall, the ingestion rate tended to decrease over time (Fig. 4C, D).

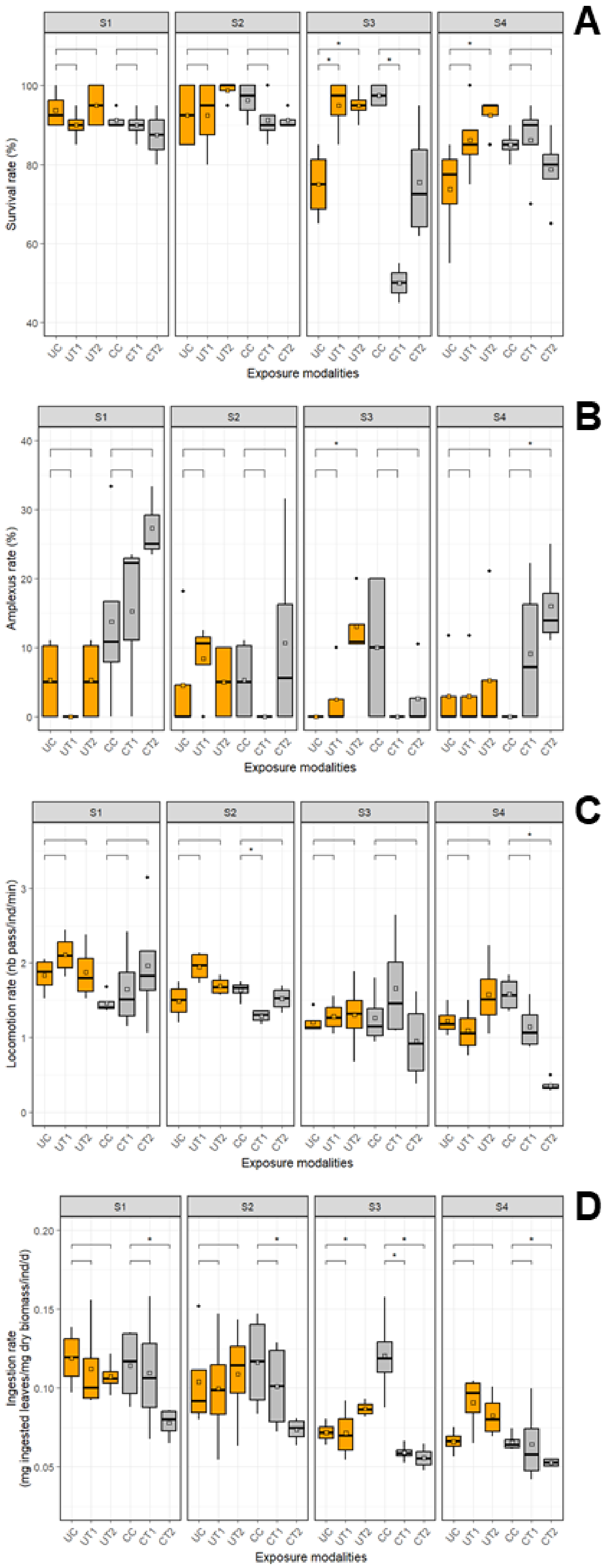

A
B
C
D
S1
S2
S3
S4
Survival rate (%)
Amplexus rate (%)
Locomotion rate (nb pass/ind/min)
Ingestion rate
(mg ingested leaves/mg dry biomass/ind/d)
UC
UT1
UT2
CC
CT1
CT2
Exposure modalities

Fig. 4 *G. fossarum* biological responses depending on exposure modalities. (A) Survival rate (%), (B) Amplexus rate (%), (C) Locomotor activity (number of passages per individual per minute), (D) Ingestion rate (mg of ingested leaves per mg of dry biomass of gammarids per individual per day). Points represent means and lines represent standard deviation (N = 4). * indicates significant differences when compared to unexposed controls (Mann-Whitney U-test, $P < 0.05$). In orange: UC = Uncovered Control, UT1 / UT2 = Uncovered Treatment. In grey: CC = Covered Control, CT1 / CT2 = Covered Treatment.

Enzymatic responses

As for individual responses, no clear pattern were found between control and treated mesocosms regardless of the enzymatic response studied (Fig. 5). NAG activity, specifically in the uncovered mesocosm UT2, in which the water did not recirculate, was systemically lower than UC and UT1 (Fig. 5D). In the same way, PAC activity, specifically in treated mesocosms UT1 and CT1, in which the water did recirculate, was lower than the other exposure modalities (Fig. 5E).

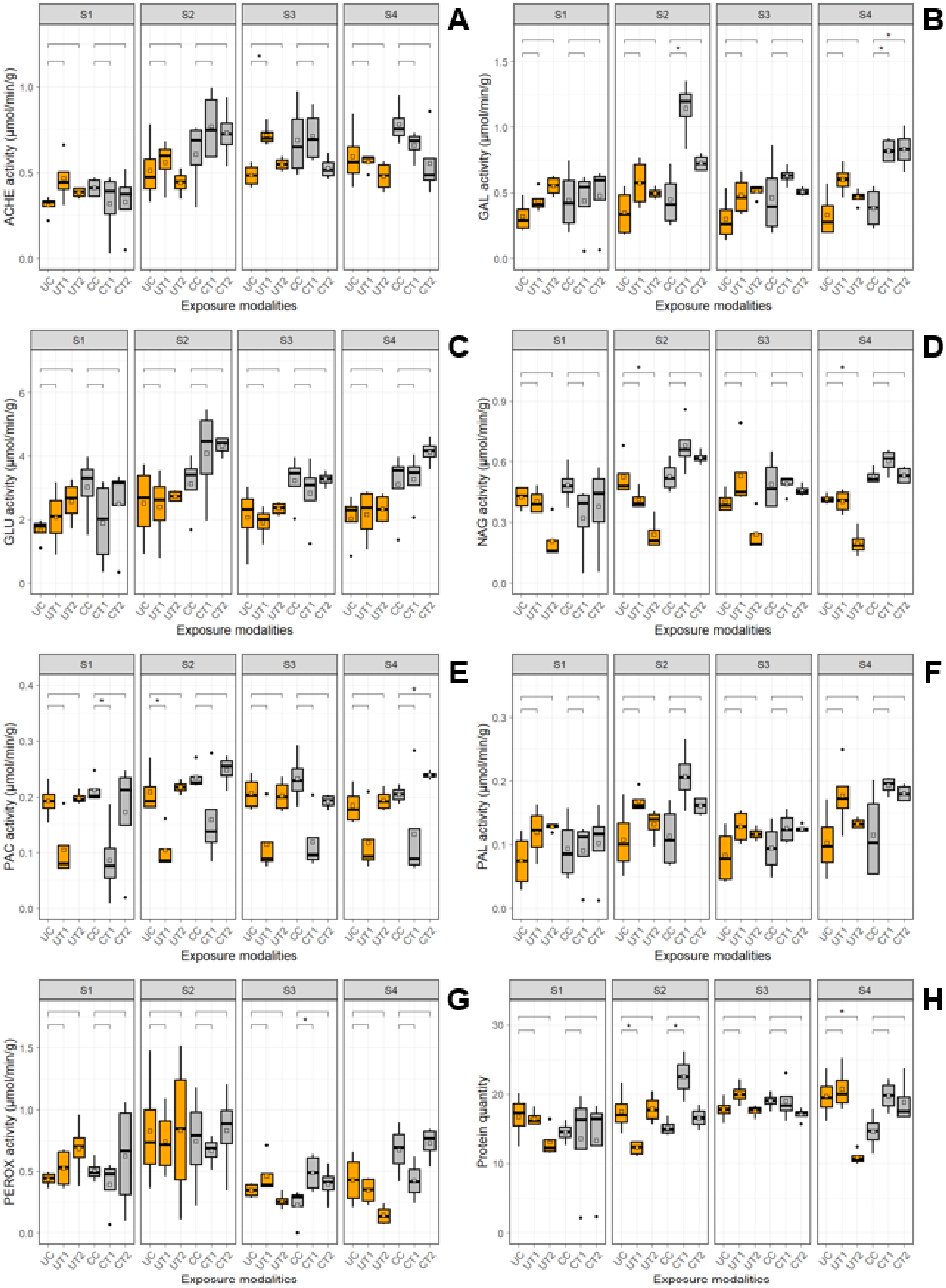


Fig. 5 Enzymatic (µmol/min/g) and protein levels in *G. fossarum* exposed in mescocoms to pesticide mixture depending on exposure modalities. (A) Acetylcholinesterase (ACHE), (B) β-galactosidase (GAL), (C) β-glucosidase (GLU), (D) chitobiase (NAG), (E) Acid phosphatase (PAC), (F) Alkaline phosphatase (PAL), (G) Peroxidase (PEROX), (H) Protein quantity (PROT). Points represent means and lines represent standard deviation (N = 4). * indicates

significant differences when compared to unexposed controls (Mann-Whitney U-test, $P < 0.05$). In orange: UC = Uncovered Control, UT1 / UT2 = Uncovered Treatment. In grey: CC = Covered Control, CT1 / CT2 = Covered Treatment.

Link between pesticides and the individual responses

Only amplexus and locomotor activity were significantly associated with at least one of the pesticide variable (Fig. 6). Some correlations between responses and environmental variables were quite strong (Fig. 6). In particular, oxygen saturation and nitrate concentration were strongly positively correlated with survival rate, locomotor activity, and ingestion rate. In contrast, ammonium and nitrite concentration were highly negatively correlated with these same response variables. Temperature was almost uncorrelated with any of the 4 response variables (Fig. 6).

Fig. 6 Correlation plot between individual response variables and environmental conditions. Individual response variables: sur = survival rate, amp = amplexus rate, loc = locomotor activity, ali = ingestion rate. Environmental conditions: temp = temperature, O2 = oxygen saturation, cond = conductivity, ph = pH, ray = cumulated ray, NO3 = nitrate NO_3^- concentration, NH4 = ammonium NH_4^+ concentration, N2 = nitrite NO_2^- concentration, pest_sum = total pesticides concentration, pest_tu = sum of TU (ΣTU). Pearson correlation coefficient is illustrated by the colored circles and by the values. * indicates significant relationships between individual response variables and pesticide variables (function *lm*, {stats}).

Link between pesticides and the enzymatic responses

The activity of ACHE, NAG, and PAC were significantly negatively correlated with at least one of the pesticide variable (Fig. 7). In contrast, the activity of GAL and PAL were significantly positively correlated with at least one of the pesticide variable (Fig. 7). The strongest negative correlations between pesticides and enzymatic activities were found for NAG and PAC, and the strongest positive correlations were found for GAL and PAL (Fig. 7). Some correlations between responses and environmental variables were quite strong (Fig. 7). In particular, the group of environmental variables oxygen saturation, pH, and cumulated ray were negatively correlated with AChE, GAL, GLU, and NAG activity. In parallel, the group of variables conductivity, NH_4^+, and NO_2^- were positively correlated with AChE, GAL, GLU, and NAG activity (Fig. 7).

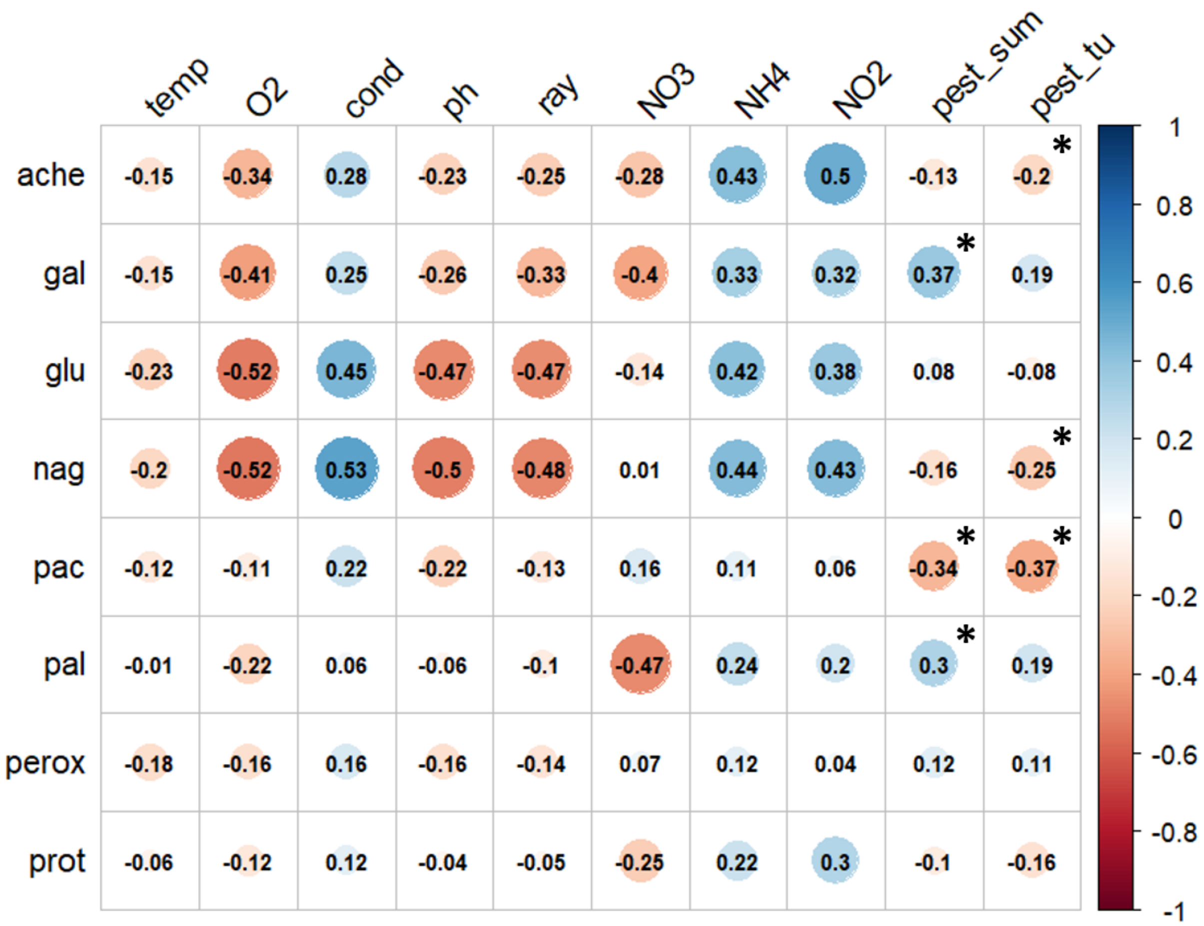


Fig. 7 Correlation plot between enzymatic response variables and environmental conditions. Enzymatic response variables: ache = Acetylcholinesterase activity, gal = β-galactosidase activity, glu = β-glucosidase activity, nag = Chitobiase activity, pac = Acid phosphatase activity, pal = Alkaline phosphatase activity, perox = Peroxidase activity, prot = Protein quantity. Environmental conditions: temp = temperature, O2 = oxygen saturation, cond = conductivity, ph = pH, ray = cumulated ray, NO3 = nitrate NO_3^- concentration, NH4 = ammonium NH_4^+ concentration, N2 = nitrite NO_2^- concentration, pest_sum = total pesticides concentration, pest_tu = sum of TU (ΣTU). Pearson correlation coefficient is illustrated by the colored circles and by the values. * indicates significant relationships between enzymatic response variables and pesticide variables (function *lm*, {stats}).

Discussion

The aim of our study was to determine the effects of a mixture of pesticides on *G. fossarum* under mesocosm conditions, in order to investigate the potential for an agricultural constructed wetland to affect the aquatic fauna it hosts. The strongest observed effects that can most probably be attributed to the consequences of the pesticide mixture were a positive effect on the locomotor activity, and on the amplexus rate of *G. fossarum*, and a stimulation of β-galactosidase, alkaline phosphatase, and an inhibition of acetylcholinesterase, chitobiase, and acid phosphatase, which could have significant ecological repercussions in the natural environment. However, the realism of the transferability of this finding to the natural environment of the CWR deserves further refinement.

Firstly, our results suggest that, although probably without lethal effects, the pesticide mixture studied may have had a stimulating effect on locomotor activity and amplexus rate, possibly reflecting a state of intense stress in *G. fossarum*. *G. fossarum* locomotor activity and amplexus rate were indeed the two most responsive individual responses to pesticide variables in this study. Stimulation of pairing has already been observed in *G. fossarum* and *G. pulex* in response to exposure to pesticides (Lebrun et al., 2020) and wastewater effluent (Love et al., 2020) respectively. An increased amplexus rate can be interpreted as a reproductive strategy in a population under stress, aiming to ensure the production of offspring before the effects of the stress in question are too pronounced on the reproductive individuals. However, in the case of pesticides, disruption of reproducing pairs has also been observed (Cold & Forbes, 2004). Similarly, increased locomotor activity in *G. fossarum* or *G. pulex* can be induced by different pesticide pressures and is interpreted as an avoidance strategy (Lebrun et al., 2020; Soose et al., 2023). Disruptions of reproductive behavior and triggering of avoidance behaviors in these organisms is likely to have repercussions on their population dynamics in the wild (Cold & Forbes, 2004; Nørum et al., 2011). Consequently, the stimulation of amplexus formation and locomotor activity observed in *G. fossarum* in our study could correspond to a response to the stress caused by the pesticide pressure studied. The application of such strategies by other native invertebrates in the CWR can or may have had crucial effects on the dynamics and structuring of the aquatic invertebrate community living there. However, such a level of result transposition is not possible at this stage.

The mixture of pesticides studied may indeed have had specific effects on the activity of certain enzymes. GAL and PAL activity appeared to be stimulated by pesticides, while AChE, NAG and PAC activity appeared to be inhibited by pesticides. These results are in line with those obtained in toxicity tests on pesticide binary mixtures for NAG, whose activity can be reduced by binary mixtures of fungicides and insecticides (Lebrun et al., 2020, 2021). However, our results seem rather contradictory with these studies, especially for GAL (Lebrun et al., 2020) and PAL (Lebrun et al., 2021), whose activity tends to be reduced by certain pesticides, and for PAC, whose activity can be stimulated (Lebrun et al., 2020, 2021). These differences in the enzymatic responses observed may be linked to the differences of complexity in the experimental design employed in our study (e.g., more complex pesticide mixture in our study, more unpredictable environmental conditions in mesocosms). Nevertheless, these results suggest that the pesticide mixture used in our study may be responsible for changes in enzymatic responses in *G. fossarum*, with potential effects on molting (NAG), and non-specific immunity (PAC, PAL).

However, the interpretation of our results is complicated by the experimental design proposed. Between the different conditions, a number of environmental variables evolved differently within the mesocosms. In particular, nitrite and ammonium appear to be the variables responsible for the excess mortality observed in mesocosms from exposure series S2 onwards. In addition, the fragility of our dataset, coupled with the complexity of interaction effects between environmental variables and pesticides (Howe et al., 1994), and with the complexity of studying the biological responses of organisms in the natural environment, greatly complicates the interpretation of our results. Thus, the potential effects attributable to the pesticide mixture observed in *G. fossarum* may not be directly transferable to what might be observed in CWR. Moreover, we have concentrated on acute effects, but chronic effects are largely likely to occur in CWR. Although this experiment would benefit from further refinement, the results obtained in our study are nevertheless particularly interesting and provide answers to some of the grey areas surrounding the potential impacts of agricultural constructed wetlands on aquatic fauna.

Conclusion

We caged *Gammarus fossarum* in outdoor mesocosms within an agricultural constructed wetland, to assess the potential impacts of this wetland on aquatic fauna by studying the effects of a mixture of pesticides representative of the average pesticide pressure found naturally within it. Despite the absence of lethal effects in *G. fossarum*, our results may show stimulatory effects of the pesticide mixture on the reproductive and locomotor behavior of the studied organism, suggesting the implementation of a defense strategy against the pesticide pressure. Moreover, the activity of certain enzymes intervening in non-specific immunity and molting seemed to have been influenced by the pesticide mixture. If such an influence occurs naturally in constructed wetlands, it suggests that agricultural constructed wetlands could have an influence on the aquatic invertebrate populations that inhabit them. However, the complexity of how natural systems function in the field makes interpretation of our results difficult. Nevertheless, this study provides new insights into the potential impacts of agricultural constructed wetlands on aquatic fauna.

Appendix

Appendix 1 Quantified pesticides and corresponding acute and chronic toxicity data for aquatic invertebrates.

Pesticide	CAS	Type	Toxicity									
			Acute toxicity					Chronic toxicity				
			EC50	Unit	Model organism	Duration	Reference	NOEC/LOEC	Unit	Model organism	Duration	Reference
Aclonifen	74070-46-5	Herbicide	1.2	mg/L	*Daphnia magna*	48h	PPDB	0.016	mg/L	*Daphnia magna*	21d	PPDB
Atrazine	1912-24-9	Herbicide	85	mg/L	*Daphnia magna*	48h	PPDB	0.25	mg/L	*Daphnia magna*	21d	PPDB
Bentazone	25057-89-0	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	> 101	mg/L	*Daphnia magna*	21d	PPDB
Chloridazon	1698-60-8	Herbicide	132	mg/L	*Daphnia magna*	48h	PPDB	6.23	mg/L	*Daphnia magna*	21d	PPDB
Chlorotoluron	15545-48-9	Herbicide	67	mg/L	*Daphnia magna*	48h	PPDB	11.2	mg/L	*Daphnia magna*	21d	PPDB
Dimethenamid	87674-68-8	Herbicide	16	mg/L	*Daphnia magna*	48h	PPDB	1.25	mg/L	*Daphnia magna*	21d	PPDB
Flufenacet	142459-58-3	Herbicide	30.9	mg/L	*Daphnia magna*	48h	PPDB	3.26	mg/L	*Daphnia magna*	21d	PPDB
Fluroxypyr	69377-81-7	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	56	mg/L	*Daphnia magna*	21d	PPDB
Glyphosate	1071-83-6	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	12.5	mg/L	*Daphnia magna*	21d	PPDB
Imazamox	114311-32-9	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	137	mg/L	*Daphnia magna*	21d	PPDB
Metazachlor	67129-08-2	Herbicide	33	mg/L	*Daphnia magna*	48h	PPDB	0.1	mg/L	*Daphnia magna*	21d	PPDB
Metolachlor	51218-45-2	Herbicide	> 23.5	mg/L	*Daphnia magna*	48h	PPDB	> 0.707	mg/L	*Daphnia magna*	21d	PPDB
Prosulfocarb	52888-80-9	Herbicide	0.51	mg/L	*Daphnia magna*	48h	PPDB	0.045	mg/L	*Daphnia magna*	21d	PPDB
Quinmerac	90717-03-6	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	> 100	mg/L	*Daphnia magna*	21d	PPDB
4,6-Dinitro-O-cresol	534-52-1	Herbicide/Insecticide	> 1.1	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Boscalid	188425-85-6	Fungicide	5.33	mg/L	*Daphnia magna*	48h	PPDB	1.3	mg/L	*Daphnia magna*	21d	PPDB
Epoxiconazole	133855-98-8	Fungicide	> 3.13	mg/L	*Daphnia magna*	48h	PPDB	0.63	mg/L	*Daphnia magna*	21d	PPDB
Fluxapyroxad	907204-31-3	Fungicide	6.78	mg/L	*Daphnia magna*	48h	PPDB	0.5	mg/L	*Daphnia magna*	21d	PPDB
Oxadixyl	77732-09-3	Fungicide	> 530	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Pentachlorophenol	87-86-5	Fungicide	> 0.45	mg/L	*Daphnia magna*	48h	PPDB	> 0.18	mg/L	*Daphnia magna*	21d	PPDB
Tebuconazole	107534-96-3	Fungicide	2.79	mg/L	*Daphnia magna*	48h	PPDB	0.01	mg/L	*Daphnia magna*	21d	PPDB
2-Hydroxyatrazine	2163-68-0	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Acetochlor ESA	187022-11-3	Metabolite	> 120	mg/L	*Daphnia magna*	48h	PPDB	120	mg/L	*Daphnia magna*	21d	PPDB
Acetochlor OXA	194992-44-4	Metabolite	> 120	mg/L	*Daphnia magna*	48h	PPDB	120	mg/L	*Daphnia magna*	21d	PPDB
Alachlor ESA	142363-53-9	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Alachlor OXA	171262-17-2	Metabolite	7.2	mg/L	Invertebrate	48h	INERIS	NA	NA	NA	NA	NA
AMPA	77521-29-0	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	15	mg/L	*Daphnia magna*	21d	PPDB
Atrazine-desethyl-2-hydroxy	19988-24-0	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Deethylatrazine	6190-65-4	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Dimethachlor OXA	1086384-49-7	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Dimethenamid ESA	205939-58-8	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Dimethenamid OXA	380412-59-9	Metabolite	95	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Flufenacet ESA	201668-32-8	Metabolite	> 87.3	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Flufenacet OXA	201668-31-7	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Metazachlor ESA	172960-62-2	Metabolite	93.8	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Metazachlor OXA	1231244-60-2	Metabolite	100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Metolachlor ESA	171118-09-5	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Metolachlor OXA	152019-73-3	Metabolite	> 16.6	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Propachlor ESA	123732-85-4	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Propachlor OXA	70628-36-3	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

CAS refers to the CAS Registry Number of the substance considered. EC_{50} = Effective Concentration 50, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration. The model organism refers to the species or the taxon on which the toxicity test was performed and the duration refers to the duration of the toxicity test (h = hour, d = day). For the references: PPDB = Pesticide Properties DataBase website (https://sitem.herts.ac.uk/aeru/ppdb/), INERIS = Substances chimiques INERIS website (https://substances.ineris.fr/).

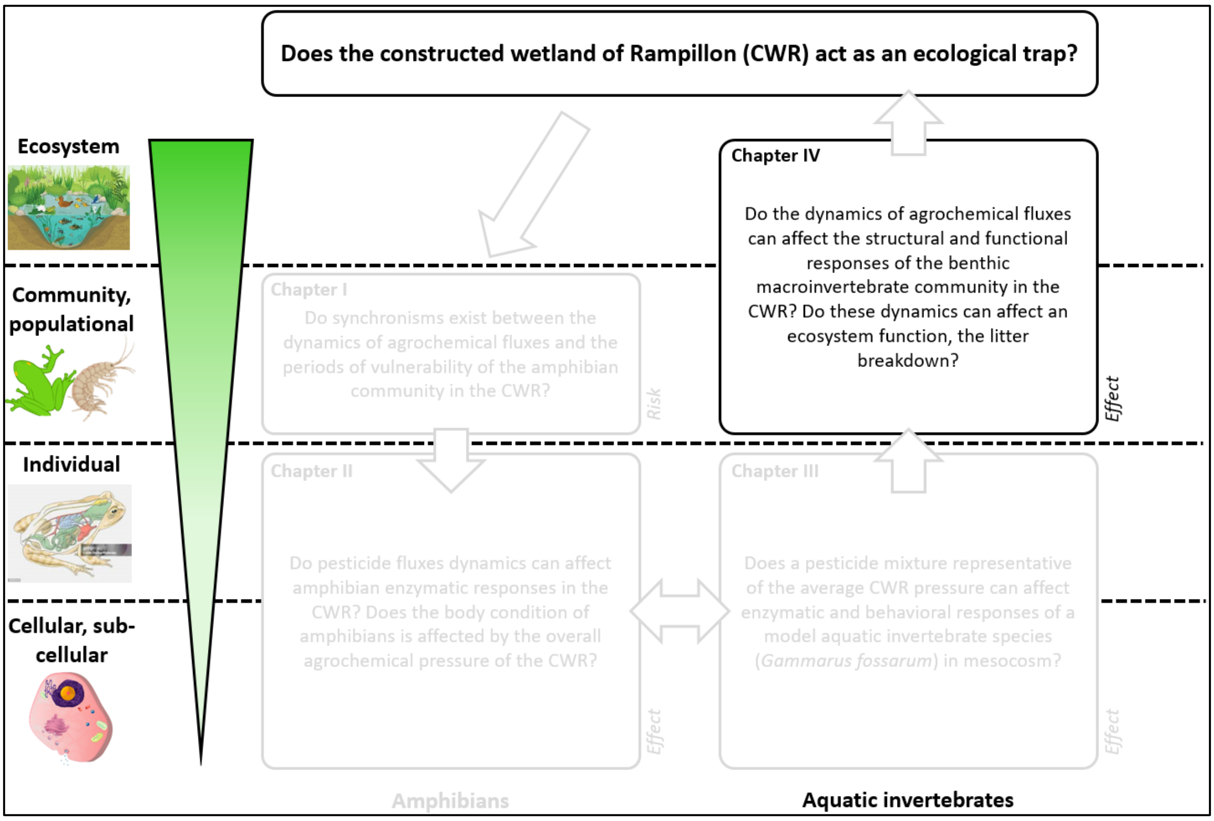
Epilog of Chapter III

Chapter III, as Chapter II on amphibians with the study of the natural pesticide pressure in the CWR, highlight the significant effects of the pesticide mixture studied on *G. fossarum*. The traits affected, namely the activity of certain enzymes, notably chitobiase, involved in moulting and therefore in gammarid growth, and certain behavioral traits, namely locomotor activity and reproductive rate, reflecting chemical stress, can have repercussions on the structure and dynamics of native populations. The link between effects observed in *G. fossarum* under mesocosm conditions and potential effects observed in native aquatic invertebrates remains hypothetical, given the degree of complexity of the natural environment. It is also worth noting the influence of environmental factors on some of the responses studied. This underlines the complexity of the natural environment and the importance of characterizing it and its influences on the responses of living organisms to specific stresses. However, the results obtained in this study reinforce the CWR's status as a potential ecological trap. Chapter IV will attempt to take a leap forward in the study of the biological levels of aquatic invertebrates, focusing then on community and ecosystem levels.

Chapter IV

Benthic macroinvertebrate diversity and function in an agricultural constructed wetland affected by agrochemical pressure (Seine-et-Marne, France)

Research article (accepted and published in Environmental Science and Pollution Research, January 2025, https://link.springer.com/article/10.1007/s11356-024-35722-4)



Chapter IV: Benthic macroinvertebrate diversity and function in an agricultural constructed wetland affected by agrochemical pressure (Seine-et-Marne, France)

Head of Chapter IV

This fourth chapter, entitled "*Benthic macroinvertebrate diversity and function in an agricultural constructed wetland affected by agrochemical pressure (Seine-et-Marne, France)*" is based on the use of a very common method for studying benthic macroinvertebrate communities and the associated leaf-litter breakdown function, namely litterbags. In this study, we deployed several series of litterbags in the CWR, as well as in a comparison pond, over 4 months of the year (March - June, i.e., including heavy contamination periods in the CWR, as highlighted in Chapter I). The aim of the study was to assess the potential effects of agrochemical flux dynamics on the CWR's benthic macroinvertebrate community, and on an ecosystem function partly assured by these aquatic invertebrates. Having studied the risk posed by the CWR to amphibians on a community level, and the effects of agrochemicals on the biological traits of amphibians and aquatic invertebrates, in Chapters I, II and III, which was intended to take a holistic approach, Chapter IV is the logical conclusion to this work, bringing to a close the study of the major levels of biological organization that may be affected by agrochemicals in the CWR. The research questions of Chapter IV are the following: *Do the dynamics of agrochemical fluxes can affect the structural and functional responses of the benthic macroinvertebrate community in the CWR? Do these dynamics can affect an ecosystem function, the litter breakdown?*

Title

Benthic macroinvertebrate diversity and function in an agricultural constructed wetland affected by agrochemical pressure (Seine-et-Marne, France)

(Research article accepted and published in *Environmental Science and Pollution Research*, *https://link.springer.com/article/10.1007/s11356-024-35722-4*)

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Abstract

Constructed wetlands (CWs), originally designed to mitigate chemical water pollution, often host noticeable aquatic fauna. However, little is known about the impact of the contaminants circulating within CWs on this local fauna, questioning the role of CWs as ecological refuges or traps. We aimed to assess the potential of an agricultural CW in northern France to act as an ecological trap for aquatic fauna and the potential consequences on wetland functioning. We made faunistic inventories of benthic macroinvertebrates, using litterbags, from March to June 2022 in two zones within the CW with contrasting levels of agrochemical contamination and in one unpolluted comparison pond. We calculated community diversity and sensitivity indices (e.g., species at risk, $SPEAR_{pesticides}$ index). We measured wetland functioning by monitoring the leaf-litter breakdown. Results showed that pesticide fluxes were related to community composition changes and had negative effects on taxonomic diversity (Shannon index) and functional traits (shredder/scraper feeding mode). The negative link between pesticides and the leaf-litter breakdown was less clear, mainly because of the high level of integration of this response. This study reveals that CWs under agrochemical pressure may act as potential ecological traps for benthic macroinvertebrates and highlights the relevance of studying this group as an early-warning indicator of chemical risk in nature-based solutions.

Keywords

Constructed wetland, Macroinvertebrates, Diversity, Pesticides, Leaf-litter breakdown, $SPEAR_{pesticides}$

Virginie Archaimbault and Alienor Jeliazkov are co-senior authors.

Statements and Declarations

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Alexandre Michel, Aliénor Jeliazkov, Virginie Archaimbault, Jérémie D. Lebrun, Cédric Chaumont, Mathieu Girondin and Julien Tournebize. The first draft of the manuscript was written by Alexandre Michel and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Introduction

The decline of biodiversity is undoubtedly linked to intensified agriculture practices, which are responsible for the destruction and the replacement of natural ecosystems (Attwood et al., 2008; Dudley & Alexander, 2017). As an aggravating factor, the use of pesticides and nitrate (hereafter grouped under the term "agrochemical") accentuates this impact (Dhananjayan et al., 2020). In particular, pesticides have been implicated in invertebrate decline through direct acute or chronic effects, and indirect effects (e.g., by reducing available resources) (Leenhardt et al., 2022). Therefore, agrochemicals pose a threat to biodiversity as (i) pesticides have become ubiquitous in air, water, soil, and biota (Carvalho, 2017; Leenhardt et al., 2022), and (ii) nitrate concentrations in aquatic ecosystems are continuously increasing due to human activities (Banerjee et al., 2023b). The entry of agrochemicals into the hydrosphere is mainly mediated by spraying, flowing, or drainage systems (Meite et al., 2018; Tournebize et al., 2017), facilitating the emergence of toxic effects on aquatic organisms.

Aquatic invertebrates are crucial for many ecosystem functions such as water purification or recycling of nutrients, but they are threatened by several pressures, including water pollution, that endanger the sustainability of these functions (Collier et al., 2016; Macadam & Stockan, 2015). In freshwater invertebrates, under controlled conditions, agrochemicals can indeed cause neurological (Cossi et al., 2015), behavioral (Augusiak & Van Den Brink, 2016), genetic (Martínez-Paz et al., 2013), immunological (Chang et al., 2013; Russo & Lagadic, 2000), reproductive (Bacchetta et al., 2002; A. P. Moore & Bringolf, 2018), and possibly endocrine (Bacchetta et al., 2002) as well as developmental impairments (Gomez Isaza et al., 2020). However, linking the effects observed in the laboratory to those in the field can be challenging due to the complexity of ecosystems. Nevertheless, there is increasing evidence that agrochemicals are responsible for disturbing freshwater invertebrate communities. For instance, pesticides, especially some insecticides, that target acetylcholinesterase (AChE) and gamma-aminobutyric acid (GABA) receptors, have been linked to the absence of specific invertebrate families from rivers in the UK (Poyntz-Wright et al., 2024). More broadly, pesticides can cause losses in regional species and family richness (Beketov et al., 2013). Besides, nitrate may be responsible for the selection of pollution-tolerant species as observed for instance, in lowland agricultural streams in New Zealand (T. P. Moore et al., 2021).

Cutter/chewer- and biofilm-eater-specialized aquatic invertebrates, called “shredders” and “scrapers,” respectively (Ramírez and Gutiérrez-Fonseca, 2014), play an important role in litter decomposition (Gingerich et al., 2015; Rezende et al., 2018). In particular, crustaceans are considered key species for this function (Piscart et al., 2009). Therefore, by inducing changes in community structure, agrochemicals can disrupt ecosystem functions such as the leaf-litter breakdown that is normally ensured by freshwater invertebrates and microbiocenosis, but a clear causality is rarely established (Brosed et al., 2016; Fernández et al., 2015; Flores et al., 2014; Schäfer et al., 2007). The link between the pesticide-induced changes in aquatic invertebrate community structure and function has been successfully demonstrated under lotic conditions (Auber et al., 2011), emphasizing the tangibility of the threat that agricultural freshwater body contamination represents for ecosystem functioning conservation.

According to the International Union for Conservation of Nature (IUCN), nature-based solutions are “actions to protect, sustainably manage and restore natural or modified ecosystems that address societal challenges effectively and adaptively, simultaneously providing human well-being and biodiversity benefits” (Cohen-Shacham et al., 2016). As a nature-based solution and landscape interface, agricultural constructed wetlands (CWs) are conceived to mitigate contaminants originating from runoff and drainage. To a certain extent, they enable the remediation of nitrate- and pesticide-contaminated waters (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Tournebize et al., 2013, 2017; Vymazal & Březinová, 2015) through biological (e.g., metabolization by plants and bacteria), physical, and chemical processes (e.g., photolysis) (Gregoire et al., 2009) while being able to support biodiversity (Zhang et al., 2020). In particular, agricultural CWs seem to contribute to enhancing aquatic invertebrate diversity within agricultural landscapes (Becerra-Jurado et al., 2014; Huikkonen et al., 2020). However, due to their purification abilities, CWs can also act as ecological traps because they store contaminants likely to affect the wild species they shelter (Stillway et al., 2019; Zhang et al., 2020), such as amphibians (Sievers et al., 2018). According to Hale & Swearer (2016), ecological traps "occur when animals mistakenly prefer habitats where their fitness is lower than in other available habitats following rapid environmental change." Although agricultural CWs could also act as ecological traps for aquatic invertebrates (Duchet et al., 2018), empirical *in situ* evidence is missing, and targeted fieldwork is needed to assess the ecological status of agricultural CWs.

The present study aims to determine whether the French agricultural constructed wetland of Rampillon (CWR), under agrochemical pressure, acts as a safe environment or as an ecological trap for aquatic invertebrates. To this end, we studied the effects of pesticides and nitrate on community and ecosystem function dynamics in three zones in two ponds with contrasting levels of contamination: in the CWR (two zones) and in a low-contaminated comparison pond (one zone). Aquatic invertebrate diversity was assessed using coarse mesh litterbags, which were immersed during six to eight sessions of 2 weeks each in the three zones from early March to late June 2022. We calculated diversity, ecological sensitivity indices, shredder/scraper frequencies, and leaf-litter breakdown rates for the three zones and assessed their response to pesticides and nitrate dynamics. We expected a negative relationship between diversity and sensitivity indices, leaf-litter breakdown rates, and pesticides and nitrate dynamics in view of the ability of these agrochemical pressures to impair aquatic invertebrate community structures and functions (Beketov et al., 2013; T. P. Moore et al., 2021; Münze et al., 2015). In addition, knowing the link between the following two variables, we expected a combined negative response of the frequencies of use of the shredder/scraper modality and leaf-litter breakdown rates to pesticides.

Material and methods

The Constructed Wetland of Rampillon

The present study focuses on the Constructed Wetland of Rampillon (CWR) located in the Seine-et-Marne department in France, about 60 km southeast of Paris (48°32′19.5″N; 3°03′46.7″E). The 5,600 m^2 CWR, situated in derivation about the stream "le ru des gouffres" (i.e., off-stream interception), collects runoff and drainage water from a 355-hectare agricultural catchment area subject to intensive crop rotation, mainly wheat, corn, and beets (85% of average crop rotation).

The CWR mitigates pesticides and nitrogen pollution before direct infiltration into sinkholes of the Champigny limestone aquifer that supplies 1.5 million Île-de-France inhabitants with water (Tournebize et al., 2012). The CWR intercepts an average of 40% of the mean runoffs, namely, 800,000 m^3 per year. The CWR has been equipped since 2012 to monitor agrochemical fluxes using three hydrological measurement stations (HMSs) situated upstream and downstream of the pond and at the outlet of the catchment basin. Each HMS includes a flow rate measurement (Doppler probe) coupled with a composite sampling system controlled by the water volume that enables measurement of the incoming and outgoing flows of pesticides and nitrate. There is a contamination gradient from upstream to downstream due to the purification action of the pond (Lebrun et al., 2019; Letournel, Chaumont, et al., 2021). A 10-year period of continuous monitoring (2012–2022) and analysis of 531 pesticides and their metabolites showed that the CWR intercepts 40–700 g of pesticides per year (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Letournel, Chaumont, et al., 2021). The CWR has been extensively described in previous works (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Bahi, Sauvage, Payraudeau, & Tournebize, 2023; Lebrun et al., 2019; Mander et al., 2021; Tournebize et al., 2012, 2017).

The average pesticide concentration of incoming water is about 1 µg/L from January to December, with peak concentrations of approximately 10 µg/L observed in May and June following the application of herbicides and fungicides that takes place from April to June (Fig. 1). Over the year, nitrate concentrations are above the threshold of 30.1 mg/L, corresponding to a bad ecological quality class (Ministère de la transition écologique et solidaire, 2019) (Fig. 1) (C. Chaumont, unpublished). The average decrease in concentrations due to the purification abilities of the CWR is about 37% depending on the properties of the different molecules in pesticides (50% are herbicides) and about 11 mg/L for nitrate concentration (Letournel, Chaumont, et al., 2021). The major pesticides retrieved in the pond are the herbicides metamitron, quinmerac, mesotrione, metolachlor, ethofumesate, terbuthylazine, bentazone, isoproturon, nicosulfuron, imazamox, glyphosate, and its metabolite AMPA as well as the insecticide thiamethoxam, the fungicide tebuconazole, and the molluscicide metaldehyde.

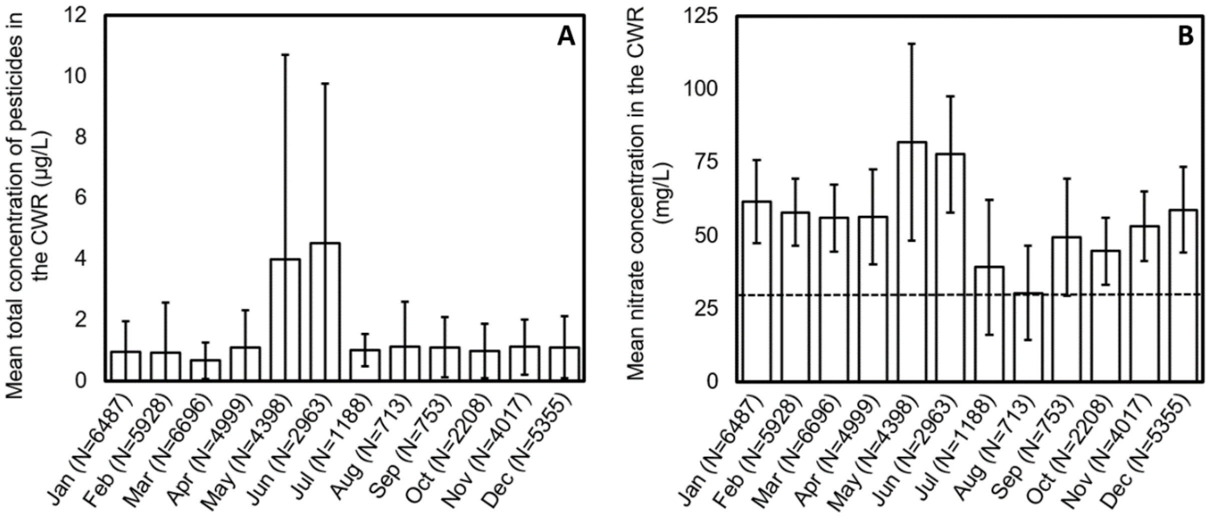


Fig. 1 (A) Pesticide concentration (µg/L) (531 molecules analyzed) (mean ± SD) and (B) nitrate (NO_3^-) concentration (mg/L) (mean ± SD) per month at the inlet of the CWR based on continuous sampling from 2012 to 2020. The dotted line represents the threshold of nitrate concentration for the poor–bad water quality class (≥ 30.1 mg/L) according to the "Guide to Assessing the State of Continental Surface Waters" (rivers, canals, bodies of water) (Ministère de la transition écologique et solidaire, 2019).

Selection of the comparison pond and study design

To have a baseline with which to compare the CWR, we looked for a pond with similar characteristics in terms of physiognomy, and landscape, geographically close to the CWR but potentially unexposed to agrochemical pressure. Using interactive cartography software, such as Geoportail (https://www.geoportail.gouv.fr/) and Google Earth (https://www.google.fr/earth/), we targeted ponds in Rampillon and the surrounding towns and prospected directly on the field. We found a pond (COMP) located approximately 2 km from the CWR, with a surrounding environment similar to that of the CWR (Fig. 2), i.e., located next to a meadow, crops, and a woodland. The configuration of this COMP pond, fed by forest run-off, not connected with a drainage network, and slightly raised above the crops, presumed no link with the surrounding crops and, thus, no or low contamination. Based on the long-term monitoring of the CWR, a contamination gradient has been identified within the CWR from upstream to downstream due to the self-purification action of the wetland (Lebrun et al., 2019; Letournel, Chaumont, et al., 2021). Consequently, we defined three zones of study following a potential decreasing gradient of contamination: (i) upstream CWR (US), (ii) downstream CWR (DS), and (iii) COMP. US and DS were separated by 80 m (Fig. 2). Pesticide analysis confirmed this chemical gradient (see the "Results" section: "Agrochemical dynamics and toxic units sum").

The study was conducted from March 7, 2022, to June 28, 2022, i.e., during the period when drainage is active, linked with agrochemical flows. We sampled water and took chemical and physical measurements, described below, in the three zones every fortnight from March 7, 2022, i.e., eight sessions (exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28). We deployed litterbags from March 7, 2022, in the CWR (US and DS) and from April 6, 2022, in COMP due to limited access to the pond in March.

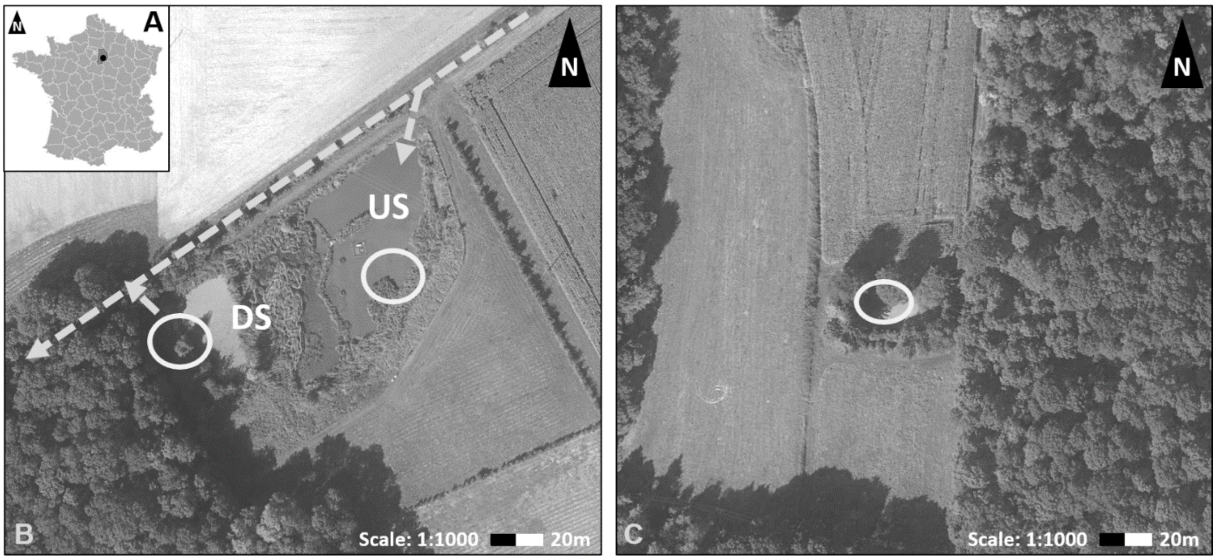


Fig. 2 (A) Location of the CWR (black dot) within the Seine-et-Marne department (France). (B) The CWR with differentiation into upstream (US) and downstream (DS) areas. The dotted arrow represents the stream "le ru des gouffres" that flows into sinkholes giving access to the Champigny aquifer. (C) The pond COMP. Circles represent deployment zones of litterbags. US and DS were separated by 80 m.

Land cover and characteristics of the ponds

To check environmental similarities between the CWR and COMP, the proportion of land cover types surrounding each pond (i.e., woodlands, meadows/grasslands, crops, urban/industrial lands) was determined in a 500-m radius buffer using QGIS 3.24.0-Tisler with the data package Theia OSO Land Cover Map 2021 (Thierion et al., 2022). General characteristics of the ponds considered important regarding the ecology of benthic macroinvertebrates (surface, presence or absence of fish, aquatic plant density, and level of tree occupancy along the bank) were determined directly in the field or using the mapping tool Geoportail (Table 1). Despite efforts to find a comparable site, significant differences remained between the two ponds; notably, COMP was characterized by a higher percentage of woodlands in a buffer of 500 m (55.3%) compared with the CWR (7.1%) (Table 1), but their adjacent environment was similar in structure (located next to a meadow and a woodland). The two ponds are permanent in terms of water level. Given these differences between the ponds, we sought to consider them a posteriori in the statistical analysis (see the “Statistical analyses” section).

Table 1 Environmental characteristics of the constructed wetland (CWR) and the comparison pond COMP in 2022.

		CWR	COMP
Land occupancy (500m radius)	Woodlands (%)	7.1	55.3
	Meadows/grasslands (%)	29.5	10.8
	Crops (%)	62.0	33.9
	Urban/industrial (%)	1.0	--
Pond characteristics	Surface (m^2)	5,600	1,000
	Fish	Presence	Absence
	Aquatic plants density (%)	40	1
	Bank afforestation (%)	5	95

The values for the percentage of aquatic plant density and bank afforestation were approximated in the field and using the mapping tool Geoportail

Physical and chemical conditions and water quality

The CWR is instrumented with monitoring stations at the inlet and the outlet that comprise automatic samplers managed for bi-monthly flow-weight sampling allowing us to estimate the average concentrations of pesticides and nitrate circulating upstream and downstream (Bahi, Sauvage, Payraudeau, Imfeld, et al., 2023; Bahi, Sauvage, Payraudeau, & Tournebize, 2023). Temperature was also monitored continuously upstream and downstream. For COMP, samples were taken manually for every session. All water samples followed a similar protocol by using 500-mL amber flasks, preserved at −20°C until pesticide analyses, and 15-mL Falcon tubes for nitrogen measures preserved at 4°C. Bi-monthly flow-weight samples from the CWR were also stored at −20°C for pesticide analyses. We also proceeded to do ad hoc water sampling in the CWR (US) for pesticide analysis to estimate the comparability and adequacy of the ad hoc pesticide measurements in COMP compared with the continuous monitoring in the CWR (US and DS). Although the continuous monitoring in the CWR is more integrative in terms of pollution events for total pesticide concentrations (integration of contamination data that can be higher during a flood, for example), the ad hoc measures were generally good proxies for the continuous measures. Thus, we could use the contamination data from COMP similarly to those from the CWR. In the three zones, multiparametric probes were used sporadically to measure temperature (°C), oxygen saturation (%), pH (no unit), and conductivity (µS/cm). Due to technical limitations, oxygen saturation, pH, and conductivity were not available for DS.

Overall, 531 pesticides and their metabolites were quantified in the samples by a subcontractor laboratory with a typical limit of detection of 0.01 µg/L. Of the 531 pesticides, 450 were below the quantification threshold (i.e., almost 85%). Concentrations of total pesticides, herbicides, fungicides, insecticides, and metabolites were determined. The mean of the physical and chemical parameters was calculated across the sessions of exp3, exp4, exp5, exp6, exp7, and exp8 and compared using histograms. We did not have contamination data (i.e., nitrate and pesticides) for the last session (exp8) for the CWR (US and DS), and thus we excluded the contamination measures of exp8 in COMP from the calculation of the mean contamination. We compared the concentrations of pesticides that were detected in at least one zone and that were listed in the "Guide to Assessing the State of Continental Surface Waters" (Ministère de la transition écologique et solidaire, 2019) with their respective environmental quality standard (EQS) values to report on the water quality of the study zones for each session.

Toxic units sum calculation

Since the total pesticide concentration of a given cocktail does not always correspond to its toxicity or toxic potential for wildlife (Rizzati, 2016), we also calculated the sum of toxic units (ΣTU). ΣTU is based on the relative toxicity of the pesticides quantified in our samples. Although pesticide cocktails are complex mixtures whose effects are difficult to predict because of synergistic or antagonistic interactions (Flores et al., 2014; Weisner et al., 2021), the calculation of ΣTU assumes additive toxic effects between pesticides. This approach is widely accepted and provided satisfactory results in several studies (Hela et al., 2005; C. S. Qu et al., 2011; Weber et al., 2018). The calculation was performed for each zone and each session as follows (1):

$$\sum_{i=1}^{n} \mathrm{TU} = \frac{C_i}{\mathrm{TV}_i} \qquad \text{Sum of toxic units} \qquad (1)$$

where ΣTU is the summed toxic unit for the pesticides quantified in our samples, for a given zone and session; C_i is the concentration of the pesticide i; TV is a toxicity value (Effective Concentration 50, EC_{50}, or No Observed Effect Concentration, NOEC, or Lowest Observed Effect Concentration, LOEC) of the pesticide i (see below). Corresponding levels of ecological risk are as follows: ΣTU = 0.01 low risk, ΣTU = 0.1 medium risk, ΣTU = 1 high risk, and ΣTU > 1 = very high risk (Hela et al., 2005; C. S. Qu et al., 2011).

We collected toxicity data for invertebrates (Appendix 1-2) and algae (Appendix 3-4) on the websites Pesticide Properties DataBase (https://sitem.herts.ac.uk/aeru/ppdb/) and Substances chimiques INERIS (https://substances.ineris.fr/). As in Brosed et al. (2016), we distinguished between invertebrate TU and algae TU in order to study the effects of pesticides on ecological variables for benthic macroinvertebrates (see the “Diversity and sensitivity indices” section) and microorganisms respectively (see the “Leaf-litter breakdown” section). Although decomposer microbes are not algae, the use of TU algae is currently one of the most widely used method for studying the effects of pesticides on the microbe-driven leaf-litter breakdown (Brosed et al., 2016; Rossi et al., 2019). In addition, we distinguished acute and chronic toxicity data, because in our case, we expect different responses depending on the type of toxicity. Aquatic invertebrates living in the CWR have been chronically exposed to pesticides for several years. On the other hand, short but intense pesticide flows can occur in the system due to rainfall events notably. The CWR is therefore supposedly an environment where both types of toxicity can affect aquatic organisms and we aimed to understand how each of them affect those organisms.

Too few toxicity data were available for pesticide metabolites, especially for chronic toxicity data, so we had to exclude metabolites from the calculation of toxic units in further analyses (see the “Statistical analyses” section).

Benthic macroinvertebrate and leaf-litter breakdown monitoring

We monitored benthic macroinvertebrate and leaf-litter breakdown every fortnight using litterbags, commonly used in aquatic and terrestrial studies (Gartner & Cardon, 2004; Pascoal et al., 2005), from March to June 2022, when high pollution flow events occur.

We collected fallen alder leaves (*Alnus glutinosa* (Gaertn., 1790)), known for their attractiveness for shredders (Hladyz et al., 2009), in autumn 2021 on the shore of a pond at Aulnoy (France). Leaves were washed with tap water and dried in the oven at 60°C for 48 h. Two types of mesh were used. Coarse mesh and fine mesh, respectively, are designed to assess the contribution of both invertebrates and microorganisms or only invertebrates (i.e., total degradation or invertebrate-driven litter breakdown) and only microorganisms (i.e., microbe-driven litter breakdown) to the litter decomposition (Tiegs et al., 2009). Thus, before each field session, leaves were weighed and bagged, with 2.5 g of leaves in coarse-mesh bags (“macrobags”: 18 × 18 cm, 5 mm mesh size) and 0.5 g of leaves in fine-mesh bags (“microbags”: 10 × 10 cm, 0.5 mm mesh size). Each microbag was inserted into one matched macrobag and each microbag–macrobag pair corresponded to one litterbag.

The litterbags were immersed in triplicate and anchored to the pond-bed in each zone (US, DS, and COMP). The mean duration of the immersion of each litterbag was 15 ± 2 days per session, over the eight sessions (exp1 to exp8) from March 7 to June 28, 2022, for US and DS

(CWR). For COMP, it was over six sessions only (exp3 to exp8) from April 6 to June 28, 2022. A total of 66 litterbags were used (8 sessions × 3 litterbags × 2 zones for the CWR and 6 sessions × 3 litterbags for COMP). Litterbags from the same session were thus all immersed in water for about 15 days, then all removed and completely replaced by litterbags from the new session, at the same place, making the litterbags independent of each other between sessions. Colonization time is an important factor determining the structure and composition of invertebrate assemblages, in both lotic (Ligeiro et al., 2010) and lentic hydrosystems (Poi et al., 2017; Silveira et al., 2013). However, in our study, an immersion time of about 15 days seemed to allow sufficient colonization of litterbags by benthic macroinvertebrates to assess litter breakdown. In addition, the similar duration of exposure between the different sessions makes the results all the same comparable. At the end of each immersion session, the litterbags were carefully lifted one at a time from the pond-bed and put in a fine-mesh sieve (0.5-mm mesh size), all in the water, to avoid invertebrate leaks. Then, the litterbag was rinsed with pond water to retrieve benthic macroinvertebrates that were preserved in 70% ethanol for later identification. Leaves were gently washed, dried in the oven at 60°C for 48 h, and weighed. The difference between the weight before bagging and the weight after drying was used to quantify the litter degradation (see the "Leaf-litter breakdown" section). Macroinvertebrates were sorted, counted, and identified to the lowest taxonomic level possible following the method of Tachet et al. (2010).

Community composition

To evaluate the potential spatial and temporal differences in community composition according to zones, sessions, and zones–sessions, we analyzed the benthic macroinvertebrate community composition through correspondence analysis (CA) (ADE-4, 2001) (see Thioulouse et al., 1997) using the abundances of the 57 identified taxa within the three zones (US, DS, and COMP) and the eight sessions (exp1 to exp8). We transformed abundances according to a logarithm transformation (i.e., ln(X+1), with X corresponding to the abundance of a given taxon) to limit the skewness of abundance distribution due to the overrepresentation of the few most common and highly abundant species (Legendre & Legendre, 2012).

Diversity and sensitivity indices

To assess community diversity and sensitivity, we calculated the following indices: the total richness as the number of taxa (S), the Shannon index (H'), the $SPEAR_{pesticides}$ index (Liess & Ohe, 2005), and the relative abundance of GOLD (Gastropoda, Oligochaeta, and Diptera) (Czerniawski et al., 2020; Timm & Haldna, 2019).

The Shannon index (H') allowed us to assess the diversity of species in the environment while accounting for their relative abundances (see Appendix 5 for the formula).

The $SPEAR_{pesticides}$ index ($SPEciesAtRisk_{pesticides}$ index) characterizes the sensitivity of freshwater invertebrate communities to pesticides (Beketov et al., 2009) and is considered pertinent in studying the effects of pesticide exposure on community composition (Schäfer, 2019). In our study, we expected lower values of $SPEAR_{pesticides}$ than the values classically obtained in lotic ecosystems, since freshwater bodies generally support less sensitive species compared with lotic ecosystems (Biggs et al., 2007). The $SPEAR_{pesticides}$ index was determined using the software SPEAR Calculator on the web application Indicate (version 2.3.1) (https://www.systemecology.de/indicate/) of the UFZ Helmholtz Centre for Environmental Research (Becker & Liess, 2023) (see Appendix 5 for the formula).

The GOLD index was calculated as the percentage of Gastropoda, Oligochaeta, and Diptera in the total abundance of macroinvertebrates. A high GOLD index is generally linked with poor ecological status (Czerniawski et al., 2020; Timm & Haldna, 2019).

Leaf-litter breakdown

The leaf-litter breakdown rate (grams of dry weight per day) was calculated, respectively, for the microbial ($k_{microbes}$, in microbags) and the invertebrate ($k_{invertebrates}$, in macrobags) compartments, for each zone, each replicate, and each session. We subtracted the microbial contribution from litter degradation to determine the macroinvertebrate contribution in accordance with microbag-macrobag pairs. $k_{microbes}$ and $k_{invertebrates}$ were determined as follows (2, 3):

$$k_{microbes} = \left(\frac{W_0 - W_t}{t}\right)_{microbag}$$ Microbe-driven leaf-litter breakdown rate (2)

$$k_{invertebrates} = \frac{(W_0 - W_t)_{macrobag} \times \left(\frac{W_t}{W_0}\right)_{microbag}}{t}$$ Invertebrate-driven leaf-litter breakdown rate (3)

where k is the leaf-litter breakdown rate (g_{dw}/day), W_t is the remaining dry weight at time t, W_0 is the initial dry weight, and t is the number of days litterbags were immersed in water over the session.

Calculation of functional trait frequencies

To account for the influence of shredders and scrapers on leaf-litter breakdown rates, we used the bioecological trait database from Tachet et al. (2010). We determined the relative frequency of use of the two modalities "shredder" and "scraper" of the feeding habits trait in the leaf-litterbags community (see Usseglio-Polatera et al., 2000 and Tachet et al., 2010 for further information). The relative frequency of shredders/scrapers was calculated for each zone and each session as follows (4):

$$S_{\mathrm{shredder/scraper}} = \frac{\sum_{k=1}^{S_k} Q_k X_{(s/s)k}}{\sum_{k=1}^{S_k} \sum_{i=1}^{n_i} Q_k X_{ik}} \qquad \text{Shredder/scraper trait modality frequency (4)}$$

where $S_{\mathrm{shredder/scraper}}$ is the relative frequency of the shredder/scraper modality compared with the n_i modalities of the feeding mode trait in the sample, Q_k is the abundance of the sampled taxon k, S_k is the total number of taxa in the sample, $X_{(s/s)k}$ is the affinity rating of the modality shredder/scraper of the taxon k, X_{ik} is the affinity rating of the modality i of the taxon k, and n_i is the total number of feeding mode modalities.

Statistical analyses

We aimed to assess the average response of community diversity and litter decomposition to pollution level across all observation sessions. It is important to note that these observation sessions were considered as independent in time because the bags were replaced every 15 days (see the “Benthic macroinvertebrate and leaf-litter breakdown monitoring” section), so the community observed in the bags had undergone a reset at every session. In order to assess the average response of community diversity and litter decomposition while accounting for other uncontrolled environmental factors, we used general linear modeling in which each statistical unit corresponds to a different zone-session-bag community.

Pollution levels were estimated through two complementary approaches, pesticide concentration and sums of toxic units. As we lacked of toxicity data on pesticide metabolites, and to avoid a bias in the calculation of toxic units, we considered that the use of total pesticide concentrations (taking into account metabolites) could provide valuable information, given the completeness of our sampling methodology (531 pesticides analyzed). The total concentrations of the different families of pesticides (i.e., herbicides, fungicides, insecticides, and metabolites) were inter-correlated. Thus, we did not study the effects of the different pesticide families on our ecological variables in our models to limit collinearity issues.

We ran general linear models (GLM) using R software (version 4.3.3, R Core Team 2024, package {stats}) generally built as follows: Y ~ X1 + X2 + (…) + Xn, where Y was the response variable (taxonomic richness, Shannon index, $\mathrm{SPEAR}_{\mathrm{pesticides}}$, % GOLD, invertebrate-driven leaf-litter breakdown ($k_{\mathrm{invertebrates}}$), microbe-driven leaf-litter breakdown (k_{microbes}), shredder/scraper modality frequencies), and X1 to Xn were the explanatory variables reflecting pesticides pollution levels and environmental conditions (see below). We ran four groups of these GLMs depending on the pollution level estimation we used, namely: (i) concentrations of total pesticides (Model 1: $[pesticide]_{tot}$), (ii) concentrations of pesticides without metabolites (Model 2: $[pesticide]_{wo\ metab}$), (iii) toxic units sum based on acute toxicity data, without metabolites (Model 3: $\Sigma TU_{acu,\ wo\ metab}$), and (iv) toxic units sum based on chronic toxicity data, without metabolites (Model 4: $\Sigma TU_{chr,\ wo\ metab}$) (resulting in a total of 7 × 4 = 28 models). The explanatory variables from X1 to Xn were the variable related to pesticides pollution levels (either (i), (ii), (iii), or (iv)), concentrations of nitrate (NO_3^-), and temperature. For the model of the $k_{invertebrates}$ response, we added the shredder/scraper frequencies as an explanatory variable given the potential role of these organisms in the ecosystem function. In order to account for potential environmental differences between the two ponds – CWR vs. COMP – that we could not measure or control for in the model (e.g., pond age, history, habitat diversity), we included the pond as an additional factor of effect in the model (pond factor with two levels: CWR and COMP, with COMP put in the intercept of the model). Finally, for each of the 28 models, we highlighted and summarized the explanatory variables that significantly explained the different biological responses. The significance threshold was set at $P < 0.05$ for all tests.

Before using GLM, Shapiro–Wilk (version 4.3.3, R Core Team 2024, package {stats}) and Levene's tests (R package {car}; Fox & Weisberg (2019)) were performed to test, respectively, data normality and the homogeneity of variance for the continuous variables Shannon index, $SPEAR_{pesticides}$ index, and leaf-litter breakdown rates. As the variables %GOLD and shredder/scraper trait modality frequency were proportions, they were transformed according to the arcsine square root transformation before applying the GLM (see Appendix 6-9). The collinearity among explanatory variables was evaluated through variance inflation factor (VIF) analysis (R package {car}; Fox & Weisberg (2019)) using a tolerance threshold of 3 (Zuur et al., 2010). Four explanatory variables posed significant collinearity issues (VIF > 3), namely, the oxygen saturation, the conductivity, the pH, and the presence of fish. We ran GLMs as described above, for each response variable, with one of the four pesticide variables, and nitrate, temperature, oxygen saturation, conductivity, and pH as explanatory variables, but without the pond effect, to highlight the high VIF values specifically for oxygen saturation, conductivity, and pH. We did not include the "presence of fish" variable, as it considerably increased the VIF of all the other variables, which was already high without the addition of this variable (Appendix 10). Consequently, we had to exclude these four variables from the models but considered that their effect should be to some extent covered by the "pond effect" introduced as an explanatory factor. McFadden's pseudo-R-squared was calculated to assess the GLM goodness of fit (Appendix 6-9) as follows (5):

$$R^2 = 1 - \frac{\log \text{likelihood}_{\text{model}}}{\log \text{likelihood}_{\text{null}}} \quad \text{McFadden's R-squared} \quad (5)$$

where log likelihood$_{\text{model}}$ is the log likelihood value of the current fitted model and log likelihood$_{\text{null}}$ is the log likelihood value of the null model (the null model includes only an intercept as predictor).

Although we considered the different sessions to be independent of each other, indirect temporal autocorrelation of litterbags among sessions is possible if we consider the total CWR community as the source of litterbag colonizers. Although this link is indirect, and thus, likely to be negligible, we checked the temporal autocorrelation of all our response variables to limit any interpretation bias. To do this, we performed an autocorrelation function estimation (acf) (version 4.3.3, R Core Team 2024, package {stats}). We calculated and used the average values of each variable, by session and by zone for this analysis (N = 8 for US and DS, N = 6 for COMP). None of the response variables studied was temporally autocorrelated (Appendix 11). Temporal autocorrelation was thus confirmed as negligible, so we did not consider it in our GLMs.

To further explore responses within particular observation sessions, we graphically represented the patterns of the different ecological responses described above according to zones and sessions (Appendix 12-13).

Results

Agrochemical dynamics and toxic units sum

All water quality data (pesticide concentrations, nitrate concentrations, and toxicity pesticide cocktails) by zone and session are summarized in Appendix 14. The mean pesticide contamination, calculated from exp3 to exp7, was about 4 times higher in US (25.11 ± 18.10 µg/L) than in DS (6.78 ± 3.82 µg/L), respectively, and more than 20 times higher than COMP (0.30 ± 0.04 µg/L) (Fig. 3). The mean nitrate concentration was relatively high in the CWR (92.83 ± 34.50 mg/L for US and 42.74 ± 50.37 mg/L for DS), while it was about 33 times lower in COMP (2.83 ± 0.57 mg/L) compared with US. The most widely represented pesticide families in the samples were herbicides and metabolites, with peaks observed in the CWR from exp5 (Fig. 3A). No insecticides were detected in COMP. Nitrate concentrations were generally high in US and DS, and the highest concentrations were found in US and DS during exp3 (linked with heavy rainfall events during this period, see Appendix 15), and during exp5, exp6, and exp7 in US (Fig. 3B). The peak pesticide concentration in US during exp5 was particularly high

(46.58 µg/L). These data allowed us to confirm that the zones followed a decreasing gradient of agrochemical contamination: US > DS > COMP (Fig. 3).

Environmental quality standard (EQS) values of some pesticides were surpassed for US and DS during exp3-exp7 (Table 2). The pesticides that exceeded the EQS were chlorotoluron, metazachlor, nicosulfuron, and diflufenican. For nitrate, the threshold value of 30.1 mg/L above which the ecological status is considered poor–bad was surpassed during all sessions for US and during exp1 and exp3 for DS (Fig. 3B).

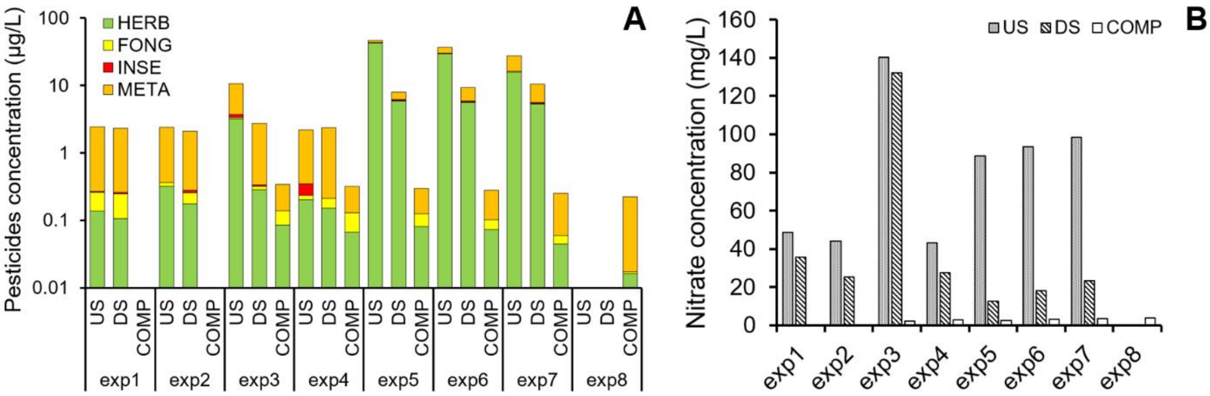


Fig. 3 (A) Pesticide concentration (µg/L) in the three zones (logarithmic scale), and (B) nitrate NO_3^- concentration (mg/L). See Appendix 14 for detailed values. HERB = herbicides, FONG = fungicides, INSE = insecticides, META = metabolites. Zones: US = CWR upstream, DS = CWR downstream, and COMP = comparison pond. Sessions: exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28.

Table 2 Concentrations (µg/L) of 12 pesticides detected (considering a limit of quantification of 0.01 µg/L) in at least one zone and their respective environmental quality standard values (EQS) (µg/L) (see Ministère de la transition écologique et solidaire (2019)). Concentration values in bold correspond to an exceedance of the respective EQS.

Pesticide	EQS - Annual mean (µg/L)	exp1			exp2			exp3			exp4			exp5			exp6			exp7		
		US	DS	COMP	US	DS	COMP	US	DS	COMP	US	DS	COMP	US	DS	COMP	US	DS	COMP	US	DS	COMP
Chlorotoluron	0.1	--	--	NA	0.073	0.032	NA	**0.633**	0.019	--	--	--	--	--	0.012	--	--	0.006	--	--	--	--
Metazachlor	0.019	--	--	NA	--	--	NA	--	--	--	--	--	--	**0.028**	--	--	0.014	--	--	--	--	--
Nicosulfuron	0.035	--	--	NA	--	--	NA	--	--	--	--	--	--	--	--	--	**0.365**	**0.125**	--	**0.730**	**0.250**	--
AMPA	452	--	0.017	NA	--	0.016	NA	0.045	0.018	NA	--	0.015	NA	0.067	0.043	NA	0.069	0.042	NA	0.070	0.041	NA
Glyphosate	28	--	--	NA	--	--	NA	0.033	--	--	--	--	--	0.130	0.012	--	0.083	0.006	--	0.036	--	--
Bentazone	70	0.082	0.058	NA	0.063	0.052	NA	0.089	0.059	--	0.060	0.048	--	0.061	0.034	--	0.057	0.017	--	0.053	--	--
Diflufenican	0.01	--	--	NA	--	--	NA	**0.220**	**0.039**	--	**0.022**	**0.025**	--	**0.023**	--	--	**0.024**	--	--	**0.024**	--	--
Imidacloprid	0.2	--	--	NA	--	--	NA	--	--	--	--	--	--	0.012	--	--	0.006	--	--	--	--	--
Azoxystrobin	0.95	--	--	NA	--	--	NA	--	--	--	--	--	--	0.071	0.013	--	0.111	0.019	--	0.150	0.024	--
Tebuconazole	1	--	--	NA	--	--	NA	--	--	--	--	--	--	0.014	--	0.010	0.087	0.011	0.010	0.160	0.022	--
Pendimethalin	0.02	--	--	NA	--	--	NA	0.016	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Simazine	1	--	--	NA	--	--	NA	--	--	0.010	--	--	0.020	--	--	0.020	0.006	--	0.020	0.011	--	0.020

The sum of toxic units based on invertebrate and algae acute toxicity data (ΣTU_{acute}) and on algae chronic toxicity data ($\Sigma TU_{chronic}$) followed this decreasing gradient, just as the pesticide contamination gradient: US > DS > COMP (Figs. 3A vs. 4A, C, D). However, invertebrate chronic toxicity data followed this gradient: US < COMP < DS (metolachlor, ethofumesate, terbuthylazine, and tebuconazole were downgrading for DS while prosulfocarb and tebuconazole were for COMP). $\Sigma TU_{chronic}$ for invertebrates in DS were particularly high from exp5 (Fig. 4B). ΣTU for algae were overall higher than ΣTU for invertebrates (Fig. 4A, B, C, D).

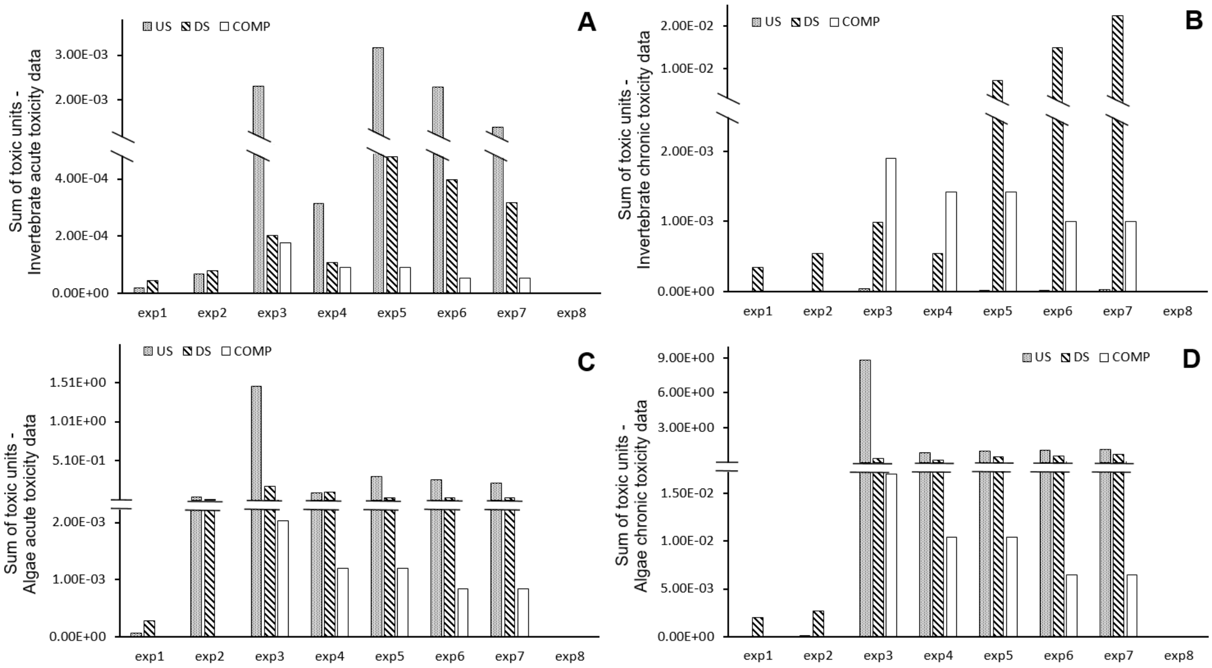


Fig. 4 Sum of toxic units (ΣTU, without unit) (A) based on invertebrate acute toxicity data, (B) based on invertebrate chronic toxicity data, (C) based on algae acute toxicity data, and (D) based on algae chronic toxicity data, in the three zones, for the eight sessions. See Appendix 14 for detailed values. Zones: US = CWR upstream, DS = CWR downstream, and COMP = comparison pond. Sessions: exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28.

Water physical and chemical conditions

Water temperature was slightly lower in COMP than in the CWR (Fig. 5A). The oxygen saturation was significantly lower in COMP than in the CWR (Fig. 5B). The same trend as for oxygen saturation was also observed for conductivity and pH (Appendix 16).

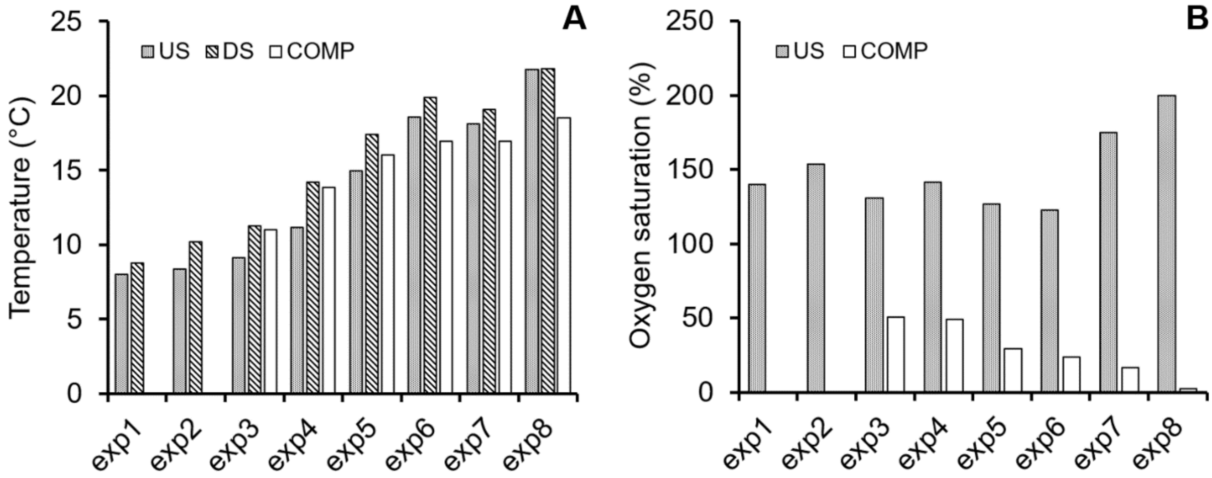


Fig. 5 Evolution of water (A) temperature (°C) and (B) oxygen saturation (%) over time during the 2022 survey (means). Zones: US = CWR upstream, DS = CWR downstream, and COMP = comparison pond. Sessions: exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28.

Community composition

In total, 57 taxa were identified in all of the samples taken into account. See the Appendix section for the list of identified taxa (Appendix 17). In terms of total abundances, the most widely represented taxa of macroinvertebrates in the CWR were Oligochaeta in US (57% of the total abundance) and Diptera (42% of the total abundance) in DS, while no order was as strongly dominant in COMP (see Appendix 18). Elmidae, Hydraenidae, Culicidae, Neritidae, Naucoridae, Nepidae, and Sialidae were present only in DS. Certain taxa, such as Hygrobiidae, Stratiomyidae, Ameletidae, and Libellulidae, were present only in COMP (see Appendix 18). Based on the results of the CA, there was a clear separation of the three zones according to F1 axis, with a gradient ranging from US, which was characterized by taxa represented in the negative part of the first factorial axis, to COMP, characterized by taxa in the positive part of the first factorial axis (Fig. 6A, B). DS was represented by Hirudinea and Odonata more so than US and COMP were. From exp5, there was a community shift in US, and particularly in COMP (Fig. 6A, D). Community composition of exp5, exp6, and exp7 seems very close (Fig. 6C) and exp8 was separated from the others (see Appendix 17 for the correspondence between tags and taxa).

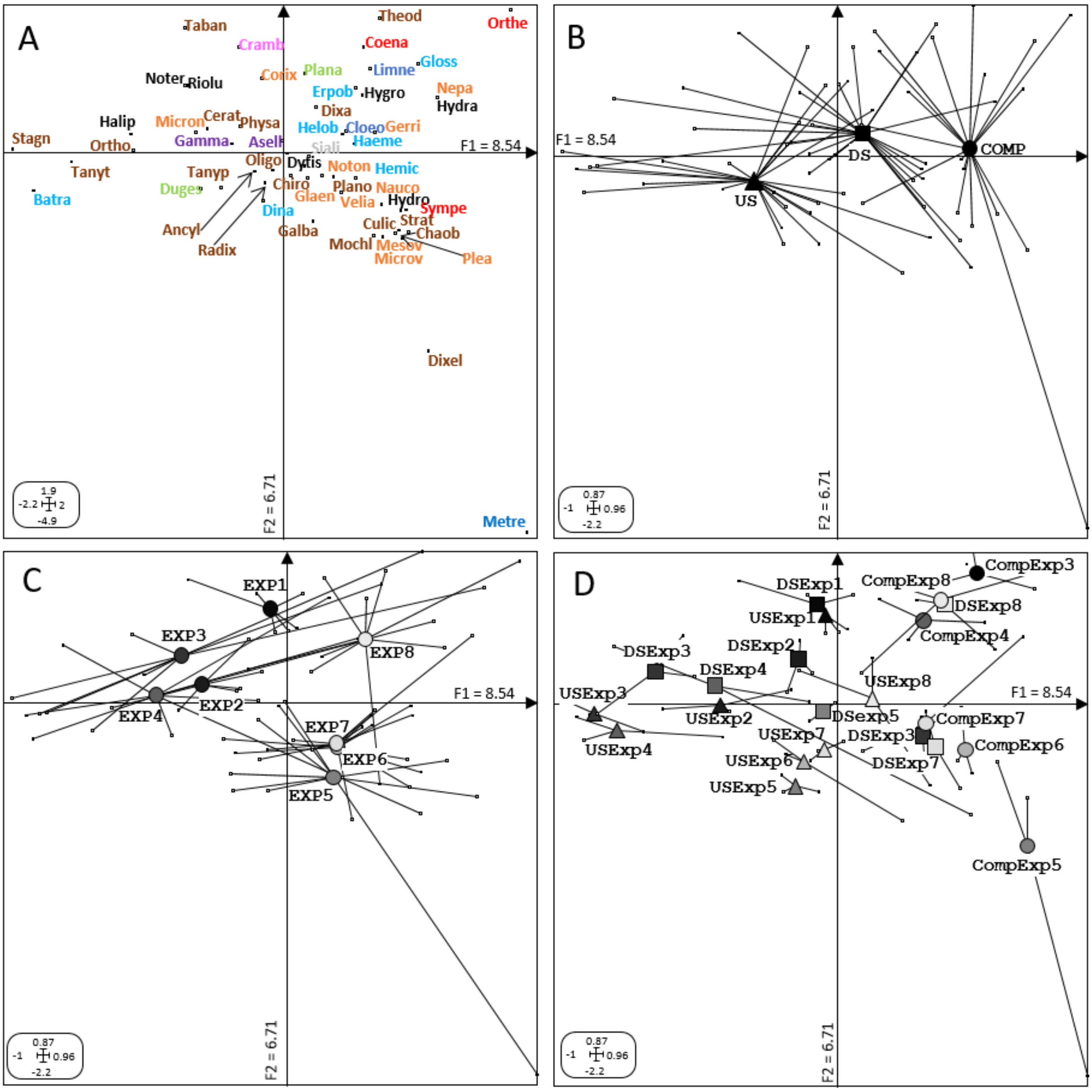


Fig. 6 Results of the correspondence analysis (CA). (A) Distribution of taxa on the F1–F2 factorial map. Taxa tags are colored according to the order that taxa belong to: Coleoptera = black, Crustacea = purple, Ephemeroptera/Trichoptera = dark blue, GOLD (Gastropoda/Oligochaeta/Diptera) = brown, Heteroptera = orange, Hirudinea = sky blue, Lepidoptera = pink, Megaloptera = grey, Odonata = red, and Turbellaria = green. See the Appendix section for the correspondence between tags and taxa (Appendix 17). (B) Ordination of zones (solid triangle, square, and circle) and litterbags (small squares) on the F1–F2 factorial map. Zones: US = CWR upstream (solid triangle), DS = CWR downstream (solid square), and COMP = comparison pond (solid circle). Zones are located at the weighted average of the corresponding litterbags. (C) Ordination of sessions (solid circles, from black to light grey) and litterbags (small squares) on the F1–F2 factorial map. Sessions: exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28 (from black to light gray). Sessions are located at the weighted average of the corresponding litterbags distribution of litterbag replicate on the F1–F2 factorial map. (D) Ordination of sessions × zones (solid triangles, squares, and circles, from black to light gray) and litterbags (small squares) on the F1–F2 factorial map. Sessions × zones are located at

the weighted average of the corresponding litterbags. The numbers shown along axes F1 and F2 correspond to the percentage of inertia.

General linear models

The explanatory variables used in the GLM had no collinearity issue (Appendix 6-9). For a given ecological response, the four models showed generally similar results (Table 3; see detailed results from GLMs in Appendix 6-9). All responses were positively linked to temperature except the SPEAR index and the shredder/scraper trait modality frequency. Pesticides had a significant negative influence on the Shannon index and on the shredder/scraper trait modality frequency in all models except the Model 4 using chronic toxicity as pesticide level indicator. Model 4 ($\Sigma TU_{chr,\ wo\ metab}$) particularly differed from the others in that it showed: (i) a negative effect of pesticides on the invertebrate-driven leaf-litter breakdown ($k_{invertebrates}$) that was not observed in the other models, and (ii) a qualitative shift in the significant variables explaining the shredder/scraper trait modality, from a positive effect of pond factor and negative effect of pesticides to negative effects of temperature and nitrate. The SPEAR index did not respond to pesticide level whatever the model but responded negatively to the pond factor (CWR) (Table 3).

Table 3 Summary of the GLM results (detailed in Appendix 6-9) highlighting the explanatory variables significantly correlated with the ecological responses. For the sake of clarity, the generic term "Pesticides" is used for all model groups in this table. However, this term refers to total pesticide concentration in Model 1, pesticide concentration without metabolites in Model 2, ΣTU_{acute} without metabolites in Model 3, and $\Sigma TU_{chronic}$ without metabolites in Model 4. The color indicates the sign of the relationship: blue = positive, red = negative. For each model and ecological response, the explanatory variables are reported in each cell from top to bottom in the decreasing order of their regression coefficients.

	Model 1 ([pesticide]tot)	Model 2 ([pesticide]wo metab)	Model 3 (ΣTUacu, wo metab)	Model 4 (ΣTUchr, wo metab)
Taxonomic richness	**Temperature**	**Temperature** **NO_3^-**	**Temperature**	**NO_3^-**
Shannon index	**Pesticides** **Temperature**	**Pesticides** **Temperature**	**Pesticides** **Temperature**	
SPEAR pesticides index	**Pond(CWR)** **Temperature**	**Pond(CWR)** **Temperature**	**Pond(CWR)**	**Pond(CWR)** **Temperature**
% GOLD (Abundance)	**NO_3^-** **Temperature**	**NO_3^-** **Temperature**	**Temperature**	**Temperature** **NO_3^-**
k invertebrates	**Temperature**	**Temperature**	**Temperature**	**Temperature** **Pesticides**
k microbes	**Temperature**	**Temperature**	**Temperature**	**Temperature**
Shredder + scraper trait modality	**Pond(CWR)** **Pesticides**	**Pond(CWR)** **Pesticides**	**Pond(CWR)** **Pesticides**	**Temperature** **NO_3^-**

Discussion

In this study, we tested the influence of pesticides and nitrate on the structure and function of benthic macroinvertebrates living in a contaminated agricultural constructed wetland compared with a low-impacted/uncontaminated comparison pond in order to determine the potential of the constructed wetland to act as either an ecological trap or a refuge for aquatic fauna. Overall, our results showed that pesticides in the CWR possibly in combination with nitrate were related with disruptions in the structure of the benthic macroinvertebrate community by decreasing diversity significantly. In addition, pesticides appeared to be responsible for disruption in the invertebrate-driven leaf-litter breakdown, and tend to support the hypothesis that the CWR may act as an ecological trap.

The CWR as an ecological trap for benthic macroinvertebrates due to pesticides

In our study, the negative response of three ecological variables, namely, the Shannon index, the shredder/scraper trait modality frequency, and $k_{invertebrates}$, suggests that the pesticide pressure was able to disrupt both community structure and function in benthic macroinvertebrates, and that the CWR could act as an ecological trap. Pesticides have been shown to be able to decrease taxonomic richness both in the field (Ito et al., 2020) and in mesocosm conditions (Bhattacharyya et al., 2023), even at low doses (Ward et al., 1995). Meanwhile, the Shannon index has been described as unreliable in several studies when studying effects of pesticides on freshwater invertebrate because of its lack of specificity in multi-stress environments (Ippolito et al., 2012; Knillmann et al., 2018; Schäfer et al., 2011). Thus, the fact that most approaches to quantifying pesticide levels showed a negative effect on the Shannon index could suggest that the pesticide pressure was substantial enough to drive the response of this particular index. In addition, the negative effect of pesticides on shredders/scrapers, possibly linked with the negative effect observed on the Shannon index, may have repercussions on leaf-litter breakdown as highlighted by Schäfer et al. (2007). We further showed the negative effect of pesticides on $k_{invertebrates}$ with the chronic toxicity approach. To explain this result, a negative structure-function response of benthic macroinvertebrates to pesticides in the CWR, we expected a link between the disruption of shredders/scrapers and the litter breakdown impairment.

However, we did not detect a significant link between the frequency of shredder/scraper organisms and the leaf-litter breakdown rate, which effectively calls into question the potential combined structure-function effect described above. The link between shredders and litter breakdown is well established in the literature, for streams (Jonsson et al., 2001) or a wetland context (Gingerich et al., 2015). Our result may be due to biological reasons, such as other feeding modes playing a major role in the process depending on the decomposition stage of the litter. Indeed, Gingerich et al. (2015) showed that oligochaetes and omnivores had a strong association with the second phase of litter decomposition. This result may also be due to methodological limitations, in that our data and model may not allow us to detect such a complex, indirect link. Notably, bioecological trait databases are not always sufficient for describing these processes in such detail: these species-level traits cannot integrate inter-individual trait variability within the community and rarely allow establishing direct links with ecosystem functions (see Jeliazkov & Chase, 2024).

In parallel, although no effect of pesticides was detected on the $SPEAR_{pesticides}$ index, the negative pond effect on this index may suggest that the long-term agrochemical stress has already exerted a selective pressure on pesticide-sensitive taxa (SPEAR). In general, the $SPEAR_{pesticides}$ index is negatively associated with pesticides (Hunt et al., 2017; Münze et al., 2015). Moreover, it has been shown that long-term occurrence of agricultural stressors in ponds could reduce the sensitivity of benthic invertebrate communities (Trau et al., 2024). Thus, if no pesticide effect on sensitive species occurred during our study, but the pond exerts a negative effect on the $SPEAR_{pesticides}$ index, then this could be the observation of a long-term negative effect of pesticide pressure on these sensitive species in the CWR (i.e., SPEciesAtRisk disadvantaged). In addition, the pressure would still be able – despite tolerance acquisition by organisms with long-term exposure – to affect the equitability of the community, as highlighted by the negative relation between pesticides and the Shannon index. This lends credence to the hypothesis that CWR is acting as an ecological trap. However, it is important to keep in mind that other environmental characteristics or pressures can influence the $SPEAR_{pesticides}$ index, such as geochemical conditions (e.g., nutrients, sediment) (Bray et al., 2021), habitat characteristics (Rasmussen et al., 2011; Rico et al., 2016), the presence of riparian forests (Orlinskiy et al., 2015), and the presence of fish in ponds (Moura E Silva et al., 2023). Hence, specific characteristics of the CWR could have also affected SPEAR for a long period, possibly in the place of or in combination with pesticides, such as the presence of fish or the smaller proportion of woodland compared with COMP. Besides, $SPEAR_{pesticides}$ index was developed for lotic water bodies, and its application for lentic hydrosystems is limited (Trau et al., 2024), so our conclusions on the response of this index need to be weighed, although it seemed sensitive, at least to the pond effect, in our study.

Finally, failure to detect an adverse effect on $k_{microbes}$ may be due either to the model not being able to detect an effect that actually exists or, for example, to differences in sensitivity between invertebrates and microbes, with microbes potentially being more tolerant to pesticides than invertebrates. For instance, Brosed et al. (2016) showed that the leaf-litter breakdown rate decrease due to pesticides in agricultural streams was mainly due to a decline in the invertebrate-driven breakdown, whereas the microbe-driven breakdown was unaffected. Some authors even showed the innocuousness of multiple pesticide loads on aquatic fungal communities and the associated leaf-litter breakdown process (Talk et al., 2016). In our study, nonetheless, the ΣTU calculation seems to indicate the opposite ($\Sigma TU_{algae} > \Sigma TU_{invertebrates}$). However, although ΣTU_{algae} has been used in several studies to investigate the effects of pesticides on microbe-driven leaf-litter breakdown (Brosed et al., 2016; Rossi et al., 2019), the calculation is based on algal toxicity data, and the very high proportion of pesticides quantified in our samples correspond to herbicides (to which algae are very sensitive), which may overestimate the toxicity of the cocktail. Calculated toxicity may not fully reflect the actual toxicity for the microbial community. Nevertheless, even if not all the causal links are demonstrated, and the reality of the field remains complex, our results tend to show that CWR could act as an ecological trap.

Potential synergistic effects between nitrate and pesticides

In addition to the pesticides that induced direct disruptions in benthic macroinvertebrates, possibly in combination with pesticides, nitrate tended to influence positively certain pollution-tolerant taxa as evidenced by its positive link with %GOLD. The high nitrate concentrations in US, which can be associated with important primary production, may explain the proliferation of GOLD in this zone. In fact, nutrients have already been shown to be positively correlated with the abundance of certain taxa such as Diptera (Jahnke et al., 2001), which are generally considered pollution-tolerant invertebrates (Boudeffa et al., 2020), but also with the density and richness of Gastropoda (Ghosh & Panigrahi, 2018). Although nitrate was not capable of generating acute toxic effects on benthic macroinvertebrates, it was likely to favor GOLD in combination with pesticides. In fact, the highest nitrate concentration measured in the CWR (about 140 mg/L) was far from the acute toxicity values determined by Soucek & Dickinson (2012) for some sensitive freshwater invertebrates (lethal concentration – LC_{50} of more than 1.5×10^3 mg/L NO_3^- even for the most sensitive species studied, i.e., the bivalve *Lampsilis siliquoidea* (Barnes, 1823)). However, the CWR was still characterized by the poor–bad ecological status linked with the nitrate contamination (in CWR-US mainly). Moreover, several authors have shown that the combined use of pesticides and fertilizers tended to favor the dominance of pollution-tolerant species in semi-natural conditions (Barmentlo et al., 2019; Polazzo et al., 2022). Thus, considering the high nitrate concentrations in the CWR, and knowing the existence of the link between fertilizers and pesticides, the positive effect of nitrate detected on %GOLD could be due to a synergistic effect between nitrate and pesticides. In the CWR, the joint presence of nitrate and pesticides is thus likely to be able to favor the dominance of pollution-tolerant taxa.

Benefits of the four approaches to pollution level

Comparing our results from the four approaches to pollution level ((i) total pesticide concentration, (ii) pesticide concentration without metabolites, (iii) ΣTU acute without metabolites, (iv) ΣTU chronic without metabolites) allowed us to challenge the robustness of our results and to identify the most consistent responses across our models. Pesticides, the pond effect, and temperature were particularly redundant between models, reinforcing the likelihood that these variables really did play a role in our study. The contribution of metabolites in our case seemed secondary given the similarity between the two first models (total pesticides vs. pesticides without metabolites). Weighting by acute toxicity data also appeared to carry the same information as models using pesticide concentrations, but the model using chronic toxicity data was the most dissimilar but not the least robust (see McFadden's R-squared for $k_{invertebrates}$ for example). The two types of model (acute vs. chronic toxicity) did not account for the same types of effects. Thus, the combined use of these two models made it possible to distinguish between acute effects (on the Shannon index notably) and chronic effects (on $k_{invertebrates}$) of pesticides on benthic macroinvertebrates. However, the bias inherent in the use of TUs (notably, cocktail effects, structural complexity of invertebrate communities) may also limit our ability to distinguish between real acute and chronic toxic effects. Apart from these difficulties, the use of TUs in our context is interesting in that it allows us to highlight specific effects related to pesticide pressure, but given the complexity of the cocktail studied, the use of total pesticide concentrations also remains relevant.

Limitations of the study and perspectives

Evaluating the effects of pesticides on aquatic macroinvertebrate communities remains a complicated task in view of the fact that cause–effect relationships are not easy to demonstrate in field conditions (Schäfer, 2019). First, the two study ponds differed in terms of environmental characteristics (surface, oxygen saturation, soil occupancy, fish, etc.), which could constitute a bias for the interpretation of our results. In particular, the difference in available habitats between the two ponds may influence the interpretability of the results, given that macrophytes, as sources of invertebrate colonizers of litterbags, can influence abundance and richness of the communities observed (Papas, 2007). However, this potential bias was partially taken into account in the statistical analyses, which allowed us to shed light on the important role the pond's singularity can play in the relationships we studied. By the way, temperature influenced almost all of the responses studied, which in certain cases may have masked some of the effects of focal interest. Notably, the positive effect of temperature on leaf-litter breakdown observed in our study is consistent with the existing literature that underlines the influence of temperature on this function (Boyero et al., 2016), but it can mask certain effects.

Secondly, cocktail effects, indirect effects, and the complexity of the responses of the organisms (Sánchez-Bayo, 2021), notably due to the existence of tolerance and resistance acquisition, or delays in the responses of populations and communities, are all processes that may complicate the interpretation of some of our results. In addition, the degree of uncertainty surrounding the use of ΣTU and the bias induced by the lack of toxicity data for metabolites have to be acknowledged for an accurate environmental impact assessment of pesticides. Nonetheless, we were able to identify effects that are likely attributable to the pesticide pressure of the CWR in benthic macroinvertebrates. Moreover, although studies on the impact of pesticides on aquatic invertebrates and litter breakdown in laboratory or mesocosm conditions are numerous, studies focusing on lotic ecosystems in field conditions are missing. This study is thus important in that it contributes to our understanding of the effects of pesticides and their dynamics on aquatic fauna structure and function in ponds.

Conclusion

Litterbags were used to study the potential effects of pesticides and nitrate dynamics on benthic macroinvertebrates and the leaf-litter breakdown in an agricultural constructed wetland in comparison with a low-contaminated/uncontaminated comparison pond. We showed that pesticides had negative effects on benthic macroinvertebrate community structure, and possibly on function, in the CWR. The existence of potential interactive, synergistic effects between nitrate and pesticides is possible but could not be tested in this study due to limited sample size. We suggest that the CWR may be an ecological trap for benthic macroinvertebrates, but further evidence on the ability of pesticides/nitrate in the CWR to reduce benthic macroinvertebrates' fitness would be needed to confirm this finding. This study opens the way for further investigations in agricultural constructed wetlands, aiming to better predict and remedy the adverse effects agricultural pollution can have on aquatic ecosystems in nature-based solutions.

Appendix

Appendix 1 Quantified pesticides and corresponding acute and chronic toxicity data for aquatic invertebrates (1/2) – Herbicides and fungicides.

Pesticide	CAS	Type	Toxicity									
			Acute toxicity					Chronic toxicity				
			EC50	Unit	Model organism	Duration	Reference	NOEC/LOEC	Unit	Model organism	Duration	Reference
2,4-MCPA	94-74-6	Herbicide	> 190	mg/L	*Daphnia magna*	48h	PPDB	56	mg/L	*Daphnia magna*	21d	PPDB
Aclonifen	74070-46-5	Herbicide	1.2	mg/L	*Daphnia magna*	48h	PPDB	0.016	mg/L	*Daphnia magna*	21d	PPDB
Benoxacor	98730-04-2	Herbicide	4.8	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Bentazone	25057-89-0	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	> 101	mg/L	*Daphnia magna*	21d	PPDB
Chloridazon	1698-60-8	Herbicide	132	mg/L	*Daphnia magna*	48h	PPDB	6.23	mg/L	*Daphnia magna*	21d	PPDB
Chlorotoluron	15545-48-9	Herbicide	67	mg/L	*Daphnia magna*	48h	PPDB	11.2	mg/L	*Daphnia magna*	21d	PPDB
Clomazone	81777-89-1	Herbicide	12.7	mg/L	*Daphnia magna*	48h	PPDB	2.2	mg/L	*Daphnia magna*	21d	PPDB
Dicamba	1918-00-9	Herbicide	> 41	mg/L	*Daphnia magna*	48h	PPDB	≥ 97	mg/L	*Daphnia magna*	21d	PPDB
Diflufenican	83164-33-4	Herbicide	> 0.24	mg/L	*Daphnia magna*	48h	PPDB	0.052	mg/L	*Daphnia magna*	21d	PPDB
Dimethenamid	87674-68-8	Herbicide	16	mg/L	*Daphnia magna*	48h	PPDB	1.25	mg/L	*Daphnia magna*	21d	PPDB
Ethofumesate	26225-79-6	Herbicide	13.52	mg/L	*Daphnia magna*	48h	PPDB	0.25	mg/L	*Daphnia magna*	21d	PPDB
Flufenacet	142459-58-3	Herbicide	30.9	mg/L	*Daphnia magna*	48h	PPDB	3.26	mg/L	*Daphnia magna*	21d	PPDB
Foramsulfuron	173159-57-4	Herbicide	100	mg/L	*Daphnia magna*	48h	PPDB	> 100	mg/L	*Daphnia magna*	21d	PPDB
Glyphosate	1071-83-6	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	12.5	mg/L	*Daphnia magna*	21d	PPDB
Imazamox	114311-32-9	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	137	mg/L	*Daphnia magna*	21d	PPDB
Lenacil	2164-08-1	Herbicide	> 8.4	mg/L	*Daphnia magna*	48h	PPDB	0.48	mg/L	*Daphnia magna*	21d	PPDB
Mesotrione	104206-82-8	Herbicide	> 622	mg/L	*Daphnia magna*	48h	PPDB	180	mg/L	*Daphnia magna*	21d	PPDB
Metamitron	41394-05-2	Herbicide	5.7	mg/L	*Daphnia magna*	48h	PPDB	10	mg/L	*Daphnia magna*	21d	PPDB
Metazachlor	67129-08-2	Herbicide	33	mg/L	*Daphnia magna*	48h	PPDB	0.1	mg/L	*Daphnia magna*	21d	PPDB
Metolachlor	51218-45-2	Herbicide	> 23.5	mg/L	*Daphnia magna*	48h	PPDB	> 0.707	mg/L	*Daphnia magna*	21d	PPDB
Nicosulfuron	111991-09-4	Herbicide	90	mg/L	*Daphnia magna*	48h	PPDB	5.2	mg/L	*Daphnia magna*	21d	PPDB
Pendimethalin	40487-42-1	Herbicide	0.147	mg/L	*Daphnia magna*	48h	PPDB	0.0145	mg/L	*Daphnia magna*	21d	PPDB
Prosulfocarb	52888-80-9	Herbicide	0.51	mg/L	*Daphnia magna*	48h	PPDB	0.045	mg/L	*Daphnia magna*	21d	PPDB
Prosulfuron	94125-34-5	Herbicide	> 120	mg/L	*Daphnia magna*	48h	PPDB	148	mg/L	*Daphnia magna*	21d	PPDB
Quinmerac	90717-03-6	Herbicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	> 100	mg/L	*Daphnia magna*	21d	PPDB
Simazine	122-34-9	Herbicide	1.1	mg/L	*Daphnia magna*	48h	PPDB	2.5	mg/L	*Daphnia magna*	21d	PPDB
Sulcotrione	99105-77-8	Herbicide	> 848	mg/L	*Daphnia magna*	48h	PPDB	> 75	mg/L	*Daphnia magna*	21d	PPDB
Sulfosate	81591-81-3	Herbicide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Terbuthylazine	5915-41-3	Herbicide	21.2	mg/L	*Daphnia magna*	48h	PPDB	0.019	mg/L	*Daphnia magna*	21d	PPDB
Triflusulfuron-methyl	126535-15-7	Herbicide	> 960	mg/L	*Daphnia magna*	48h	PPDB	11	mg/L	*Daphnia magna*	21d	PPDB
2,4-Dinitrophenol	51-28-5	Fungicide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Azoxystrobin	131860-33-8	Fungicide	0.23	mg/L	*Daphnia magna*	48h	PPDB	0.044	mg/L	*Daphnia magna*	21d	PPDB
Cyproconazole	94361-06-5	Fungicide	> 22	mg/L	*Daphnia magna*	48h	PPDB	0.023	mg/L	*Daphnia magna*	21d	PPDB
Epoxiconazole	133855-98-8	Fungicide	> 3.13	mg/L	*Daphnia magna*	48h	PPDB	0.63	mg/L	*Daphnia magna*	21d	PPDB
Fluopicolide	239110-15-7	Fungicide	> 1.8	mg/L	*Daphnia magna*	48h	PPDB	0.37	mg/L	*Daphnia magna*	21d	PPDB
Fluopyram	658066-35-4	Fungicide	> 100	mg/L	*Daphnia magna*	48h	PPDB	1.25	mg/L	*Daphnia magna*	21d	PPDB
Fluxapyroxad	907204-31-3	Fungicide	6.78	mg/L	*Daphnia magna*	48h	PPDB	0.5	mg/L	*Daphnia magna*	21d	PPDB
Metalaxyl	57837-19-1	Fungicide	3.47	mg/L	*Daphnia magna*	48h	PPDB	> 1	mg/L	*Daphnia magna*	21d	PPDB
Oxadixyl	77732-09-3	Fungicide	> 530	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Sedaxane	874967-67-6	Fungicide	6.1	mg/L	*Daphnia magna*	48h	PPDB	0.75	mg/L	*Daphnia magna*	21d	PPDB
Tebuconazole	107534-96-3	Fungicide	2.79	mg/L	*Daphnia magna*	48h	PPDB	0.01	mg/L	*Daphnia magna*	21d	PPDB
Tetraconazole	112281-77-3	Fungicide	3	mg/L	*Daphnia magna*	48h	PPDB	0.19	mg/L	*Daphnia magna*	21d	PPDB
Tolytriazole	29878-31-7	Fungicide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

CAS refers to the CAS Registry Number of the substance considered. EC_{50} = Effective Concentration 50, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration. The model organism refers to the species or the taxon on which the toxicity test was performed and the duration refers to the duration of the toxicity test (h = hour, d = day). For the references: PPDB = Pesticide Properties DataBase website (https://sitem.herts.ac.uk/aeru/ppdb/), INERIS = Substances chimiques INERIS website (https://substances.ineris.fr/).

Appendix 2 Quantified pesticides and corresponding acute and chronic toxicity data for aquatic invertebrates (2/2) – Insecticides, molluscicides, and metabolites.

Pesticide	CAS	Type	Toxicity									
			Acute toxicity					Chronic toxicity				
			EC50	Unit	Model organism	Duration	Reference	NOEC/LOEC	Unit	Model organism	Duration	Reference
Chlorantraniliprole	500008-45-7	Insecticide	0.0116	mg/L	*Daphnia magna*	48h	PPDB	0.00447	mg/L	*Daphnia magna*	21d	PPDB
Flonicamid	158062-67-0	Insecticide	> 100	mg/L	*Daphnia magna*	48h	PPDB	3.1	mg/L	*Daphnia magna*	21d	PPDB
Imidacloprid	138261-41-3	Insecticide	85	mg/L	*Daphnia magna*	48h	PPDB	1.8	mg/L	*Daphnia magna*	21d	PPDB
Piperonyl butoxide	51-03-6	Insecticide	0.51	mg/L	*Daphnia magna*	48h	PPDB	0.12	mg/L	*Daphnia magna*	21d	PPDB
Thiamethoxam	153719-23-4	Insecticide	> 100	mg/L	*Daphnia magna*	48h	PPDB	> 100	mg/L	*Daphnia magna*	21d	PPDB
Metaldehyde	9002-91-9	Molluscicide	> 78.4	mg/L	*Daphnia magna*	48h	PPDB	90	mg/L	*Daphnia magna*	21d	PPDB
2-Aminosulfonyl-N,N-dimethylnicotinamide	112006-75-4	Metabolite	> 954	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
2-Hydroxyatrazine	2163-68-0	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methoxy-6-(trifluoromethyl)-1,3,5-triazin-2-amine	5311-05-7	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Alachlor OXA	171262-17-2	Metabolite	7.2	mg/L	Invertebrate	48h	INERIS	NA	NA	NA	NA	NA
AMPA	77521-29-0	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	15	mg/L	*Daphnia magna*	21d	PPDB
Atrazine-desethyl-2-hydroxy	19988-24-0	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chlorothalonil-4-hydroxy	28343-61-5	Metabolite	19	mg/L	*Mysidopsis bahia*	96h	PPDB	NA	NA	NA	NA	NA
Deethylatrazine	6190-65-4	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Deisopropylatrazine	1007-28-9	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Desethylterbuthylazine	30125-63-4	Metabolite	42	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Desmethyl chlorotoluron	22175-22-0	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Dimethenamid ESA	205939-58-8	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Dimethenamid OXA	380412-59-9	Metabolite	95	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Flufenacet ESA	201668-32-8	Metabolite	> 87.3	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Flufenacet OXA	201668-31-7	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Metazachlor ESA	172960-62-2	Metabolite	93.8	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Metazachlor OXA	1231244-60-2	Metabolite	100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Methyl-3-hydroxylphenylcarbamate	13683-89-1	Metabolite	1.1	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Methyldesphenylchloridazon	17254-80-7	Metabolite	100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Metolachlor ESA	171118-09-5	Metabolite	> 100	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Metolachlor OXA	152019-73-3	Metabolite	> 16.6	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
N-acetyl-AMPA	57637-97-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Prosulfocarb sulfoxide	51954-81-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Prothioconazole-desthio	120983-64-4	Metabolite	10	mg/L	*Daphnia magna*	48h	PPDB	0.1	mg/L	*Daphnia magna*	21d	PPDB
S-Metolachlor CGA 357704	1217465-10-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S-Metolachlor CGA 368208	1173021-76-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S-Metolachlor NOA 413173	1418095-19-8	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Terbutylazine-2-hydroxy	66753-07-9	Metabolite	> 2.8	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA
Terbutylazine-desethyl-2-hydroxy	66753-06-8	Metabolite	> 15	mg/L	*Daphnia magna*	48h	PPDB	NA	NA	NA	NA	NA

CAS refers to the CAS Registry Number of the substance considered. EC_{50} = Effective Concentration 50, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration. The model organism refers to the species or the taxon on which the toxicity test was performed and the duration refers to the duration of the toxicity test (h = hour, d = day). For the references: PPDB = Pesticide Properties DataBase website (https://sitem.herts.ac.uk/aeru/ppdb/), INERIS = Substances chimiques INERIS website (https://substances.ineris.fr/).

Appendix 3 Quantified pesticides and corresponding acute and chronic toxicity data for algae (1/2) – Herbicides and fungicides.

Pesticide	CAS	Type	Toxicity									
			Acute toxicity					Chronic toxicity				
			EC50	Unit	Model organism	Duration	Reference	NOEC/LOEC	Unit	Model organism	Duration	Reference
2,4-MCPA	94-74-6	Herbicide	79.8	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	60	mg/L	Unknown species	96h	PPDB
Aclonifen	74070-46-5	Herbicide	0.47	mg/L	*Navicula pelliculosa*	72h	PPDB	0.0012	mg/L	NA	NA	INERIS
Benoxacor	98730-04-2	Herbicide	0.63	mg/L	*Scenedesmus subspicatus*	72h	PPDB	NA	NA	NA	NA	NA
Bentazone	25057-89-0	Herbicide	10.1	mg/L	*Anabaena flos-aquae*	72h	PPDB	25.7	mg/L	Unknown species	96h	PPDB
Chloridazon	1698-60-8	Herbicide	> 3	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.73	mg/L	Unknown species	96h	PPDB
Chlorotoluron	15545-48-9	Herbicide	0.082	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	> 0.8	mg/L	*Pseudokirchneriella subcapitata*	96h	PPDB
Clomazone	81777-89-1	Herbicide	0.136	mg/L	*Navicula pelliculosa*	72h	PPDB	0.05	mg/L	*Navicula pelliculosa*	96h	PPDB
Dicamba	1918-00-9	Herbicide	> 87	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	25	mg/L	Unknown species	96h	PPDB
Diflufenican	83164-33-4	Herbicide	0.00025	mg/L	*Scenedesmus subspicatus*	72h	PPDB	0.0001	mg/L	*Scenedesmus subspicatus*	96h	PPDB
Dimethenamid	87674-68-8	Herbicide	0.062	mg/L	*Scenedesmus subspicatus*	72h	PPDB	0.002	mg/L	*Lemna gibba*	NA	INERIS
Ethofumesate	26225-79-6	Herbicide	3.9	mg/L	*Raphidocelis subcapitata*	72h	PPDB	9.68	mg/L	*Pseudokirchneriella subcapitata*	96h	PPDB
Flufenacet	142459-58-3	Herbicide	0.00204	mg/L	*Raphidocelis subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Foramsulfuron	173159-57-4	Herbicide	8.1	mg/L	*Anabaena flos-aquae*	72h	PPDB	NA	NA	NA	NA	NA
Glyphosate	1071-83-6	Herbicide	19	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	2	mg/L	Unknown species	96h	PPDB
Imazamox	114311-32-9	Herbicide	> 29.1	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Lenacil	2164-08-1	Herbicide	0.0077	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.01	mg/L	Unknown species	96h	PPDB
Mesotrione	104206-82-8	Herbicide	3.5	mg/L	*Raphidocelis subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Metamitron	41394-05-2	Herbicide	0.4	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.1	mg/L	Unknown species	96h	PPDB
Metazachlor	67129-08-2	Herbicide	0.0162	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.34	mg/L	Unknown species	96h	PPDB
Metolachlor	51218-45-2	Herbicide	> 57.1	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	3	mg/L	*Anabaena sp.*	96h	PPDB
Nicosulfuron	111991-09-4	Herbicide	71.2	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	100	mg/L	Unknown species	96h	PPDB
Pendimethalin	40487-42-1	Herbicide	0.004	mg/L	*Selenastrum capricornutum*	72h	PPDB	0.003	mg/L	*Pseudokirchneriella subcapitata*	96h	PPDB
Prosulfocarb	52888-80-9	Herbicide	0.049	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.005	mg/L	*Pseudokirchneriella subcapitata*	72h	INERIS
Prosulfuron	94125-34-5	Herbicide	0.0089	mg/L	*Raphidocelis subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Quinmerac	90717-03-6	Herbicide	48.5	mg/L	*Chlorella fusca*	72h	PPDB	20	mg/L	Unknown species	96h	PPDB
Simazine	122-34-9	Herbicide	0.04	mg/L	*Scenedesmus subspicatus*	72h	PPDB	0.6	mg/L	Unknown species	96h	PPDB
Sulcotrione	99105-77-8	Herbicide	1.2	mg/L	*Raphidocelis subcapitata*	72h	PPDB	0.051	mg/L	*Lemna gibba*	7d	INERIS
Sulfosate	81591-81-3	Herbicide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Terbuthylazine	5915-41-3	Herbicide	0.012	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.0006	mg/L	*Selenastrum capricornutum*	5d	INERIS
Triflusulfuron-methyl	126535-15-7	Herbicide	2.02	mg/L	*Anabaena flos-aquae*	72h	PPDB	NA	NA	NA	NA	NA
2,4-Dinitrophenol	51-28-5	Fungicide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Azoxystrobin	131860-33-8	Fungicide	0.36	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.8	mg/L	Unknown species	96h	PPDB
Cyproconazole	94361-06-5	Fungicide	0.099	mg/L	*Scenedesmus subspicatus*	72h	PPDB	0.021	mg/L	*Scenedesmus subspicatus*	96h	PPDB
Epoxiconazole	133855-98-8	Fungicide	> 10.69	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	0.0078	mg/L	*Pseudokirchneriella subcapitata*	96h	PPDB
Fluopicolide	239110-15-7	Fungicide	0.029	mg/L	*Navicula pelliculosa*	72h	PPDB	NA	NA	NA	NA	NA
Fluopyram	658066-35-4	Fungicide	> 1.13	mg/L	*Skeletonema costatum*	72h	PPDB	NA	NA	NA	NA	NA
Fluxapyroxad	907204-31-3	Fungicide	0.7	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Metalaxyl	57837-19-1	Fungicide	0.42	mg/L	*Scenedesmus subspicatus*	72h	PPDB	> 20	mg/L	*Chlorella pyrenoidosa*	96h	PPDB
Oxadixyl	77732-09-3	Fungicide	> 46	mg/L	*Scenedesmus subspicatus*	72h	PPDB	3.06	mg/L	Pseudokirchneriella subcapitata	96h	PPDB
Sedaxane	874967-67-6	Fungicide	1.9	mg/L	NA	72h	PPDB	NA	NA	NA	NA	NA
Tebuconazole	107534-96-3	Fungicide	1.96	mg/L	*Scenedesmus subspicatus*	72h	PPDB	0.1	mg/L	Unknown species	96h	PPDB
Tetraconazole	112281-77-3	Fungicide	2.4	mg/L	*Ankistodesmus bibaiamus*	72h	PPDB	0.032	mg/L	*Lemna gibba*	7d	INERIS
Tolytriazole	29878-31-7	Fungicide	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

CAS refers to the CAS Registry Number of the substance considered. EC_{50} = Effective Concentration 50, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration. The model organism refers to the species or the taxon on which the toxicity test was performed and the duration refers to the duration of the toxicity test (h = hour, d = day). For the references: PPDB = Pesticide Properties DataBase website (https://sitem.herts.ac.uk/aeru/ppdb/), INERIS = Substances chimiques INERIS website (https://substances.ineris.fr/).

Appendix 4 Quantified pesticides and corresponding acute and chronic toxicity data for algae (2/2) – Insecticides, molluscicides, and metabolites.

Pesticide	CAS	Type	Toxicity									
			Acute toxicity					Chronic toxicity				
			EC50	Unit	Model organism	Duration	Reference	NOEC/LOEC	Unit	Model organism	Duration	Reference
Chlorantraniliprole	500008-45-7	Insecticide	> 4.0	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Flonicamid	158062-67-0	Insecticide	> 100	mg/L	*Raphidocelis subcapitata*	72h	PPDB	> 100	mg/L	*Raphidocelis subcapitata*	96h	PPDB
Imidacloprid	138261-41-3	Insecticide	> 10	mg/L	*Scenedesmus subspicatus*	72h	PPDB	10	mg/L	*Scenedesmus subspicatus*	96h	PPDB
Piperonyl butoxide	51-03-6	Insecticide	0.24	mg/L	Unknown species	72h	PPDB	NA	NA	NA	NA	NA
Thiamethoxam	153719-23-4	Insecticide	> 100	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Metaldehyde	9002-91-9	Molluscicide	75.9	mg/L	*Scenedesmus subspicatus*	72h	PPDB	NA	NA	NA	NA	NA
2-Aminosulfonyl-N,N-dimethylnicotinamide	112006-75-4	Metabolite	> 336	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
2-Hydroxyatrazine	2163-68-0	Metabolite	10	mg/L	*Scenedesmus quadricauda*	NA	NA	NA	NA	NA	NA	NA
4-Methoxy-6-(trifluoromethyl)-1,3,5-triazin-2-amine	5311-05-7	Metabolite	NA	NA	NA	NA	NA	> 100	mg/L	*Pseudokirchneriella subcapitata*	96h	PPDB
Alachlor OXA	171262-17-2	Metabolite	0.0019	mg/L	NA	NA	INERIS	0.00035	mg/L	NA	NA	INERIS
AMPA	77521-29-0	Metabolite	191	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	8.3	mg/L	*Scenedesmus subspicatus*	72h	INERIS
Atrazine-desethyl-2-hydroxy	19988-24-0	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chlorothalonil-4-hydroxy	28343-61-5	Metabolite	13000	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Deethylatrazine	6190-65-4	Metabolite	0.1	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Deisopropylatrazine	1007-28-9	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Desethylterbuthylazine	30125-63-4	Metabolite	0.14	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Desmethyl chlorotoluron	22175-22-0	Metabolite	NA	NA	NA	NA	NA	0.115	mg/L	*Desmodesmus subspicatus*	96h	PPDB
Dimethenamid ESA	205939-58-8	Metabolite	> 208	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Dimethenamid OXA	380412-59-9	Metabolite	94	mg/L	*Raphidocelis subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Flufenacet ESA	201668-32-8	Metabolite	> 86.7	mg/L	*Scenedesmus subspicatus*	72h	PPDB	NA	NA	NA	NA	NA
Flufenacet OXA	201668-31-7	Metabolite	> 100	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Metazachlor ESA	172960-62-2	Metabolite	93.8	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Metazachlor OXA	1231244-60-2	Metabolite	25.7	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Methyl-3-hydroxylphenylcarbamate	13683-89-1	Metabolite	2.72	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Methyldesphenylchloridazon	17254-80-7	Metabolite	18.6	mg/L	*Scenedesmus subspicatus*	72h	PPDB	NA	NA	NA	NA	NA
Metolachlor ESA	171118-09-5	Metabolite	> 100	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Metolachlor OXA	152019-73-3	Metabolite	77.6	mg/L	*Desmodesmus subspicatus*	72h	PPDB	NA	NA	NA	NA	NA
N-acetyl-AMPA	57637-97-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Prosulfocarb sulfoxide	51954-81-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Prothioconazole-desthio	120983-64-4	Metabolite	0.07	mg/L	*Scenedesmus subspicatus*	72h	PPDB	NA	NA	NA	NA	NA
S-Metolachlor CGA 357704	1217465-10-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S-Metolachlor CGA 368208	1173021-76-5	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S-Metolachlor NOA 413173	1418095-19-8	Metabolite	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Terbutylazine-2-hydroxy	66753-07-9	Metabolite	> 3.8	mg/L	*Pseudokirchneriella subcapitata*	72h	PPDB	NA	NA	NA	NA	NA
Terbutylazine-desethyl-2-hydroxy	66753-06-8	Metabolite	> 15.0	mg/L	Unknown species	72h	PPDB	NA	NA	NA	NA	NA

CAS refers to the CAS Registry Number of the substance considered. EC_{50} = Effective Concentration 50, NOEC = No Observed Effect Concentration, LOEC = Lowest Observed Effect Concentration. The model organism refers to the species or the taxon on which the toxicity test was performed and the duration refers to the duration of the toxicity test (h = hour, d = day). For the references: PPDB = Pesticide Properties DataBase website (https://sitem.herts.ac.uk/aeru/ppdb/), INERIS = Substances chimiques INERIS website (https://substances.ineris.fr/).

Appendix 5 Formulas for Shannon and $SPEAR_{pesticides}$ indices.

$$H' = -\sum_{i=1}^{S} p_i . \log 2(p_i)$$ Shannon index

where p_i is the proportion of species i and S is the total number of species (Jost, 2006)

$$SPEAR_{pesticides} = \frac{\sum_{i=1}^{n} \log(x_i+1) \times y}{\sum_{i=1}^{n} \log(x_i+1)}$$ $SPEciesAtRisk_{pesticides}$ index

where n is the number of taxa, x_i is the abundance of the taxon i, and y ranges from 0 for taxa not at risk to 1 for taxa at highest risk according to the taxa list of Liess & Ohe (2005) (in addition, see Beketov et al. (2009)). The ecological status quality classes according to $SPEAR_{pesticides}$ are as follows: < 0.2 = bad, 0.2–0.4 = poor, 0.4–0.6 = moderate, 0.6–0.8 = good, 0.8–1.0 = high (see Liess et al. (2021)).

Appendix 6 GLM results - Model 1: Total pesticide concentration ([pesticide]$_{tot}$).

Variables	Transformation	GLM family	Explanatory variables	Estimate	z / t-value	Pr(>|t|)	VIF	R-squared
Taxonomic richness	None	Poisson	Pesticides	-0.046	-0.702	0.483	2.338	0.177
			NO_3^-	0.120	1.835	0.066	2.360	
			Temperature	**0.172**	**2.684**	**0.007**	1.703	
			Pond(CWR)	-0.023	-0.184	0.854	1.641	
Shannon index	None	Gaussian	**Pesticides**	**-0.337**	**-3.370**	**0.001**	2.296	0.262
			NO_3^-	0.192	1.921	0.060	2.307	
			Temperature	**0.295**	**3.116**	**0.003**	1.687	
			Pond(CWR)	-0.165	-0.894	0.375	1.639	
SPEAR pesticides index	None	Gaussian	Pesticides	0.036	0.858	0.395	2.296	0.464
			NO_3^-	-0.069	-1.653	0.104	2.307	
			Temperature	**-0.093**	**-2.374**	**0.021**	1.687	
			Pond(CWR)	**-0.361**	**-4.709**	**<0.001**	1.639	
% GOLD (Abundance)	Arcsine	Gaussian	Pesticides	0.048	0.772	0.443	2.296	0.320
			NO_3^-	**0.173**	**2.763**	**0.008**	2.307	
			Temperature	**0.132**	**2.221**	**0.031**	1.687	
			Pond(CWR)	-0.090	-0.777	0.441	1.639	
k invertebrates	None	Gaussian	Pesticides	0.001	0.937	0.353	2.489	0.468
			NO_3^-	-0.001	-1.053	0.297	2.397	
			Temperature	**0.004**	**4.557**	**<0.001**	1.815	
			Shredder + scraper trait modality	0.001	0.895	0.375	1.682	
			Pond(CWR)	<0.001	0.041	0.968	2.010	
k microbes	None	Gaussian	Pesticides	<0.001	0.182	0.856	2.277	0.455
			NO_3^-	<-0.001	-0.929	0.357	2.292	
			Temperature	**0.001**	**4.473**	**<0.001**	1.682	
			Pond(CWR)	<-0.001	-0.498	0.620	1.618	
Shredder + scraper trait modality	Arcsine	Gaussian	**Pesticides**	**-0.094**	**-2.233**	**0.030**	2.296	0.345
			NO_3^-	-0.053	-1.256	0.215	2.307	
			Temperature	-0.066	-1.656	0.103	1.687	
			Pond(CWR)	**0.228**	**2.929**	**0.005**	1.639	

Appendix 7 GLM results - Model 2: Pesticide concentration without metabolites ($[pesticide]_{wo\ metab}$).

Variables	Transformation	GLM family	Explanatory variables	Estimate	z / t-value	Pr(>|t|)	VIF	R-squared
Taxonomic richness	None	Poisson	Pesticides (without metabolites)	-0.061	-1.053	0.292	1.780	0.188
			NO_3^-	**0.124**	**2.049**	**0.040**	2.031	
			Temperature	**0.179**	**2.971**	**0.003**	1.506	
			Pond(CWR)	-0.022	-0.184	0.854	1.587	
Shannon index	None	Gaussian	**Pesticides (without metabolites)**	**-0.353**	**-4.223**	**<0.001**	1.766	0.329
			NO_3^-	0.176	1.976	0.053	1.999	
			Temperature	**0.293**	**3.443**	**0.001**	1.492	
			Pond(CWR)	-0.196	-1.129	0.264	1.589	
SPEAR pesticides index	None	Gaussian	Pesticides (without metabolites)	0.008	0.210	0.834	1.766	0.457
			NO_3^-	-0.052	-1.344	0.185	1.999	
			Temperature	**-0.077**	**-2.074**	**0.043**	1.492	
			Pond(CWR)	**-0.348**	**-4.584**	**<0.001**	1.589	
% GOLD (Abundance)	Arcsine	Gaussian	Pesticides (without metabolites)	0.065	1.196	0.237	1.766	0.330
			NO_3^-	**0.168**	**2.909**	**0.005**	1.999	
			Temperature	**0.124**	**2.246**	**0.029**	1.492	
			Pond(CWR)	-0.090	-0.796	0.430	1.589	
k invertebrates	None	Gaussian	Pesticides (without metabolites)	0.001	1.503	0.139	1.959	0.482
			NO_3^-	-0.001	-1.177	0.244	2.121	
			Temperature	**0.003**	**4.765**	**<0.001**	1.640	
			Shredder + scraper trait modality	0.001	1.109	0.273	1.718	
			Pond(CWR)	<-0.001	-0.052	0.959	1.940	
k microbes	None	Gaussian	Pesticides (without metabolites)	<0.001	0.526	0.601	1.754	0.457
			NO_3^-	<-0.001	-1.134	0.262	1.985	
			Temperature	**0.001**	**4.605**	**<0.001**	1.488	
			Pond(CWR)	<-0.001	-0.546	0.588	1.569	
Shredder + scraper trait modality	Arcsine	Gaussian	**Pesticides (without metabolites)**	**-0.092**	**-2.515**	**0.015**	1.766	0.359
			NO_3^-	-0.061	-1.569	0.122	1.999	
			Temperature	-0.071	-1.898	0.063	1.492	
			Pond(CWR)	**0.217**	**2.865**	**0.006**	1.589	

Appendix 8 GLM results - Model 3: Toxic units sum (acute toxicity, without metabolites) ($\Sigma TU_{acu,\ wo\ metab}$).

Variables	Transformation	GLM family	Explanatory variables	Estimate	z / t-value	Pr(>|t|)	VIF	R-squared
Taxonomic richness	None	Poisson	ΣTU (acute toxicity, without metabolites)	-0.039	-0.597	0.551	2.206	0.191
			NO_3^-	0.125	1.720	0.085	2.794	
			Temperature	**0.171**	**2.962**	**0.003**	1.287	
			Pond(CWR)	-0.089	-0.703	0.482	1.491	
Shannon index	None	Gaussian	**ΣTU (acute toxicity, without metabolites)**	**-0.275**	**-2.568**	**0.013**	2.279	0.168
			NO_3^-	0.209	1.750	0.086	2.855	
			Temperature	**0.186**	**2.059**	**0.045**	1.307	
			Pond(CWR)	-0.270	-1.369	0.177	1.478	
SPEAR pesticides index	None	Gaussian	ΣTU (acute toxicity, without metabolites)	-0.025	-0.571	0.570	2.279	0.466
			NO_3^-	-0.028	-0.586	0.561	2.855	
			Temperature	-0.057	-1.565	0.124	1.307	
			Pond(CWR)	**-0.371**	**-4.650**	**<0.001**	1.478	
% GOLD (Abundance)	Arcsine	Gaussian	ΣTU (acute toxicity, without metabolites)	0.097	1.515	0.136	2.279	0.348
			NO_3^-	0.127	1.777	0.082	2.855	
			Temperature	**0.129**	**2.384**	**0.021**	1.307	
			Pond(CWR)	-0.081	-0.682	0.498	1.478	
k invertebrates	None	Gaussian	ΣTU (acute toxicity, without metabolites)	<0.001	0.503	0.617	2.764	0.449
			NO_3^-	-0.001	-0.827	0.412	2.817	
			Temperature	**0.004**	**5.147**	**<0.001**	1.438	
			Shredder + scraper trait modality	0.001	0.803	0.426	1.823	
			Pond(CWR)	<0.001	0.246	0.807	1.687	
k microbes	None	Gaussian	ΣTU (acute toxicity, without metabolites)	<0.001	0.628	0.533	2.133	0.449
			NO_3^-	<-0.001	-1.138	0.261	2.850	
			Temperature	**0.001**	**5.642**	**<0.001**	1.057	
			Pond(CWR)	<-0.001	-0.399	0.692	1.523	
Shredder + scraper trait modality	Arcsine	Gaussian	**ΣTU (acute toxicity, without metabolites)**	**-0.134**	**-3.205**	**0.002**	2.279	0.386
			NO_3^-	-0.003	-0.054	0.957	2.855	
			Temperature	-0.066	-1.871	0.067	1.307	
			Pond(CWR)	**0.161**	**2.088**	**0.042**	1.478	

Appendix 9 GLM results - Model 4: Toxic units sum (chronic toxicity, without metabolites) ($\Sigma TU_{chr,\ wo\ metab}$).

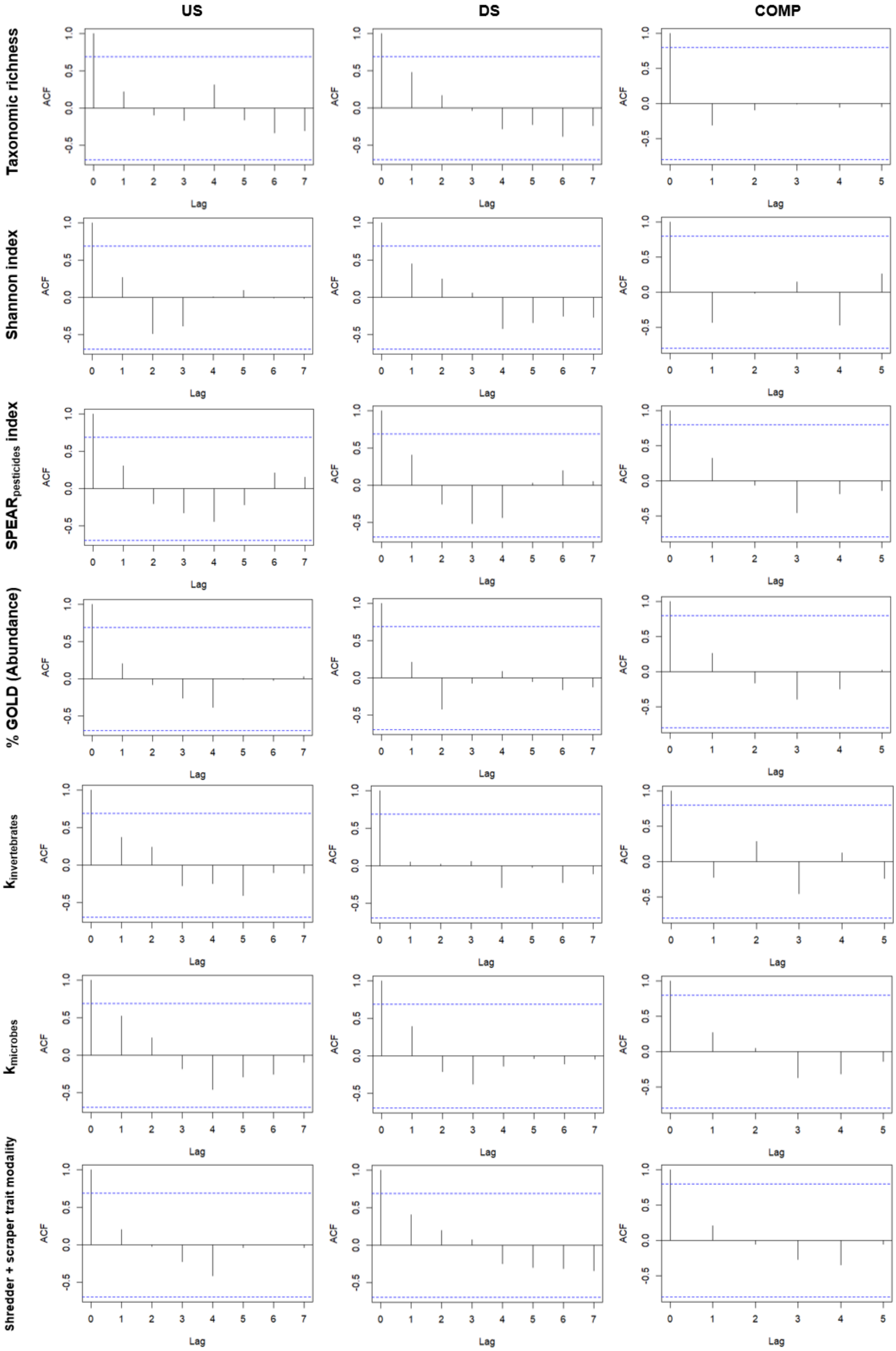
Variables	Transformation	GLM family	Explanatory variables	Estimate	z / t-value	Pr(>|t|)	VIF	R-squared
Taxonomic richness	None	Poisson	ΣTU (chronic toxicity, without metabolites)	0.083	1.448	0.148	1.991	0.220
			NO_3^-	**0.137**	**2.246**	**0.025**	2.011	
			Temperature	0.108	1.739	0.082	1.514	
			Pond(CWR)	-0.187	-1.288	0.198	1.975	
Shannon index	None	Gaussian	ΣTU (chronic toxicity, without metabolites)	0.195	1.950	0.057	1.903	0.125
			NO_3^-	0.084	0.847	0.401	1.875	
			Temperature	-0.031	-0.304	0.762	1.556	
			Pond(CWR)	-0.446	-1.946	0.057	1.895	
SPEAR pesticides index	None	Gaussian	ΣTU (chronic toxicity, without metabolites)	0.066	1.720	0.092	1.903	0.492
			NO_3^-	-0.018	-0.471	0.639	1.875	
			Temperature	**-0.105**	**-2.696**	**0.009**	1.556	
			Pond(CWR)	**-0.440**	**-4.988**	**<0.001**	1.895	
% GOLD (Abundance)	Arcsine	Gaussian	ΣTU (chronic toxicity, without metabolites)	-0.072	-1.215	0.230	1.903	0.337
			NO_3^-	**0.170**	**2.911**	**0.005**	1.875	
			Temperature	**0.207**	**3.481**	**0.001**	1.556	
			Pond(CWR)	-0.016	-0.117	0.908	1.895	
k invertebrates	None	Gaussian	**ΣTU (chronic toxicity, without metabolites)**	**-0.002**	**-2.610**	**0.012**	1.904	0.515
			NO_3^-	-0.001	-1.663	0.103	2.110	
			Temperature	**0.005**	**6.282**	**<0.001**	1.908	
			Shredder + scraper trait modality	0.001	0.886	0.380	1.493	
			Pond(CWR)	0.002	1.399	0.168	2.035	
k microbes	None	Gaussian	ΣTU (chronic toxicity, without metabolites)	<0.001	0.368	0.715	1.658	0.446
			NO_3^-	<-0.001	-1.003	0.321	2.262	
			Temperature	**0.001**	**5.605**	**<0.001**	1.054	
			Pond(CWR)	<-0.001	-0.487	0.629	1.484	
Shredder + scraper trait modality	Arcsine	Gaussian	ΣTU (chronic toxicity, without metabolites)	0.027	0.634	0.529	1.903	0.268
			NO_3^-	**-0.094**	**-2.271**	**0.027**	1.875	
			Temperature	**-0.133**	**-3.138**	**0.003**	1.556	
			Pond(CWR)	0.150	1.571	0.122	1.895	

Appendix 10 Variance Inflation Factor (VIF) of each explanatory variables for the 28 GLMs without the pond effect.

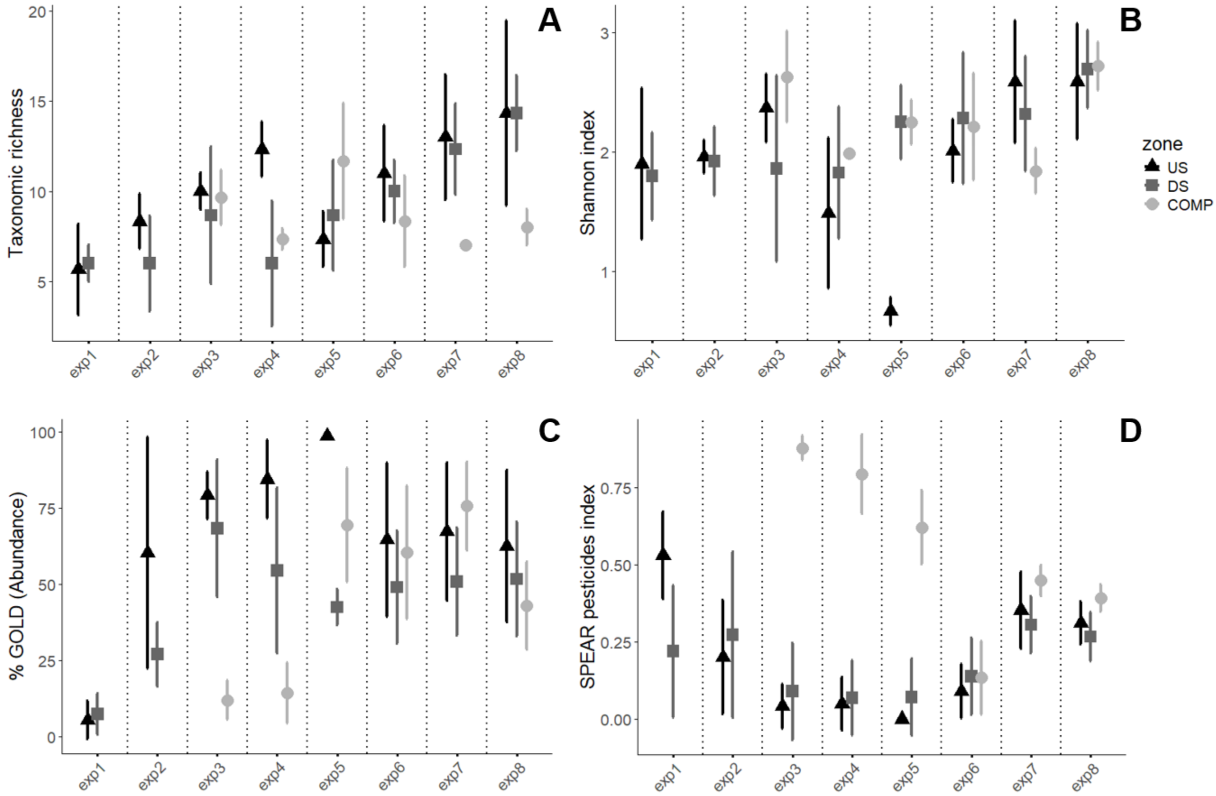
Models	Pesticides	NO_3^-	Temperature	Oxygen saturation	Conductivity	pH
Taxonomic richness ~ Pesticides	3.226	3.924	**2.239**	21.400	10.524	48.429
Taxonomic richness ~ Pesticides (without metabolites)	**2.370**	3.191	**2.122**	19.437	11.058	46.834
Taxonomic richness ~ ΣTU (acute toxicity, without metabolites)	**2.892**	4.419	**1.960**	14.256	9.781	35.336
Taxonomic richness ~ ΣTU (chronic toxicity, without metabolites)	**2.591**	**2.842**	**2.438**	13.757	9.596	35.400
Shannon index ~ Pesticides	3.131	3.916	**2.176**	23.905	11.242	53.101
Shannon index ~ Pesticides (without metabolites)	**2.382**	3.236	**2.059**	21.849	11.817	51.583
Shannon index ~ ΣTU (acute toxicity, without metabolites)	**2.852**	4.372	**1.919**	15.715	10.333	38.044
Shannon index ~ ΣTU (chronic toxicity, without metabolites)	**2.463**	**2.584**	**2.473**	14.916	10.156	36.273
SPEAR pesticides index ~ Pesticides	3.131	3.916	**2.176**	23.905	11.242	53.101
SPEAR pesticides index ~ Pesticides (without metabolites)	**2.382**	3.236	**2.059**	21.849	11.817	51.583
SPEAR pesticides index ~ ΣTU (acute toxicity, without metabolites)	**2.852**	4.372	**1.919**	15.715	10.333	38.044
SPEAR pesticides index ~ ΣTU (chronic toxicity, without metabolites)	**2.463**	**2.584**	**2.473**	14.916	10.156	36.273
% GOLD (Abundance) ~ Pesticides	3.131	3.916	**2.176**	23.905	11.242	53.101
% GOLD (Abundance) ~ Pesticides (without metabolites)	**2.382**	3.236	**2.059**	21.849	11.817	51.583
% GOLD (Abundance) ~ ΣTU (acute toxicity, without metabolites)	**2.852**	4.372	**1.919**	15.715	10.333	38.044
% GOLD (Abundance) ~ ΣTU (chronic toxicity, without metabolites)	**2.463**	**2.584**	**2.473**	14.916	10.156	36.273
k invertebrates ~ Pesticides	3.187	4.038	**2.189**	24.205	11.773	55.167
k invertebrates ~ Pesticides (without metabolites)	**2.438**	3.339	**2.075**	22.082	12.415	53.645
k invertebrates ~ ΣTU (acute toxicity, without metabolites)	**2.867**	4.442	**1.926**	15.624	10.510	38.730
k invertebrates ~ ΣTU (chronic toxicity, without metabolites)	**2.478**	**2.613**	**2.449**	14.706	10.262	36.636
k microbes ~ Pesticides	3.187	4.038	**2.189**	24.205	11.773	55.167
k microbes ~ Pesticides (without metabolites)	**2.438**	3.339	**2.075**	22.082	12.415	53.645
k microbes ~ ΣTU (acute toxicity, without metabolites)	**2.216**	**2.967**	**1.711**	15.280	8.720	32.759
k microbes ~ ΣTU (chronic toxicity, without metabolites)	**1.733**	**2.398**	**1.710**	15.221	8.848	33.147
Shredder + scraper trait modality ~ Pesticides	3.131	3.916	**2.176**	23.905	11.242	53.101
Shredder + scraper trait modality ~ Pesticides (without metabolites)	**2.382**	3.236	**2.059**	21.849	11.817	51.583
Shredder + scraper trait modality ~ ΣTU (acute toxicity, without metabolites)	**2.852**	4.372	**1.919**	15.715	10.333	38.044
Shredder + scraper trait modality ~ ΣTU (chronic toxicity, without metabolites)	**2.463**	**2.584**	**2.473**	14.916	10.156	36.273

The models presented in Appendix 10 were built just like the main GLMs, i.e., those presented in Appendix 6-9, but without the pond effect, and with the following explanatory variables: pesticides (among the four possible), nitrate (NO_3^-), temperature, oxygen saturation, conductivity and pH. Whatever the GLM, the VIF of the oxygen saturation, conductivity and pH variables were over 3, highlighting the existence of multicollinearity, hence their grouping under the pond effect. Values in bold correspond to VIFs < 3 (i.e., no collinearity).

Appendix 11 Temporal autocorrelation of the ecological variables (acf).

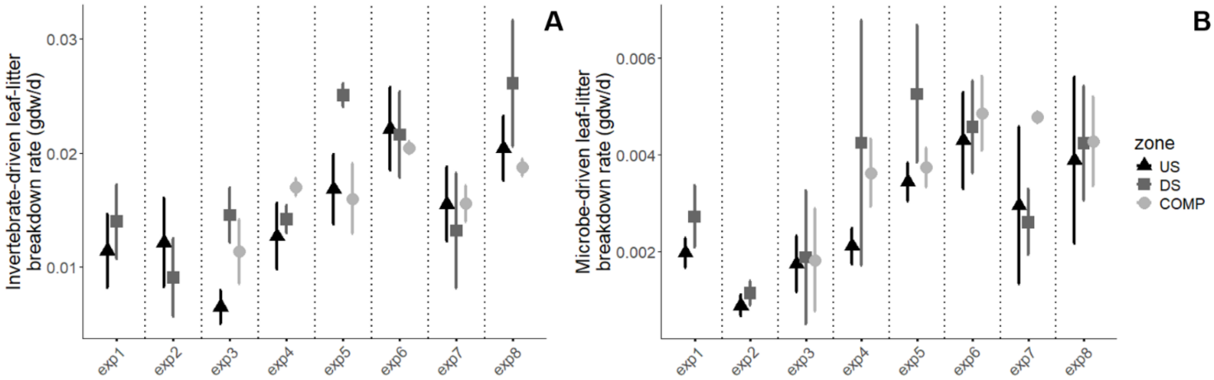


Appendix 12 Diversity and sensitivity indices of benthic macroinvertebrate communities sampled from litterbags. (A) Taxonomic richness, (B) Shannon index, (C) relative abundance of GOLD (i.e., Gastropoda, Oligochaeta, Diptera) (%), and (D) SPEAR$_{pesticides}$ index. Zones: US = CWR upstream, DS = CWR downstream, COMP = comparison pond. Sessions: exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28. *N* = 3.



Taxonomic richness tended to increase over time, except in COMP during exp6 (Appendix 12A). Taxonomic richness was generally higher in US than in DS and COMP, or it was comparable, but it was lower during exp5 in US. The Shannon index was generally similar among zones except during exp5 where US showed lower values than in DS and COMP (Appendix 12B). The relative abundance of GOLD was higher in US than in DS and COMP for almost all sessions and reached a mean of 98.53% in US during exp5 (Appendix 12C). The SPEAR$_{pesticides}$ index was overall identical between US and DS, except during exp1 and exp5 when it was, respectively, higher and lower than in DS (Appendix 12D). The SPEAR$_{pesticides}$ index was higher in COMP during exp3, exp4, exp5, and marginally higher during exp7 and exp8, than in US and DS. SPEAR was equal to 0 in the three litterbag replicates in US during exp5 (Appendix 12D).

Appendix 13 Leaf-litter breakdown rate (g_{dw}/d). (A) Invertebrate-driven leaf-litter breakdown (in macrobags) and (B) microbial-driven leaf-litter breakdown (in microbags). Zones: US = CWR upstream, DS = CWR downstream, and COMP = comparison pond. Sessions: exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28. *N* = 3.



The invertebrate-driven leaf-litter breakdown rates were generally similar among zones for almost all sessions, except for the higher level in DS during exp3, exp5, and exp8 (Appendix 13A). During the first two pesticide peaks that occurred mainly in US, namely, during exp3 and exp5, invertebrate-driven leaf-litter breakdown was lower in US than in DS (Fig. 3A; Appendix 13A). During the exp3 peak, invertebrates from US and DS were subject to the same level of high nitrate pressure (Fig. 3B; Appendix 13A). Microbe-driven leaf-litter breakdown rates followed generally the same patterns as the invertebrate-driven one (Appendix 13B). Microbe-driven leaf-litter breakdown rates were higher in DS than in US during exp1, exp4, and exp5, and they were higher in COMP during exp7 than in US and DS. Overall, both invertebrate-driven leaf-litter breakdown and microbe-driven leaf-litter breakdown rates tended to increase over time, but there was a decrease in the rates during exp7 compared with exp6 and an increase during exp8 (Appendix 13A, B).

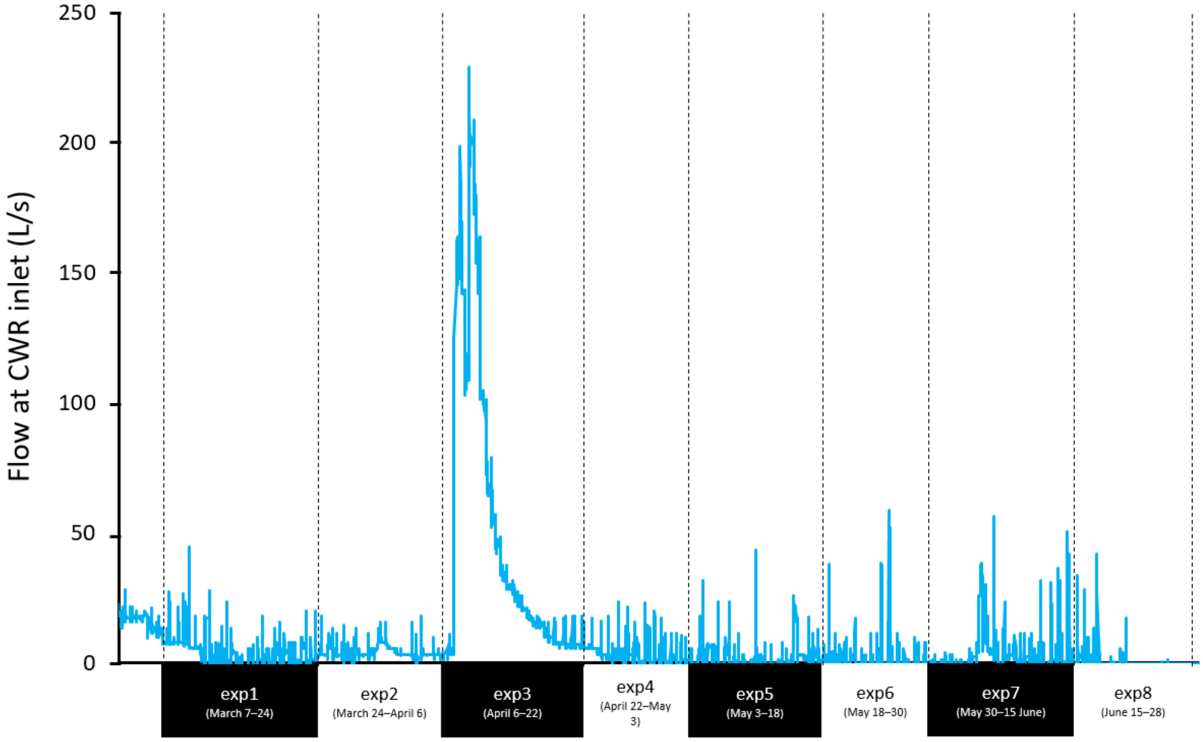
Appendix 14 Water quality data (pesticide concentrations, nitrate concentrations, and toxicity of pesticide cocktails) by zone and session.

		exp1 (March 7–24)			exp2 (March 24–April 6)			exp3 (April 6–22)			exp4 (April 22–May 3)		
		US	DS	COMP	US	DS	COMP	US	DS	COMP	US	DS	COMP
Pesticide concentration	Total pesticides (µg/L)	2.629	2.329	NA	2.614	2.081	NA	11.670	2.987	0.350	2.388	2.466	0.321
	Herbicides (µg/L)	0.136 (5.17%)	0.106 (4.55%)	NA	0.321 (12.28%)	0.176 (8.46%)	NA	3.208 (27.49%)	0.284 (9.51%)	0.085 (24.26%)	0.203 (8.51%)	0.151 (6.11%)	0.067 (20.97%)
	Fungicides (µg/L)	0.125 (4.75%)	0.143 (6.14%)	NA	0.044 (1.68%)	0.080 (3.84%)	NA	0.162 (1.39%)	0.038 (1.27%)	0.054 (15.38%)	0.032 (1.34%)	0.064 (2.60%)	0.063 (19.72%)
	Insecticides (µg/L)	0.084 (0.32%)	0.011 (0.47%)	NA	-- (0%)	0.024 (1.15%)	NA	0.326 (2.79%)	0.016 (0.54%)	-- (0%)	0.110 (4.61%)	-- (0%)	-- (0%)
	Metabolites (µg/L)	2.190 (83.29%)	2.069 (88.84%)	NA	2.049 (78.4%)	1.801 (86.55%)	NA	7.015 (60.12%)	2.427 (81.25%)	0.205 (58.72%)	1.873 (78.43%)	2.141 (86.83%)	0.191 (59.31%)
Nitrate concentration	Nitrate NO3- (mg/L)	48.68	35.55	NA	44.11	25.34	NA	140.19	132.29	2.10	43.16	27.40	2.72
Pesticide toxicity (invertebrates)	ΣTU acute	2.00E-05	4.46E-05	NA	6.86E-05	7.78E-05	NA	2.30E-03	2.02E-04	1.78E-04	3.14E-04	1.09E-04	8.92E-05
	ΣTU chronic	1.42E-07	3.50E-04	NA	1.36E-06	5.42E-04	NA	4.56E-05	9.84E-04	1.90E-03	3.59E-06	5.43E-04	1.43E-03
Pesticide toxicity (algae)	ΣTU acute	6.64E-05	2.84E-04	NA	4.67E-02	2.24E-02	NA	1.46E+00	1.83E-01	2.04E-03	9.89E-02	1.08E-01	1.19E-03
	ΣTU chronic	3.16E-10	2.02E-03	NA	1.28E-04	2.66E-03	NA	8.80E+00	3.91E-01	1.70E-02	8.80E-01	2.51E-01	1.04E-02

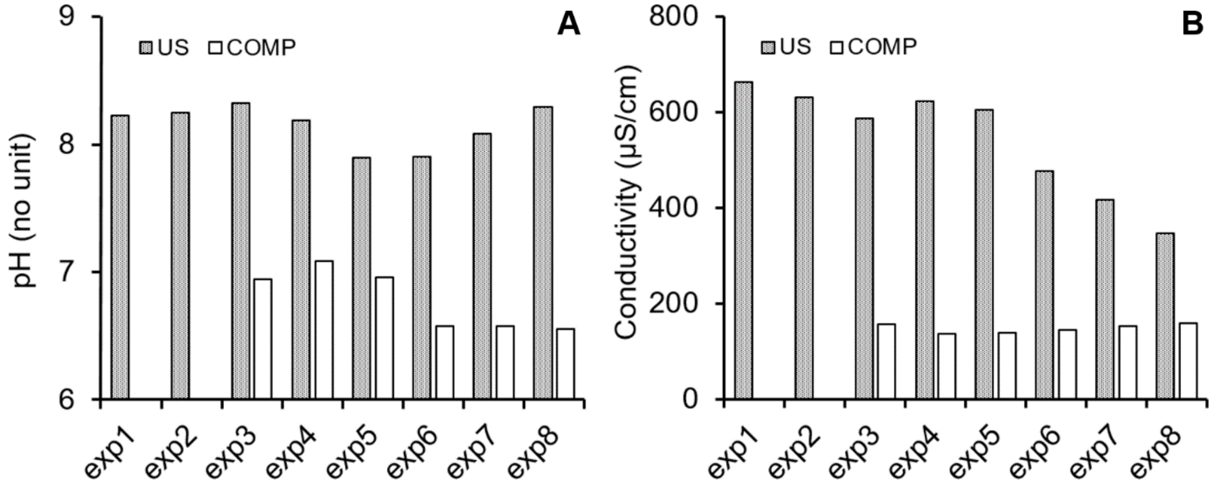
		exp5 (May 3–18)			exp6 (May 18–30)			exp7 (May 30–15 June)			exp8 (June 15–28)		
		US	DS	COMP	US	DS	COMP	US	DS	COMP	US	DS	COMP
Pesticide concentration	Total pesticides (µg/L)	46.584	8.128	0.301	37.162	9.474	0.277	27.741	10.820	0.250	NA	NA	0.222
	Herbicides (µg/L)	42.731 (91.73%)	5.918 (72.80%)	0.080 (26.74%)	29.287 (78.81%)	5.611 (59.22%)	0.073 (26.32%)	15.843 (57.11%)	5.304 (49.02%)	0.045 (18.00%)	NA	NA	0.016 (7.25%)
	Fungicides (µg/L)	0.253 (0.54%)	0.094 (1.15%)	0.046 (15.38%)	0.345 (0.93%)	0.109 (1.15%)	0.030 (10.89%)	0.438 (1.58%)	0.124 (1.15%)	0.015 (5.86%)	NA	NA	0.001 (0.58%)
	Insecticides (µg/L)	0.466 (1.00%)	0.141 (1.73%)	-- (0%)	0.236 (0.64%)	0.171 (1.80%)	-- (0%)	0.007 (0.02%)	0.200 (1.85%)	-- (0%)	NA	NA	-- (0%)
	Metabolites (µg/L)	2.704 (5.80%)	1.786 (21.97%)	0.174 (57.89%)	7.009 (18.86%)	3.361 (35.47%)	0.174 (62.79%)	11.314 (40.78%)	4.935 (45.61%)	0.191 (76.13%)	NA	NA	0.208 (93.39%)
Nitrate	Nitrate NO3- (mg/L)	88.68	12.63	2.55	93.60	18.00	3.16	98.51	23.37	3.60	NA	NA	3.69
Pesticide toxicity (invertebrates)	ΣTU acute	3.17E-03	4.78E-04	8.92E-05	2.28E-03	3.97E-04	5.22E-05	1.39E-03	3.16E-04	5.22E-05	NA	NA	NA
	ΣTU chronic	1.28E-05	7.16E-03	1.43E-03	1.95E-05	1.48E-02	9.99E-04	2.62E-05	2.25E-02	9.99E-04	NA	NA	NA
Pesticide toxicity (algae)	ΣTU acute	3.10E-01	3.53E-02	1.19E-03	2.70E-01	3.94E-02	8.38E-04	2.30E-01	4.35E-02	8.38E-04	NA	NA	NA
	ΣTU chronic	9.87E-01	5.04E-01	1.04E-02	1.07E+00	6.07E-01	6.44E-03	1.14E+00	7.11E-01	6.44E-03	NA	NA	NA

The percentage indicated for the concentrations of the various pesticide families corresponds to the proportion of the concentration of the family in question in relation to the total pesticide concentration for the given zone and session. ΣTU = Sum of toxic units.

Appendix 15 Flow at the CWR inlet (L/s) during the study.



Appendix 16 Evolution of water (A) pH (no unit) and (B) conductivity (µS/cm) over time during the 2022 survey (means). Zones: US = CWR upstream, and COMP = comparison pond. Sessions: exp1 = March 7–24, exp2 = March 24–April 6, exp3 = April 6–22, exp4 = April 22–May 3, exp5 = May 3–18, exp6 = May 18–30, exp7 = May 30–15 June, exp8 = June 15–28.



Appendix 17 Taxa identified during the study with the corresponding tags.

Order	Family	Retained taxonomic level	Tag
Coleoptera	Dytiscidae	Dytiscidae	Dytis
Coleoptera	Elmidae	Riolus	Riolu
Coleoptera	Haliplidae	Haliplus	Halip
Coleoptera	Hydraenidae	Hydraena	Hydra
Coleoptera	Hydrophilidae	Hydrophilidae	Hydro
Coleoptera	Hygrobiidae	Hygrobia	Hygro
Coleoptera	Noteridae	Noterus	Noter
Crustacea	Asellidae	Asellus	Asell
Crustacea	Gammaridae	Gammaridae	Gamma
Diptera	Ceratopogonidae	Ceratopogoninae	Cerat
Diptera	Chironomidae	Tanypodinae	Tanyp
Diptera	Chironomidae	Chironomini	Chiro
Diptera	Chironomidae	Tanytarsini	Tanyt
Diptera	Chironomidae	Orthocladiinae	Ortho
Diptera	Chaoboridae	Chaoborus	Chaob
Diptera	Chaoboridae	Mochlonyx	Mochl
Diptera	Culicidae	Culicinae	Culic
Diptera	Dixidae	Dixa	Dixa
Diptera	Dixidae	Dixella	Dixel
Diptera	Stratiomyidae	Stratiomyidae	Strat
Diptera	Tabanidae	Tabanidae	Taban
Ephemeroptera	Ameletidae	Metreletus	Metre
Ephemeroptera	Baetidae	Cloeon	Cloeo
Gastropoda	Lymnaeidae	Galba	Galba
Gastropoda	Lymnaeidae	Radix	Radix
Gastropoda	Lymnaeidae	Stagnicola	Stagn
Gastropoda	Neritidae	Theodoxus	Theod
Gastropoda	Physidae	Physa	Physa
Gastropoda	Planorbidae	Ancylus	Ancyl
Gastropoda	Planorbidae	Planorbidae	Plano
Heteroptera	Corixidae	Micronecta	Micron
Heteroptera	Corixidae	Glaenocorisa	Glaen
Heteroptera	Corixidae	Corixinae	Corix
Heteroptera	Gerridae	Gerris	Gerri
Heteroptera	Mesoveliidae	Mesovelia	Mesov
Heteroptera	Naucoridae	Naucoris	Nauco
Heteroptera	Nepidae	Nepa	Nepa
Heteroptera	Notonectidae	Notonecta	Noton
Heteroptera	Pleidae	Plea	Plea
Heteroptera	Veliidae	Microvelia	Microv
Heteroptera	Veliidae	Velia	Velia
Hirudinea	Erpobdellidae	Erpobdella	Erpob
Hirudinea	Erpobdellidae	Dina	Dina
Hirudinea	Glossiphoniidae	Glossiphonia	Gloss
Hirudinea	Glossiphoniidae	Helobdella	Helob
Hirudinea	Glossiphoniidae	Hemiclepsis	Hemic
Hirudinea	Glossiphoniidae	Haementeria	Haeme
Hirudinea	Glossiphoniidae	Batracobdella	Batra
Lepidoptera	Crambidae	Crambidae	Cramb
Megaloptera	Sialidae	Sialis	Siali
Odonata	Coenagrionidae	Coenagrionidae	Coena
Odonata	Lestidae	Sympecma	Sympe
Odonata	Libellulidae	Orthetrum	Orthe
Oligochaeta	Oligochaeta	Oligochaeta	Oligo
Trichoptera	Limnephilidae	Limnephilinae	Limne
Turbellaria	Dugesiidae	Dugesia	Duges
Turbellaria	Planariidae	Planariidae	Plana

Appendix 18 Total abundances of taxa sampled in litterbags, with every replicate and session taken into account.

Order	Family	US	DS	COMP
Hirudinea	Erpobdellidae	15	57	3
	Glossiphoniidae	86	503	106
Coleoptera	Dytiscidae	8	2	8
	Elmidae	--	1	--
	Haliplidae	7	--	2
	Hydraenidae	--	1	--
	Hydrophilidae	--	2	7
	Hygrobiidae	--	--	2
	Noteridae	1	1	1
Crustacea	Asellidae	387	716	185
	Gammaridae	321	273	--
Diptera	Ceratopogonidae	10	4	10
	Chaoboridae	3	6	8
	Chironomidae	1071	1589	249
	Culicidae	--	1	--
	Dixidae	2	--	3
	Stratiomyidae	--	--	1
Ephemeroptera	Ameletidae	--	--	3
	Baetidae	141	111	206
Gastropoda	Lymnaeidae	23	1	--
	Neritidae	--	4	--
	Physidae	17	5	--
	Planorbidae	19	34	6
Heteroptera	Corixidae	43	67	--
	Gerridae	3	3	1
	Mesoveliidae	1	6	2
	Naucoridae	--	1	--
	Nepidae	--	1	--
	Notonectidae	2	--	1
	Pleidae	1	--	3
	Veliidae	11	10	--
Lepidoptera	Pyralidae	--	1	1
Megaloptera	Sialidae	--	4	--
Odonata	Coenagrionidae	--	15	8
	Lestidae	8	18	10
	Libellulidae	--	--	1
Oligochaeta	Oligochaeta	2946	377	186
Trichoptera	Limnephilidae	--	1	15
Turbellaria	Dugesiidae	10	1	--
	Planariidae	16	3	--
	Total of orders	10	13	11
	Total of families	25	32	26
	Total abundance	5152	3819	1028

Epilog of Chapter IV

The study presented in this chapter, through the deployment of litterbags, highlighted effects of the CWR agrochemical pressure on the structure and function of the benthic macroinvertebrate community. In particular, it consisted in negative effects of pesticide fluxes in the CWR on the community's Shannon index, as well as on the frequencies of trait modalities directly involved in the leaf-litter breakdown process, namely the “shredder” and “scraper” trait modalities, and even on invertebrate-driven leaf-litter breakdown. The results of this study are therefore crucial, as they indicate that, in addition to being potentially able of exerting negative effects at the community scale in amphibians (cf. Chap. I), to being able of exerting negative effects at sub-cellular levels in amphibians (cf. Chap. II), and at sub-cellular and individual levels in aquatic invertebrates (cf. Chap. III), the agrochemical pressure of the CWR is able of having negative repercussions on the highly integrative ecological levels of aquatic invertebrates, namely, the community and ecosystem levels. The impact at the ecosystem level remains though less clear and require further investigation. Once again, it is important to note the importance of the influence of certain environmental factors on the ecological responses studied. Here, temperature appears to have a strong effect on virtually all responses. Nonetheless, this study, and all the work carried out in this thesis, tend to confirm the role of the CWR as an ecological trap for aquatic fauna. The following section will attempt to take a step back from all the results obtained and their implications in the context of assessing the potential impact of agricultural constructed wetlands on aquatic fauna.

IV. General discussion and conclusion

IV. General discussion and conclusion

With the aim of better understanding the role that agricultural constructed wetlands can play for aquatic fauna, as shelters or ecological traps, this thesis focused on the study of a pilot agricultural constructed wetland, located in the Seine-et-Marne region of France. We focused specifically on amphibians and aquatic invertebrates as key taxa in this artificial ecosystem, through a multi-level study approach. We first investigated the risk posed by agrochemical flux dynamics on the native amphibian community. We then studied the potential impacts of these fluxes on enzymatic activities and body condition in the common toad (*Bufo bufo*) and the green frog (*Pelophylax sp.*), directly in the field, and on enzymatic activities and certain behavioral traits in a model aquatic invertebrate species, *Gammarus fossarum*, in mesocosm conditions. Finally, we studied the effects of agrochemical fluxes in the CWR on the structure of the benthic macroinvertebrate community and an associated ecosystem function, the leaf-litter breakdown. In this section, we will discuss and conclude on the potential of CWR to act as an ecological trap and open up new perspectives and questions that may arise from this work.

1. The CWR is a risky environment for aquatic fauna: exploring synchronisms between agrochemical fluxes and ecological dynamics at the community level

1.1. The amphibian community is diverse, but at risk due to agrochemical flux dynamics

First, this thesis highlighted the importance of the constructed wetland of Rampillon in hosting amphibians at the community level. However, paradoxically, this suggests that the CWR can potentially act as an ecological trap for amphibians (cf. Chap. I). The CWR is characterized by a noticeable level of diversity of amphibians that regularly uses this environment for reproduction, with a high stability observed in the community composition, i.e., same species observed over time, for at least 5 years (see Letournel et al., 2021). This suggests, in the first instance, that the CWR would act as an actual shelter for amphibians, providing habitats, resources and thus, a favorable site for reproduction. The environmental stability of the CWR, and the abundance of resources provided, would also allow the amphibian community to thrive, explaining the persistence of the species over years.

Paradoxically, however, the noticeable level of amphibian richness characterizing the CWR could make this environment an ecological trap for amphibians (cf. Chap. I). Indeed, the CWR exhibits signals favorable to amphibian settlement, notably through the diversity of habitats it can offer (i.e., deep and shallow water zones, diversity of macrophyte populations, gentle slopes, etc.). Through its ecological attractiveness, CWR can therefore promote the use of this environment by amphibians. However, due to the ecotoxic effects of the contaminants circulating in this environment, whether acute or chronic, living in a contaminated environment can reduce amphibian fitness. In addition, some metapopulation[4] or metacommunity[5] processes may concurrently occur which could mask the negative effects of the CWR. For instance, regular flows of colonizing individuals to the CWR, coming from close and suitable occupied habitats (source-sink effects; see Harrison (1991)) may mask local declines in species abundance due to water toxicity linked with agrochemicals. Alternatively, effects on populations are not yet visible but may occur soon if the system we observe is not at the equilibrium, and due to potential time delays in biological responses, in particular at the population and community levels, ranging from a few days to several years (Daskalova et al., 2020; Essl et al., 2015; Sánchez-Bayo & Mann, 2011). Thus, although the CWR appears to be favorable for amphibians, the actual metacommunity dynamics into which CWR amphibians fit are unknown in our case and may nuance this conclusion (cf. perspectives of the thesis), raising questions, like this one, for example: what influence does the pond network in which the CWR is integrated have on the recruitment rate of the various amphibian species using the CWR? A long-term monitoring of the metacommunity dynamics, into which the CWR fits, with regular census of amphibians in all or part of the CWR pond network could help reduce the uncertainty about the degree of integrity of the CWR amphibian community. Thus, despite the potential high importance of the CWR for reinforcing connectivity (Préau et al., 2022), crucial for amphibian populations and communities, and despite the CWR's apparent role as an attractive shelter for amphibians, we showed that a wide range of amphibian species are likely to be affected by the pond's agrochemical pressure, making the CWR a potential ecological trap for these animals.

Beyond its attractiveness, the potential of the CWR to act as an ecological trap likely depends on the existence of synchronisms between the chemical and ecological dynamics of native amphibians. Our results allowed us to highlight potential synchronisms between dynamics of agrochemical fluxes and periods of vulnerability of the amphibian community, increasing the likelihood of CWR acting as an ecological trap for amphibians (cf. Chap. I). We observed that amphibian community vulnerability periods, i.e., periods when a maximum of amphibian species are present in the water, were indeed synchronous with the highest nitrate and pesticide fluxes within the CWR (i.e., from 86% to 100% of the species of the community depending on

[4] The metapopulation concept is definable as a "network of populations of the same species separated in space but interconnected by flows of individuals or propagules" (Hanski, 1999; Tirard et al., 2022).

[5] The metacommunity concept is definable as "a set of local communities that exchange colonists of multiple species" (Leibold et al., 2004).

the month considered) (cf. Chap I). This suggests that significant acute toxic effects could occur on individuals during May and June, with potential long-term community consequences. In particular, although species phenology differs, and therefore their exposure to agrochemicals in the CWR is different, all amphibian species present in the CWR are likely to be exposed to peak fluxes, just not all at the same life stage. In particular, according to our observations on the field (cf. Chap I), the common toad (*Bufo bufo*) will be subject to higher concentrations of agrochemicals at the time of metamorphosis and its completion, the green frog (*Pelophylax sp.*) will be at the height of the breeding season, while newts (*Lissotriton helveticus, Lissotriton vulgaris*) will be exposed to these intense fluxes during their juvenile development. Nonetheless, chronic toxicity (vs. acute toxicity) of pesticides (Jayawardena et al., 2011; Wrubleswski et al., 2018) and nitrate (Gomez Isaza et al., 2020) can also have influenced amphibians, given repeated chronic exposure even to lower concentrations of pesticides and nitrate during the rest of the year (e.g., amphibians hibernating in the water or sediment). Moreover, although the higher risk is likely to occur when agrochemical fluxes are synchronous with amphibian vulnerability periods, effects on individuals in field conditions are difficult to predict, as the vulnerability of amphibians to pesticides equally depends on both abiotic and biotic factors (Boone & James, 2003; Boone & Semlitsch, 2001, 2002; Relyea et al., 2005). In addition, the complexity of mixture effects (Weisner et al., 2021) complicates the prediction of the effects pesticides will have on organisms in the field. Thus, due to the existence of synchronisms between the agrochemical flux dynamics and amphibian community vulnerability periods in the CWR, linked with the number of amphibian species exposed to agrochemicals, in spring-early summer (cf. Chap I), and more generally, given the chronic nature of amphibian exposure, the CWR is all the more likely to act as an ecological trap.

1.2. The aquatic invertebrate community is also certainly at risk due to agrochemical flux dynamics

The study of synchronisms between agrochemicals fluxes and aquatic invertebrate community dynamics has not been carried out, with phenological data, given (i) the difficulty to identify all taxa at the species level, (ii) the scarcity of phenological data on aquatic invertebrates, (iii) the complexity of monitoring the phenology of this group, because of the diversity of life cycles, and their different durations (cycles can be very short in some taxa). All of these constraints made it complicated to develop such a fine study within the framework of the thesis. Nevertheless, given the high diversity of aquatic invertebrates living in the CWR, and their strong dependence on the quality of aquatic environment (cf. Chap. III), we can reasonably hypothesize that synchronisms between their dynamics and agrochemical fluxes may exist too. Like amphibians, aquatic invertebrates are exposed acutely and chronically to agrochemicals present in the water of the CWR. Some of their bio-ecological traits and phenological characteristics will influence their susceptibility to agrochemical fluxes in the CWR. In particular, strictly aquatic invertebrate taxa, such as Hirudinea, Gastropoda, or Crustacea, for example, will be more likely to suffer chronic exposure to CWR chemical pressure than other semi-aquatic taxa such as Odonata or Diptera. Studying the synchronisms between chemical and ecological dynamics in aquatic invertebrates could prove more complex and time-consuming than for amphibians. Indeed, the specific diversity of aquatic invertebrates, the diversity of their bio-ecological traits, and therefore the diversity of their susceptibility profile to agrochemicals, requires a more specific approach to identify such synchronisms. Moreover, on a community scale, aquatic invertebrates are extremely dependent on the aquatic environment, in the sense that their representativeness in water or sediment, all life stages combined, is very high throughout the year (see Tachet et al., 2010). The level of exposure of the aquatic invertebrate community to agrochemicals in the CWR may therefore be at least as high as for the amphibian community. In addition, like amphibians, invertebrate populations have been exposed for several years to agrochemical fluxes in the CWR, making CWR a potential ecological trap for these communities as well. This raises questions about the adaptation (i.e., tolerance, resistance profiles) of aquatic invertebrates in particular, in relation to the chronic nature of exposure to agrochemical pressures, linked with a relatively high population renewal dynamic (Becker et al., 2020; Major et al., 2018). This also raises the question of whether native aquatic invertebrates from the CWR are more resistant to agrochemicals than those living in less affected areas of the same territory.

2. The CWR is an impacting environment for aquatic fauna: testing the effects of agrochemicals from sub-cellular to ecosystem levels

2.1. Evidence for pesticide flux effects on enzymatic traits in native amphibians

First, the use of buccal swabs in amphibians for *in situ* enzymatic biomonitoring seemed conclusive, lending credence to the relevance of buccal swabbing, as a low cost non-invasive approach having limited impacts on animal welfare (cf. Chap. II). The development and democratization of non-invasive approaches for studying effects of pesticides in reptiles and amphibians (Chen et al., 2019; Mingo et al., 2016, 2017, 2019; Van Meter et al., 2024), such as buccal swabbing, that is relatively recent, apart from genetic studies, can be a powerful lever for refining experimental procedures using living animals. This approach can reduce pain and suffering in animal, while enabling biomonitoring of the quality of aquatic environments with a view to better predicting and limiting the potential harmful effects of agrochemicals on aquatic fauna, reducing, in turn, the potential suffering generated by these same effects in the field. The simplicity with which these techniques can be implemented and their limited impact on amphibians do not, however, constitute a “permit to disturb” wild amphibians, which remain protected and fragile animals.

The use of buccal swabbing and body condition to determine effects of pesticides on enzymatic and morphological traits on two amphibian species, the common toad (*Bufo bufo*), and the green frog (*Pelophylax sp.*), allowed us to point out potential pesticide effects on native amphibians (cf. Chap. II). At the scale of several ponds, including the CWR, we were able to identify significant effects of pesticides on enzymatic activities of detoxification and non-specific immunity biomarkers, including the glutathione S-transferase, and peroxidases. This suggests that such effects could occur in amphibians within the CWR, where agrochemical fluxes vary and where acute toxicity events can take place. Specifically in *Pelophylax sp.*, within the CWR, and because we were able to sample the species during two very heterogeneous periods in terms of pesticide pressure levels (i.e., greater contamination levels late June than late May), we highlighted probably synchronic-antagonistic pesticide effects, i.e., acute negative effects of pesticides, directly linked with their dynamics, on enzymatic levels of acetylcholinesterase, β-galactosidase, β-glucosidase, and peroxidases. Although statistical replication was lacking in this study, and it is therefore important to be cautious when interpreting our observations, these results are important since they suggest that pesticide effects are likely to occur at the sub-cellular level in native amphibians of the CWR. These biochemical biomarkers are useful early warning systems that can sound the alarm before any repercussions at population and community level.

Moreover, the period during which potential pesticide effects were observed in *Pelophylax sp.*, in association with significant chemical fluxes (cf. Chap. II), also corresponds to the highly vulnerable period for the amphibian community in terms of exposure to agrochemical fluxes in the CWR (cf. Chap. I). It is therefore interesting to note that the period we have identified as sensitive for amphibians at the community level, i.e., from May to June, also corresponds to the period when potential acute effects of pesticides can act on the sub-cellular level, as highlighted by the buccal swabbing monitoring. The evidence of synchronicity of exposure and effects between chemical and biological dynamics in amphibians reinforces the risky nature of the CWR.

In parallel, the study of body condition of amphibian individuals of the CWR did not allow us to identify any clear pesticide effects on this trait (cf. Chap. II). For instance, we had expected morphological abnormalities in amphibian species in the CWR (Agostini et al., 2013; David et al., 2012; Pavan et al., 2021; Peltzer et al., 2011) but did not detect any to the naked eye. This does not suggest that agrochemicals have no effect on the morpho-anatomical characteristics of CWR amphibians. This absence of effect in our case may be due to the difficulty of observing such effects in the wild, in particular if the mortality of morphologically abnormal individuals was so high (e.g., deformities generating alterations in feeding behavior, locomotion, escape responses linked with predation, increasing, as a result, mortality rates) that too few individuals would still be present and detectable at the site at the moment of the sampling. Further investigations on the health of native amphibians, through more focused morphometrical studies may shed light on the actual negative effects of agrochemicals in the CWR on these organisms. During this thesis, we began to explore the potential deforming effects of pesticides on the individuals sampled, by carrying out, in addition to total length and weight measurements, advanced morphometric measurements (length and width of head, length of arm, forearm, thigh, leg, interdigital webbing, inter-orbital distance) in the field. Multivariate morphometric analyses were envisaged, but were not pursued due to time constraints. In addition, for example, monitoring the development of juveniles of amphibians native to the CWR in the laboratory, by sampling eggs in the natural environment (which may, however, require killing the juveniles for subsequent observation with a binocular magnifying glass, which, however, runs counter to the non-invasive methods that this thesis aims to highlight) to determine rates of juveniles whose development is abnormal, or morphological description and malformation research on several dozen adult amphibians directly in the CWR, given that the normal frequency of abnormalities is estimated to be ≤ 5% (Sparling et al., 2010), are potential surveys that could be set up to take this investigation further. We can also mention tests of mobility in juveniles, such as anuran tadpoles, relatively abundant in *B. bufo* for example, directly in the field, based on video tracking, which would not require killing the individuals, and could shed light on the negative effects of pesticides on this behavioral trait (Denoël et al., 2013).

2.2. Enzymatic and behavioural responses of gammarids caged in mesocosms as proxy of toxic events on native aquatic invertebrates

Concerning the effects of pesticides on individual and sub-cellular levels in aquatic invertebrates, we showed that the pesticide mixture studied during the mesocosm experiment, which was representative of the average pressure in the CWR, could have had specific sublethal effects in *Gammarus fossarum* (cf. Chap. III). In particular, pesticides seemed to be responsible for an increasing amplexus rate and locomotor activity. Stimulation of pairing has already been observed in *G. fossarum* and *G. pulex* in response to exposure to pesticides (Lebrun et al., 2020) and wastewater effluent (Love et al., 2020) respectively, that may reflect an behavioral response related to the chemical stress. An increased amplexus rate can be interpreted as a reproductive strategy in a population under stress, aiming to ensure the production of offspring before the effects of the stress in question are too pronounced on the reproductive individuals. However, in the case of pesticides, disruption of reproducing pairs has also been observed (Cold & Forbes, 2004). Similarly, increased locomotor activity in *G. fossarum* or *G. pulex* can be induced by different pesticide pressures and is interpreted as an avoidance strategy (Lebrun et al., 2020; Soose et al., 2023), to move to more favorable local conditions. Besides, nitrate appeared to be responsible for increased survival and feeding rates, and an increase locomotor activity, that could lead to changes in intra- and interspecific interactions within the CWR ecosystem, with potential repercussions on trophic networks for example. In addition, we highlighted potential pesticide effects on certain enzymatic activities intervening in non-specific immunity (acid and alkaline phosphatases), and on molting (chitobiase). Disruptions of reproductive behavior and triggering of avoidance behaviors in these organisms is likely to have repercussions on their population dynamics in the wild (Cold & Forbes, 2004; Nørum et al., 2011), as well as disruption of enzymatic functions can be responsible for alterations of individual performances, such as impairment in growth linked with reduced chitobiase activity due to pesticide exposure for example (Duchet et al., 2011), with potential repercussions on population dynamics. The main limitation of this study lies in the difficulty to transfer these results at the population and community levels of native aquatic invertebrates of the CWR, although the fact that the effects occur using a mixture representative of CWR chemical pressure, all under local climatic conditions, enabled by mesocosm conditions, is a strong point of the experiment. It remains that, in addition to the risk presented by the chronic toxicity to which aquatic invertebrates are exposed in the CWR, it is now possible to suggest that acute toxicity events may occur and significantly affect at least some of the aquatic invertebrates living in the CWR.

In this thesis, it is still challenging to compare directly the results of pesticides effects on amphibians with those of invertebrates, because different traits, i.e., behavioral traits in *G. fossarum*, and morphological traits in amphibians, were used for the two groups (cf. Chap. II and III). We had to do so because morphometric and behavioral monitoring were the most straightforward in our case. For instance, for amphibians, it was far more feasible to combine morphometric measurements with buccal swabbing, than to set up specific behavioral monitoring directly in the field (e.g., locomotion monitoring). Conversely, for monitoring behavioral traits of *G. fossarum* in mesocosms, measuring locomotor activity, amplexus rate and ingestion rate was applied in the proposed experiment, based on the long-lasting experience of the team working with such methods (Lebrun et al., 2017, 2020, 2021). Furthermore, we would not have been able to address adequately the question of the effects of pesticides on the morphological traits of *G. fossarum*, since the individuals selected were already adult and mature, and the time of exposure was short (1 week), limiting both the impact of pesticides on the morpho-anatomical development of the organisms (that could manifest through deformities notably), and the likelihood to observe such effects. To improve the comparability across taxonomic groups, further investigations should focus, for example, on a few enzymes common to both taxa, taken from given species of native amphibians and native aquatic invertebrates, at strictly identical sampling periods, to compare their respective responses to the same global chemical pressure. Nevertheless, beyond these considerations, it is important to note that pesticide effects were identified in two very different taxa (i.e., amphibians vs. aquatic invertebrates), suggesting possible effects on the entire aquatic biocenosis.

2.3. Effects of agrochemical fluxes on community structure and ecosystem functioning in native aquatic invertebrates

Our results so far, tend to suggest that agrochemicals in the CWR could have repercussions on the structure and functions of the aquatic invertebrate community. Even if, as indicated above, it is complicated here to directly deduce the effects of pesticides on the CWR aquatic invertebrates from the observed effects in *G. fossarum*, the evidence of enzymatic and behavioral effects under semi-controlled conditions (cf. Chap. III) suggests that such effects could act on several species and the whole community. Indeed, some authors have already shown that pesticides that can have negative effects on cellular traits can be linked to observable ecological effects at the community level. For instance, pesticides, especially some insecticides, that target acetylcholinesterase (AChE) and gamma-aminobutyric acid (GABA) receptors, involved in neurotransmission, have been linked to the absence of specific invertebrate families from rivers in the UK (Poyntz-Wright et al., 2024). In the CWR, effects of agrochemicals on cellular and individual levels in aquatic invertebrates may have repercussions on ecological scales (see avenues for future research with this respect in section 3).

Furthermore, the use of litterbags in the CWR and a control pond enabled us to identify negative effects likely attributable to the agrochemical pressure (cf. Chap. IV). Indeed, structural effects (Shannon index, shredder + scraper trait modality), and possibly functional effects, were highlighted. In particular, our results showed that, in the CWR, pesticide fluxes had negative effects on Shannon index, and shredder + scraper trait modality for 3 models out 4, and on the invertebrate-driven leaf-litter breakdown for 1 model out 4. These results are important since they highlight the potential for an agricultural constructed wetland to have a negative influence, at least, on the state of the hosted benthic macroinvertebrate community, with the potential ecosystem disorders that may ensue. So, consistently with the two previous studies (cf. Chap. II and III), these results tend to support the negative effects of agrochemical fluxes at the community and ecosystem levels and underline the significant potential of the CWR to act as an ecological trap for aquatic fauna (cf. Chap. IV).

It is important to note that during this monitoring, temperature emerged as a powerful factor influencing all the response variables studied. This study highlights the importance of considering the influence of environmental factors on biological responses, even more so in uncontrolled environments such as those studied in this thesis. Having said this, we will now discuss the difficulty of linking agrochemical pressures with biological responses in the natural environment, due to the complexity of the natural environment and its influence on living organisms.

3. Natural environment complexity, limitations of the thesis, and perspectives

In this thesis, we were able to identify both synchronisms between ecological and agrochemical dynamics, and thus a significant risk for the amphibian community (cf. Chap. I), and also actual or potential antagonisms between aquatic fauna and agrochemicals, at sub-cellular, individual (cf. Chap. II and III), community and ecosystem levels (cf. Chap. IV), increasing the likelihood that the CWR is acting as a true ecological trap for aquatic fauna.

Nonetheless, as this thesis is mainly based on fieldwork, interpretation of most of our results are subject to the complexity of studying biological systems in the natural environment (i.e., in line with the logic of the "natural experiments challenge" concept). Thus, while the initial objective of the thesis was to study synchronisms and related antagonisms between aquatic fauna and agrochemicals, we faced two major limitations: (i) to find a standard method that allows us to study both synchronisms and antagonisms at all levels in a comparable way, and evaluate the effects of one level on the responses of another (i.e., hierarchy effects, "deleterious cascade effects"), and (ii) the general complexity of field ecology, with an important and uncontrolled

/ unknown contribution of various environmental factors on several of the responses studied. We detail these two aspects below.

First, we were confronted with the difficulty of studying the link between agrochemicals and biological responses in a comparable way, when these responses manifest themselves on different timescales (Sánchez-Bayo & Mann, 2011). In addition, we were confronted with the difficulty of studying causal links between the different levels of biological organization (i.e., causal links, for a given structural and/or functional biological trait, which, at a given organization level, can partly explain the response observed at the organization level just above), since the general study design of the thesis, *in fine*, does not allow us, with any certainty, to identify them. Establishing such links could have made it possible to confirm that the agrochemical pressure of the CWR is exerting a negative impact on aquatic fauna from biomolecules to ecosystem function. In our case, all the levels of biological organization studied seemed relevant for monitoring the ecotoxicity of agrochemicals, and the complementarity of the associated approaches was a strong point of the thesis. Nonetheless, it appeared particularly challenging to link molecular, cellular, individual, community and ecosystem responses in aquatic invertebrates, mainly because of the complexity of hierarchy effects and the difficulty of understanding the mechanisms underlying biological responses between different levels of biological organization (Domingues et al., 2010). For instance, we cannot be sure that, the negative effects of pesticides on the activity of the chitobiase and the acid phosphatase enzymes, and the avoidance behavior observed in *G. fossarum* in the mesocosm experiment (cf. Chap. III) reflect the negative structural effects observed during the litterbag monitoring (cf. Chap. IV), nor that the nitrate-induced increase in feeding rates in mesocosms could actually be observed at the level of the community, because the study designs are too different and the link between this individual behavior and ecological effects is far too complex to establish in this context. In aquatic invertebrates, the transferability of these individual-level effects to the community level is compromised by the strong differences between these two study settings (i.e., semi-controlled mesocosm conditions (cf. Chap. III) versus uncontrolled field conditions (cf. Chap. IV)). However, complementary studies could help improve these links. For example, we could repeat the mesocosm experiment on several other species of aquatic invertebrate natively present in the CWR, and representative of the functional diversity of this community (e.g., larval stages of a Chironomidae species, larval stage of an Ephemeroptera species, as a sensitive species, a Gastropoda species, a Hirudinea species). Expanding this panel could also highlight different effects depending on the preferred exposure route of the taxon in question, in relation to its specific bio-ecological traits (e.g., exposure through dissolved or particulate matter, sediment, etc.). We could imagine and make a focus on the study of the effects of pesticides and nitrate on the chitobiase activity (for concerned taxa, i.e., Chironomidae, and Ephemeroptera), and the cascading effects on growth, and on population structure and dynamics of each species. We could also study the cascading effects of agrochemicals on the nutrition function (possible for the 4 taxa proposed), from enzymes (β-galactosidase and β-glucosidase), through ingestion rates, right up to litter breakdown / biofilm consumption rates for example. It could also involve studying the responses of these organisms directly in the CWR, in relation to actual agrochemical flux dynamics.

Beyond the challenge of linking the different levels of organization studied, we were confronted with limitations specific to our study design and the complexity of studying biological responses in the natural environment. In particular, enzymatic monitoring by buccal swabbing was characterized by a low number of temporal replicates, particularly within the CWR (i.e., maximum of 2 sampling sessions for *Pelophylax sp.*) (cf. Chap. II). Although the ecological monitoring using litterbags was more standardized and had more replications, the robustness of the conclusions remained limited, given the short study period compared to the high level of integration and the large temporal scale of the response of the biological levels considered (i.e., community, ecosystem function) (cf. Chap. IV). In addition, if we take the example of amphibians, *in situ* toxic effects of agrochemicals are multifactorial and can be influenced by UV radiations (e.g., for genetic and morpho-anatomical impairments) (Bridges et al., 2004; da Rocha et al., 2020; Yu, Wages, et al., 2015), temperature (e.g., for developmental impairments) (Sinai et al., 2024), or else parasitic infections (e.g., for morpho-anatomical impairments) (Haas et al., 2018), making difficult to identifying effects specific to agrochemicals. At the community level, compensatory ecological effects (e.g., source-sink dynamics) may be at the root of the noticeable level of amphibian species richness characterizing the CWR, and to its sustainability, masking significant local mortality rates due to agrochemicals. Regular, long-term surveys should be set up to monitor the potential impact of CWR on its amphibian community. In addition, the comparability of comparison ponds is questionable in a sense that it was not easy to find perfectly comparable sites. Environmental factors, such as nitrite (cf. Chap. III) or temperature (cf. Chap. IV) were very influential on the responses studied. The complexity of fieldwork was thus incontestably a source of uncertainty in the interpretation of our results, linked with the unpredictability of the fieldwork, and the complex entanglement of a multitude of uncontrolled environmental factors on the responses studied. It is therefore necessary to evaluate the influence of these natural factors, in order to determine the influence of *in situ* chemical pressures on biological responses.

Given all the limitations described above and our current state of knowledge, we remain cautious with drawing any definite conclusions about the CWR's role as an ecological shelter or trap for aquatic fauna. Nevertheless, despite these limitations, we were able to identify synchronisms and antagonisms between agrochemical fluxes dynamics and aquatic fauna. In addition, the diversity of the study designs proposed in this thesis allowed us to study the responses of two major taxa of aquatic animals to agrochemicals, while covering major levels of biological organization. This enabled us to establish a holistic vision of the state of aquatic fauna in the CWR. We consequently tend to consider that the CWR is acting as an ecological trap for aquatic fauna. Perhaps to go a step further, on a non-exclusive basis, the CWR could act as: (i) an ecological shelter for some species and as an ecological trap for others, by modifying interspecific interactions, for example by modulating interspecific competition and predation (see Relyea, 2009), (ii) an ecological shelter at certain times and an ecological trap at others (e.g., depending on the hydrology and weather conditions of the watershed, on extreme pollution episodes, on phenological characteristics of species). We may even envision that (iii) some areas within the CWR could act as ecological shelters, while others would act as ecological traps.

The use of more spatial and temporal data, in the future, could allow us to refine our comprehension of the antagonistic links existing between aquatic fauna and agrochemicals within the CWR, all set within a dynamic temporal and spatial framework. Further investigations could be carried out to monitor changes in the condition of native aquatic fauna closely, through regular fauna inventories and ecotoxicological monitoring. In particular, more targeted, long-term monitoring could be used to report on changes in the health of individuals (e.g., body condition of amphibians), or to identify synchronisms and antagonisms between ecological and agrochemical dynamics (e.g., litter breakdown). Other proposals for experimentation or monitoring, and for improving the studies undertaken in this thesis, were made throughout the discussion. Of course, this list does not claim to be exhaustive, given the vast scope for exploring the question of synchronisms and antagonisms between agrochemicals and aquatic fauna in agricultural constructed wetlands.

As for the scope of this work on the potential of agricultural constructed wetlands to act as ecological traps, by virtue of their primary role of pollution mitigation, CWs present a risk for aquatic fauna. However, the specific features of each system and the complexity of the natural processes operating at these scales mean that we have to work on a case-by-case basis, by using standardized monitoring approaches to monitor the quality of aquatic environments, as the ones employed in this thesis (e.g., buccal swabbing for amphibians, litterbags to monitor litter breakdown, etc.). Although the CWR possibly acts as an ecological trap for aquatic fauna, the transferability of this finding remains relatively limited to the other agricultural CWs. However, it might be possible to assemble a dataset over several CWs where responses to pesticides could be measured over a sufficient period of time, and where these responses could be studied taking into account statistically confounding factors.

In parallel, it is important to note that, in this thesis, emphasis was placed on the well-being of the amphibians studied, through the use of non-invasive approaches (faunistic census, buccal swabbing, non-lethal morphometric measurements). However, the asymmetry of the treatment inflicted on aquatic invertebrates in the course of our work obviously raises non-negligible ethical questions. Although the question of the existence of nociception in invertebrates is open to debate (Adamo, 2019; Gibbons, Crump, et al., 2022; Gibbons, Sarlak, et al., 2022; Walters, 2018), without detracting from the vast and fascinating character of this science front, the potential for aquatic invertebrates to feel pain compels consideration and questioning of the treatment inflicted on them during faunal surveys (ethanol fixation), or during the various experiments carried out as part of this thesis (pesticide exposure). In addition to these ethical considerations, it also seems important to be aware of the ecological impact of our practices, however negligible it might be (disturbance of amphibians during inventories, disruption of the pond bed during net use, destruction of part of benthic macroinvertebrate populations, etc.). Similarly, although being a non-invasive method, buccal swabbing is not an impactless procedure for amphibians, as fragile vertebrates. The use of approaches such as this must be carefully considered, and must strive to have as little impact as possible on the animals. Consideration could be given to the development and implementation of non-lethal monitoring (e.g., DNA-based approaches) (Duarte et al., 2021; T.-T. Wang et al., 2023), or experiments where the use of aquatic invertebrates is refined, as part of the study of the harmful effects of agrochemicals on aquatic fauna.

Finally, in order to meet the challenges of biodiversity conservation in agricultural landscapes, we believe that the creation of agricultural constructed wetlands can reinforce the structure of the blue network, so necessary for aquatic fauna, and thus provide shelters for aquatic organisms. If the ecotoxic pressure is too influent within agricultural CWs, they could, at least, act as stepping stones between favorable habitats, thus helping to improve ecological connectivity in the highly fragmented agricultural landscapes (Jeliazkov et al., 2019; Préau et al., 2022; Y. Qu et al., 2024). As already highlighted by several authors (Piha, 2006; Stillway et al., 2019; Zhang et al., 2020), and supported by our results, the ecotoxic risk in these ecohydrosystems is far from nil, and should be taken into account in their implementation, and be biomonitored, in order to limit as far as possible the negative repercussions they could have on aquatic fauna.

4. General conclusion

In a context of both biodiversity under pressure from the intensification of agriculture and agrochemicals, and aquatic fauna conservation issues within agricultural landscapes, this thesis aimed to determine the potential of a French agricultural constructed wetland (CWR) to act as an ecological trap for native amphibians and aquatic invertebrates, through a multi-site, multi-taxon, multi-level, and multi-response biomonitoring approach. Overall, we showed that the potential for the CWR to act as an ecological trap for aquatic fauna was notable. However, the complexity of study designs in uncontrolled conditions across different levels of organization has challenged us in drawing definite conclusions with respect to the status of CWR. Nonetheless, for now, given the evidence of synchronisms between amphibian community vulnerability periods and agrochemical flux dynamics in the CWR, of pesticide (and sometimes nitrate) effects on sub-cellular, individual and ecological traits of aquatic fauna, we propose to define the CWR as an ecological trap for aquatic fauna. Further investigations, at all levels of biological organization, on the state of health of individuals and, more broadly, on the state of health of the ecosystem, will make it possible to refine and specify the results of this thesis. The holistic approach employed in this thesis has nevertheless enabled us to address this issue to bring new insights concerning the *in situ* effects of agrochemicals on aquatic fauna within agricultural landscapes. Given the potential role of agricultural constructed wetlands in contributing to the blue network in fragmented agricultural landscapes, investigating in more details their potential to act as ecological traps could permit to prevent and limit as far as possible the potential adverse effects they could have on aquatic fauna, while enhancing landscape connectivity. This would help sustain biodiversity in agricultural landscapes. In this sense, a better understanding of the role played by agricultural constructed wetlands within agricultural landscapes, and the promotion of actions in favor of both the remediation of chemical pollutions and the preservation of aquatic fauna, can act as an important lever for the good of human societies, environment and biodiversity.

V. References

V. References

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