Ponds for biodiversity and conservation : context, design and evaluation of restorative measures in Europe

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Ponds for biodiversity and conservation: context, design and evaluation of restorative measures in Europe

Petits plans d'eau pour la biodiversité et conservation : contexte, conception et évaluation des mesures de restauration en Europe

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- https://doi.org/10.15468/qcqnub
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Résumé étendu en français

I. Introduction

La biodiversité des écosystèmes d'eau douce est en grave déclin. La perte et la dégradation des habitats comptent parmi les principales menaces qui pèsent sur la biodiversité d'eau douce. La protection et la restauration des habitats aquatiques doivent donc être intégrées aux efforts visant à enrayer ce déclin. La nécessité de protéger et de restaurer des habitats est reconnue dans les initiatives mondiales et européennes, notamment le cadre mondial de la biodiversité de Kunming-Montréal, qui vise à ce qu'au moins 30% des écosystèmes dégradés fassent l'objet d'une restauration d'ici 2030, et le règlement de l'Union européenne (UE) sur la restauration de la nature, qui est juridiquement contraignant et fixe comme objectif de restaurer 20% des types d'habitats protégés d'ici 2030, puis l'ensemble des habitats nécessitant une restauration d'ici 2050.

La restauration écologique a été définie par la Society for Ecological Restoration comme « le processus qui assiste le rétablissement d'un écosystème qui a été dégradé, endommagé ou détruit ». Outre la restauration écologique au sens strict de cette définition, d'autres « activités restauratives », telles que la réhabilitation, font partie d'un continuum d'activités visant à améliorer le potentiel de rétablissement écologique. Dans le cadre mondial de la biodiversité de Kunming-Montréal et dans le règlement de l'UE sur la restauration de la nature, le terme « restauration » n'est pas clairement défini et peut désigner l'ensemble des activités de ce continuum.

La création de petits plans d'eau peu profonds (mares) et de réseaux de mares pourraient contribuer à atténuer le déclin de la biodiversité d'eau douce. Les mares sont des plans d'eau d'une superficie maximale de 5 ha, d'une profondeur maximale de 5 m et dont la couverture végétale émergente est inférieure à 30%. Les mares peuvent être d'origine anthropique ou naturelle. Ce n'est que récemment que les mares ont été reconnues comme des habitats d'eau douce importants. Les mares abritent des espèces qui sont absentes d'autres plans d'eau et présentent une grande diversité bêta. En raison de leur biodiversité et des services écosystémiques qu'elles rendent, la conservation des mares de haute qualité et la restauration des mares dégradées sont des priorités en matière de conservation. La création de nouvelles mares, en particulier lorsqu'elles sont placées en réseau, peut également être bénéfique pour la biodiversité et la conservation. Les projets de restauration et de création de mares sont largement mis en œuvre en Europe, et la plupart des projets combinent les deux approches. La création des réseaux de mares fait partie du continuum des activités restauratives et, avec la mise en œuvre du règlement de l'UE sur la restauration de la nature, on peut s'attendre à ce que le nombre de projets de création de mares augmente.

Pour que ces projets améliorent effectivement l'état de la biodiversité des eaux douces, il est essentiel qu'ils utilisent des méthodes fondées sur des données probantes. Cependant, les projets de restauration et de création de mares ne font souvent l'objet d'aucun suivi ni d'aucune évaluation. Il existe donc encore d'importantes lacunes dans les connaissances concernant la conception la plus efficace des réseaux de mares et les méthodes de suivi et d'évaluation adéquates. La théorie écologique est nécessaire pour fournir un contexte aux études sur la création de réseaux de mares pour la biodiversité et la conservation, et pour intégrer les résultats de ces études dans les connaissances. Les cadres pertinents pour la création de mares comprennent les concepts de la succession écologique, des filtres environnementaux, de la complexité des habitats, des causes et des impacts des invasions d'espèces exotiques et des processus d'assemblage déterministes et stochastiques, ainsi que la théorie des états stables alternatifs des lacs peu profonds et le concept de métacommunauté. Le concept de

métacommunauté intègre les processus locaux et régionaux et leurs interactions. Les métacommunautés sont des ensembles de communautés liées par la dispersion de multiples espèces en interaction. Lorsque des mares sont proches les unes des autres, leurs communautés peuvent se disperser entre les mares, formant ainsi des métacommunautés. Les mares ne sont donc pas indépendantes les unes des autres, ni de leurs entourages terrestres. Elles devraient être étudiées dans leurs « pondscapes », c'est-à-dire des réseaux de mares répartis spatialement dans une matrice terrestre, ainsi que leur connectivité.

Il est essentiel d'évaluer le succès des activités restauratives afin de savoir si les ressources ont été utilisées efficacement ou si elles auraient pu être mieux employées dans d'autres efforts de conservation et de restauration. De plus, si les activités restauratives ne sont pas évaluées, les méthodes non testées risquent d'être réutilisées dans des projets futurs, et ces méthodes ne pourront pas être optimisées. Les projets actuels de création et de restauration de mares ne sont souvent pas évalués, ou seulement sur la base d'un ensemble limité d'indicateurs. Il peut également être difficile de savoir comment évaluer le succès d'une restauration. Le succès peut être défini comme le degré de réalisation des objectifs fixés. Il est donc important de définir des objectifs clairs et des cibles quantifiables. Plusieurs stratégies peuvent être utilisées pour évaluer ce succès, par exemple en évaluant les trajectoires dans le temps ou la contribution des écosystèmes restaurés à la biodiversité du paysage aquatique, ou en comparant les écosystèmes avant et après la restauration sur les sites de contrôle et de restauration. Conformément aux objectifs fixés, il convient de définir des cibles, des stratégies d'évaluation, des indicateurs et des protocoles de suivi.

Les indicateurs de l'état écologique des mares sont souvent basés sur les macrophytes, les macroinvertébrés et les amphibiens. Dans le cadre de cette thèse, je me concentre sur les macrophytes et les macroinvertébrés. Pour les macroinvertébrés j'utilise le protocole d'échantillonnage et d'identification des macroinvertébrés Sampling of Small Shallow lake invertebrates (S_3i), qui a été récemment développé (Labat et al., 2022). L'étude ainsi que l'échantillonnage des macrophytes et des macroinvertébrés à l'aide de méthodes conventionnelles nécessitent une expertise taxonomique. De plus, l'échantillonnage des macroinvertébrés est invasif, et le tri et l'identification des échantillons de macroinvertébrés peuvent être très chronophage. Le métabarcoding de l'ADN environnemental (ADNe) est une technique non invasive prometteuse qui pourrait permettre le suivi de mares à des échelles spatiales et temporelles plus grandes. Le métabarcoding de l'ADN est beaucoup étudié mais surtout dans d'autres milieux, et elle doit encore être adaptée et validée pour les mares.

Objectifs de la thèse

Cette thèse porte sur la conception, le suivi et l'évaluation de réseaux de mares pour la biodiversité et la conservation. Elle s'inscrit dans le cadre du projet EMYS-R, qui vise à définir « les méthodes écologiques les plus efficaces et qui bénéficient du soutien social pour restaurer les zones humides en faveur de la réintroduction de la cistude d'Europe (*Emys orbicularis,* L., 1758) et de la biodiversité associée dans toute l'Europe » (https://emysr.cnrs.fr/). Ce projet européen implique un large éventail de partenaires académiques et non académiques français, allemands, polonais et lettons. Les sites d'étude de cette thèse font également l'objet d'autres recherches dans le cadre du projet EMYS-R, qui portent par exemple, sur l'analyse de la dynamique des populations et du régime alimentaire des cistudes réintroduites, sur les méthodes adaptatives de gestion des écrevisses envahissantes et sur les aspects sociaux des projets de restauration et de réintroduction. Les données générées dans le cadre de cette thèse, ainsi que d'autres données que j'ai collectées à cette fin, seront utilisées pour identifier les sources alimentaires potentielles pour la cistude.

Les objectifs généraux de ce doctorat sont de créer des connaissances pour améliorer la conception, le suivi et l'évaluation des réseaux de mares pour la conservation de la biodiversité d'eau douce en Europe. Le doctorat vise à apporter des réponses aux questions de recherche générales suivantes :

- Comment les variables environnementales et spatiales influencent-elles les communautés de macrophytes et de macroinvertébrés dans les réseaux de mares artificielles ?
- Quelles techniques de suivi sont adaptées aux réseaux de mares artificielles ?

II. Sites étudiés

Deux réseaux de mares artificielles sont étudiés : l'un sur le Neuburger Altrhein et le Woerr (Neu-Woerr), à la frontière entre la France et l'Allemagne ; et l'autre dans le parc naturel de Silene, au sud-est de la Lettonie. Le site du Neu-Woerr est situé sur la rive gauche du Rhin supérieur, qui était à l'origine un fleuve anastomosé avec une large plaine inondable. La section du Rhin supérieur où se trouve le Neu-Woerr a été régularisée entre 1907 et 1924. Le Neuburger Althrein est un ancien méandre du Rhin, qui était déjà naturellement coupé du cours principal du fleuve avant le début des travaux de régularisation. Il reste quelques bras-morts en eau, et des fossés antichars ont été creusés dans le méandre. L'abandon de la récolte traditionnelle des roseaux et de l'entretient des bras-morts pour la pêche a favorisé l'envahissement progressif des plans d'eau par les saules. Des zones protégées ont été mises en œuvre dans le Neuburger Altrhein depuis 1983 afin de protéger les populations d'espèces protégées qui habitent l'ancien méandre. Sur le Woerr, du gravier, du sable et des granulats ont été extraits des années 1960 à 1994. Depuis 1994, la gravière a été réhabilitée et des zones protégées ont été mises en œuvre depuis 1998. À Silene, des lacs précieux tels que les lacs Richu et Sita ont été soumis à l'eutrophisation depuis les années 1930. De plus, le drainage des terres autour des lacs a entraîné la dégradation des tourbières et des marais de transition dans la région. Afin de protéger les lacs et les tourbières, le parc naturel de Silene a été fondé en 1997.

Les deux sites d'étude présentaient des populations d'amphibiens en déclin. De plus, ces deux sites sont des lieux de réintroduction de la cistude d'Europe. Afin de fournir un habitat aux cistudes réintroduites et aux populations d'amphibiens en déclin, des mares ont été créées entre 2011 et 2018. Au total, 26 d'entre elles sont étudiés dans le cadre de cette thèse. Sur chaque site d'étude, 13 mares permanentes ont été sélectionnées dans le but de couvrir la plus grande diversité possible de macrophytes. De plus j'ai échantillonné d'autres plans d'eau pour identifier les sources alimentaires potentielles de la cistude.

III. Facteurs environnementaux et spatiaux structurant les métacommunautés de macrophytes dans des paysages aquatiques restaurés

La création de réseaux de mares pourrait être favorable à la biodiversité d'eau douce, mais des recherches supplémentaires sont nécessaires pour comprendre comment leur conception influe sur les résultats en termes de biodiversité. Cette conception de réseau de mares pourrait influencer les variables environnementales et spatiales susceptibles de structurer les métacommunautés de macrophytes qui se forment dans les réseaux de mares créés. On peut s'attendre à ce que des facteurs environnementaux, tels que la disponibilité de la lumière, des nutriments et du bicarbonate, ainsi que la présence d'écrevisses bioturbatrices, influencent la composition des communautés, la diversité et les formes de vie des macrophytes. Les variables spatiales, notamment le degré d'isolement et les distances entre les mares, pourraient aussi influencer la composition des communautés de macrophytes, en limitant leur dispersion.

Les macrophytes sont des éléments clés des écosystèmes d'eau douce, car ils stabilisent les sédiments, régulent les nutriments et fournissent un habitat physique et de la nourriture à de nombreux organismes aquatiques. Cependant, peu d'études ont été menées sur les processus qui façonnent les communautés de macrophytes dans les réseaux de mares créés pour la conservation de la biodiversité. Par ailleurs, la dimension spatiale reste négligée dans les études portant sur les facteurs influençant les communautés de macrophytes. Afin de combler ces lacunes, cette étude examine les processus environnementaux et spatiaux qui façonnent les communautés de macrophytes dans deux réseaux de mares permanentes d'origine anthropiques en Europe. Les questions de recherche sont les suivantes :

- Comment les conditions abiotiques et biotiques des mares permanentes artificielles sont-elles liées à la composition et aux métriques décrivant les communautés de macrophytes ?
- Comment l'occupation des sols environnante est-elle liée aux conditions abiotiques et biotiques des mares ?
- Comment les distances géographiques et les différences en termes de conditions abiotiques et biotiques entre les mares influencent-elles la dissimilarité des communautés de macrophytes ?

Méthodes

Les communautés de macrophytes des 26 mares sélectionnées sur les sites de Neu-Woerr et Silene ont été étudiées en juin et juillet 2023. Dans chaque mare, toutes les hélophytes et hydrophytes présentes dans la zone habituellement inondée ont été identifiées et le pourcentage de la surface de la mare couvert par chaque taxon a été estimé. Les métriques taxonomiques suivantes ont été calculées : richesse taxonomique, diversité de Hill Shannon, égalité de Simpson et distinction taxonomique ; ainsi que les métriques fonctionnelles suivantes : recouvrement total des macrophytes et recouvrement relatif des formes de vie émergentes, flottantes ancrées, submergées ancrées et flottantes non ancrées. Les variables environnementales suivantes ont été mesurées : transparence de l'eau, pH, concentration en chlorophylle a, conductivité spécifique, profondeur maximale et, à Neu-Woerr, abondance de l'écrevisse calicot (*Faxonius immunis*, Hagen, 1870). Le pourcentage de la surface de la mare ombragée par les arbres environnants à midi, ainsi que la contribution à l'occupation des sols dans la zone tampon de 5 m autour des mares par des roselières, des saules et des arbustes, des grands arbres, des zones humides et des prairies ont été estimés. L'isolement des mares, mesuré comme la distance au plan d'eau le plus proche, et la superficie de la mare correspondant au niveau d'eau maximal, ainsi que les coordonnées spatiales des mares ont été obtenues à l'aide de QGIS à partir de caractéristiques numérisées basées sur les images satellites de Google. Les coordonnées spatiales ont été utilisées pour calculer la distance géographique entre les mares. La dissimilarité de Bray-Curtis entre les communautés de macrophytes des différentes mares a été calculée sur les données de recouvrement.

Les co-inerties entre a) les variables environnementales et les métriques dérivant les communautés de macrophytes, b) les variables environnementales et le recouvrement des taxons de macrophytes et c) l'occupation des sols autour des mares et les variables environnementales, ont été analysées à l'aide des analyses de coinertie. La régression non linéaire par loi de puissance, également appelée modèle log-log, a été utilisée pour examiner la relation entre la distance géographique entre les mares et la similarité de leurs communautés de macrophytes. En outre, des modèles de dissimilarité généralisés ont été utilisés pour identifier à la fois la contribution de la distance géographique entre les mares ainsi que les contributions des dissimilarités en termes de chacune des variables environnementales aux dissimilarités de macrophytes.

Résultats et discussion

Au total, 81 espèces de macrophytes ont été identifiés, dont 35 à Neu-Woerr et 63 à Silene. Certains taxons de *Carex* n'ont été identifiés qu'au niveau du genre. Par mare, la richesse taxonomique, la diversité de Hill Shannon et le recouvrement total des macrophytes étaient plus élevés à Silene qu'à Neu-Woerr. Les macrophytes dans les mares à Neu-Woerr étaient principalement des plantes émergentes et des charophytes, tandis que le recouvrement relatif des différentes formes de vie dans les mares à Silene variait d'une mare à l'autre.

Pour les deux sites, l'analyse de co-inertie a montré des corrélations significatives entre les variables environnementales et les métriques décrivant les communautés de macrophytes, ainsi qu'entre les variables environnementales et le recouvrement des taxons de macrophytes. Les variables influençant les conditions lumineuses : la transparence de l'eau, la concentration en chlorophylle a, le degré d'ombrage par les arbres environnants et, à Neu-Woerr, l'abondance des écrevisses, ont été les variables avec les contributions les plus importantes à la co-inertie. À Neu-Woerr, les mares présentant des conditions de luminosité réduites avaient le recouvrement relatif le plus élevé d'espèces émergentes, tandis que les mares présentant des conditions de luminosité plus élevées avaient le recouvrement relatif le plus élevé de plantes submergées, et présentaient le recouvrement total et la distinction taxonomique les plus élevés. À Silene, les mares avec des conditions de luminosité plus faible montrent un recouvrement relatif plus élevé en macrophytes flottants non ancrés (principalement des *Lemnacea*), tandis que celles avec des conditions de luminosité améliorées, ainsi qu'un pH élevé et une plus grande profondeur, se caractérisaient par un recouvrement relatif élevé de plantes submergées. Pour les deux sites, la superficie des mares et la distance par rapport au plan d'eau le plus proche ont peu contribué aux co-inerties. Pour Neu-Woerr, l'âge des mares n'a pas contribué aux co-inerties non plus, et pour Silene, l'âge des mares a eu une faible contribution aux co-inerties.

L'occupation des sols dans une zone tampon de cinq mètres autour des mares présentait une corrélation significative avec les variables environnementales à Silene, mais pas à Neu-Woerr. À Silene, les mares bordées de roselières et de prairies avaient une eau relativement claire, tandis que celles entourées d'arbres présentaient des concentrations plus élevées en chlorophylle a et une transparence de l'eau plus faible. Selon la régression non linéaire, la similarité des communautés de macrophytes diminuait de manière significative avec la distance géographique entre les mares à Silene, mais pas à Neu-Woerr. Cependant, selon le modèle de dissimilarité généralisé, la dissimilarité des communautés de macrophytes à Silene s'expliquait par les différences d'ombrage par les arbres environnants et de transparence de l'eau, mais pas par la distance géographique entre les mares. Le modèle de dissimilarité généralisé pour Neu-Woerr n'était pas significatif.

Sur le site de Silene, la dominance des macrophytes flottants dans les eaux ombragées et troubles pouvait probablement être liée à une combinaison de conditions de faible luminosité, d'absence de vent et de niveaux élevés de nutriments, qui se retrouvent dans ces mares entourées d'arbres. À Neu-Woerr, l'absence de macrophytes submergés dans les mares où les écrevisses calicot étaient abondantes pourrait s'expliquer par la bioturbation des sédiments liés à l'activité de creusement des écrevisses, et par l'herbivorie directe exercée par ces écrevisses. À Silene, la diversité bêta des macrophytes était liée aux différences d'ombrage des arbres environnants et à la transparence de l'eau. Sur la base de ces résultats, il peut être recommandé de créer de nouvelles mares dans des régions sans écrevisses bioturbatrices. En outre, la diversité bêta des communautés de macrophytes peut être améliorée en créant des mares présentant des conditions environnementales diversifiés, avec notamment différents niveaux d'ombrage provenant des arbres environnants.

IV. Évaluation de la biodiversité en macroinvertébrés des mares anthropiques dans un pays balte

Bien que les mares soient reconnues comme des habitats d'eau douce importants, elles ne sont souvent pas incluses dans les programmes de suivi de la qualité écologique. Comme la plupart des États membres de l'UE, les pays baltes n'incluent que les plans d'eau d'une superficie supérieure à 50 ha dans leurs programmes de suivi pour la directive-cadre sur l'eau. Des exemples du Royaume-Uni, des pays méditerranéens et de la Suède ont montré que la création de mares peut être une mesure efficace pour renforcer la biodiversité d'eau douce. Cependant, la biodiversité des mares artificielles dans les pays baltes a reçu peu d'attention. Des réseaux de mares ont été créés dans les pays baltes pour la conservation des d'amphibiens et de la cistude d'Europe. Les populations d'amphibiens et de cistudes ont ainsi été étudiées dans ces mares, mais pas des autres groupes, comme les macroinvertébrés.

Les macroinvertébrés sont omniprésents, vivent dans une grande variété d'habitats et représentent plusieurs niveaux trophiques. Ils contribuent à la décomposition des feuilles mortes, au cycle des nutriments, au flux de nutriments à travers l'interface sédiments-eau, et fournissent de la nourriture aux consommateurs dans et hors de l'eau. Les mares sont des habitats pour des macroinvertébrés rares et protégés. Il n'est donc pas surprenant que les macroinvertébrés sont utilisés dans les quelques protocoles d'évaluation de la qualité des mares qui existent en Europe. Pour la Lettonie, des données sur les macroinvertébrés sont disponibles pour les lacs qui font l'objet d'une suivie pour la directive-cadre sur l'eau. Cependant, il n'existe aucune donnée lettone sur les macroinvertébrés des mares, du moins pas dans la littérature publiée en anglais.

Pour combler cette lacune, cette étude examine les communautés de macroinvertébrés dans les mares artificielles permanentes en Lettonie à l'aide de la méthode d'échantillonnage et d'identification des macroinvertébrés S_3i. La méthode S_3i prescrit de prélever un échantillon de macroinvertébrés par type de mésohabitat. Un mésohabitat est un habitat visuellement distinct et facilement identifiable au sein de la mare, et la méthode S_3i fournit une liste des types de mésohabitats qui peuvent être observés dans une mare. La méthode distingue les types de mésohabitats dont la profondeur est supérieure ou inférieure à 20 cm. Les objectifs de cette étude sont d'étudier l'influence du type de mésohabitat et de l'identité de la mare sur la composition des communautés de macroinvertébrés, et d'évaluer la co-inertie entre les variables environnementales à l'échelle de la mare et la composition des communautés de macroinvertébrés, ainsi que la co-inertie entre les communautés de macroinvertébrés et de macrophytes. L'étude examine en outre si la diversité des macrophytes, dont l'évaluation est moins longue que celle de la diversité des macroinvertébrés, pourrait se substituer à cette dernière, et examine les différences et les similarités entre les taxons de macroinvertébrés présents dans les mares artificielles et les lacs lettons étudiés pour la directive-cadre sur l'eau.

Méthodes

Dix des mares artificielles permanentes sur le site de Silène en Lettonie (décrites dans les sections II et III), ont été échantillonnées en juillet 2022. Les macrophytes ont été étudiés comme décrit dans la section III. Les variables environnementales décrites dans la section III ont été mesurées, ainsi que les concentrations d'oxygène dissous, d'azote total et de phosphore total. Les macroinvertébrés ont été échantillonnés selon le protocole S_3i. En fonction du type de mésohabitat, les échantillons ont été prélevés en faisant des mouvements vigoureux aller-retour avec un haveneau dans une zone d'environ un mètre carré ou en ramassant des sédiments ou des macrophytes flottants dans des zones de superficie prescrites dans le protocole. Les échantillons ont été conservés dans de l'éthanol et identifiés au niveau taxonomique le plus bas possible. L'ambiguïté taxonomique dans l'ensemble de données a été résolue en attribuant des

identifications « parents » de niveau taxonomique supérieur à leurs « enfants » des niveaux inférieurs présents dans l'échantillon, puis dans l'ensemble de données.

Pour les dix mares combinées, la complétude de l'échantillonnage a été évaluée à l'aide de courbes de raréfaction et de l'estimateur de richesse Chao2. Des courbes de raréfaction ont également été construites pour chaque mare. Les différences dans la composition des communautés de macroinvertébrés entre les types de mésohabitats et les mares ont été testées à l'aide d'une analyse des similarités et visualisées à l'aide d'un positionnement multidimensionnel non métrique basé sur l'indice de dissimilarité de Hellinger. La co-inertie entre les variables environnementales et la composition des communautés de macroinvertébrés a été analysée. Une analyse de co-inertie a également été effectuée entre les communautés de macrophytes et de macroinvertébrés, et des tests de Mantel ont été réalisés pour évaluer la corrélation entre les dissimilarités des communautés de macrophytes et de macroinvertébrés. La corrélation de Spearman entre la diversité des macrophytes et celle des macroinvertébrés a également été testée. Enfin, les taxons de macroinvertébrés trouvés dans les dix mares artificielles ont été comparés à ceux observés dans les dix lacs les plus proches suivis dans le cadre du programme de suivi de la directive-cadre sur l'eau en Lettonie.

Résultats et discussion

Dans les dix mares, 36 échantillons ont été prélevés dans 12 types de mésohabitats. Un total de 116 taxons de macroinvertébrés (89 genres, 53 familles) a été identifié. Parmi ceux-ci figuraient *Lestes virens*, *Leucorrhinia albifrons*, *Leucorrhinia pectoralis* et *Segmentina nitida*, qui sont des espèces protégées en Lettonie. L'estimateur Chao2 était de 132 avec une erreur type de 8 taxons, ce qui suggère que la grande majorité des taxons des dix mares ont été échantillonnés. La richesse taxonomique des macroinvertébrés par échantillon variait de 0 à 47 taxons et était significativement plus élevée dans les types de mésohabitats de moins de 20 cm de profondeur que dans ceux de plus de 20 cm de profondeur. L'analyse de similarité a montré que les macroinvertébrés des échantillons provenant de la même mare étaient plus similaires que ceux provenant de différentes mares. Cependant, les échantillons provenant du même type de mésohabitat n'étaient pas plus similaires que ceux provenant de différents types de mésohabitats. L'analyse de la co-inertie a montré une corrélation forte et significative entre la composition des macroinvertébrés et les variables environnementales. Les variables environnementales avec les contributions les plus importantes à la co-inertie étaient les concentrations de chlorophylle a, d'azote total, de phosphore total et d'oxygène, ainsi que l'ombrage des arbres environnants et la transparence de l'eau. L'abondance de la plupart des taxons était plus élevée dans les mares présentant des concentrations relativement faibles en nutriments et un degré d'ombrage relativement faible, ainsi que des concentrations en oxygène et une transparence de l'eau relativement élevées. Les mares à faible concentration en oxygène hébergeaient les plus fortes abondances de planorbidés, y compris l'espèce protégée *Segmentina nitida*.

Les tests de Mantel et les analyses de co-inertie ont démontré des corrélations significatives entre les communautés de macrophytes et de macroinvertébrés, mais les tests de corrélation de Spearman entre leurs diversités n'étaient pas significatifs. Les comparaisons entre les mares et les lacs ont révélé que 81 taxons étaient propres aux mares, 64 propres aux lacs et 31 taxons étaient observés à la fois dans les mares et les lacs. Les quatre espèces protégées susmentionnées n'étaient présentes que dans le jeu de données des mares, tandis que les espèces protégées en Lettonie *Anax imperator* et *Libellula fulva* n'étaient présentes que dans le jeu de données des lacs. Le jeu de données des mares contenait plus de taxons de coléoptères, d'hémiptères et d'odonates que le jeu de données des lacs, alors que ce dernier contenait plus de taxons de trichoptères et d'éphéméroptères.

Cette étude fournit un jeu de données unique sur les macroinvertébrés des mares artificielles lettones. Elle démontre que ces mares peuvent abriter des espèces protégées. De plus, la richesse taxonomique médiane par mare était comparable à celle des mares de haute qualité échantillonnées à l'aide de la méthode S_3i en France. L'échantillonnage à l'aide du protocole S_3i a permis de capturer la plupart des taxons présents dans les dix mares. Cependant, l'exhaustivité de l'échantillonnage dans chaque mare doit encore être mesurée en faisant des courbes de raréfaction à partir d'un plus grand nombre d'échantillons provenant de la même mare. Les communautés de macroinvertébrés différaient significativement d'une mare à l'autre, mais pas d'un type de mésohabitat à l'autre, ce qui pourrait indiquer que les variables environnementales au niveau des mares avaient plus d'influence sur la structure des communautés de macroinvertébrés que le type de mésohabitat. Cependant, ce résultat pourrait également s'expliquer par des différences dans les communautés végétales constituant le même type de mésohabitat dans différentes mares.

La concentration en oxygène était probablement un facteur majeur influençant les communautés de macroinvertébrés dans les mares de Silene. Les planorbidés ont probablement pu vivre dans les mares où les concentrations en oxygène dissous étaient faibles en raison de leur capacité à prélever l'oxygène à la surface, de leurs poumons et de leur hémoglobine à forte affinité pour l'oxygène. Les taxons moins adaptés aux conditions de faible concentration en oxygène n'ont probablement pas pu vivre dans ces mares. La co-inertie significative et forte entre la composition des communautés de macrophytes et de macroinvertébrés pourrait s'expliquer par les réponses de ces deux groupes aux mêmes conditions environnementales, par l'influence de ces conditions sur les macrophytes qui, à leur tour, influencent les macroinvertébrés, ou par une combinaison de ces deux possibilités.

La comparaison entre les jeux de données des mares anthropiques et des lacs constitue une première étape vers une évaluation de la complémentarité des mares artificielles par rapport aux autres habitats d'eau douce dans le paysage et, par conséquent, de leur contribution potentielle à la biodiversité régionale des eaux douces. Les différences entre les ensembles de données des mares et des lacs pourraient s'expliquer par des différences dans les années et les saisons d'échantillonnage, et dans les protocoles d'échantillonnage. Cependant, l'observation d'une richesse taxonomique des coléoptères et des hémiptères plus élevée dans les mares que dans les lacs concorde avec d'autres études. Les études futures pourraient comparer les mares artificielles à d'autres types de plans d'eau dans le paysage en utilisant des protocoles comparables pour les différents types de plans d'eau.

V. Évaluation du métabarcoding de l'ADN environnemental pour des mares européennes

Le suivi des mares avec des méthodes conventionnelles de détection des macrophytes, des macroinvertébrés et des amphibiens nécessite une expertise taxonomique. De plus, les méthodes conventionnelles pour les macroinvertébrés sont chronophages et invasives. Les mares peuvent être difficiles à accéder et, comme des mares, même proches, peuvent différer considérablement dans leurs compositions de communautés, il peut être nécessaire d'en suivre un nombre important d'entre elles pour obtenir un échantillonnage représentatif. Pour permettre le suivi des grands nombres de mares et des suivis répétés dans le temps, une méthode moins chronophage et moins invasive serait donc très avantageuse. Le métabarcoding de l'ADN environnemental a été présenté comme une révolution pour le suivi de la biodiversité aquatique. Non invasive, sensible, économique et rapide, cette méthode permettrait de détecter simultanément plusieurs groupes taxonomiques à partir d'un seul échantillon d'eau et pourrait compléter les études conventionnelles. Malgré les progrès rapides des techniques de métabarcoding de l'ADN environnemental pour la biosurveillance aquatique, les évaluations

spécifiques aux mares restent rares pour les groupes de macro-organismes autres que les amphibiens et les poissons. La dynamique de l'ADN environnemental peut varier en fonction des types de milieux aquatiques, et les protocoles doivent être adaptés aux différents types de plans d'eau et groupes taxonomique cibles. Dans les mares, l'ADN environnemental peut être réparti de manière hétérogène en raison du mélange limité de l'eau, et son échantillonnage peut être compliqué en raison de la turbidité de l'eau.

Cette étude vise à évaluer l'utilisation du métabarcoding de l'ADN environnemental pour détecter les macrophytes, les macroinvertébrés et les amphibiens à partir des mêmes échantillons d'eau prélevés dans des mares européennes. Elle vise également à détecter les poissons à partir de ces échantillons, car ceux-ci pourraient exercer une influence négative sur les communautés des mares. L'un des objectifs est d'étudier l'hétérogénéité de l'ADN environnemental dans les mares. Un autre objectif est de comparer les inventaires de taxons obtenus par métabarcoding de l'ADN environnemental à ceux obtenus par les méthodes conventionnelles de suivi des macrophytes, des macroinvertébrés et des amphibiens.

Méthodes

L'échantillonnage a été effectué en juillet 2022 dans neuf des mares artificielles à Silene. Dans chaque mésohabitat, identifié selon le protocole S_3i, deux réplicas de 250 mL d'eau de surface ont été prélevés pour collecter l'ADN environnemental, puis les macroinvertébrés ont été échantillonnés comme décrit dans la section IV. Les relevés de macrophytes ont été effectués comme décrit dans la section III. Des données de suivi des amphibiens étaient également disponibles pour cinq des mares. Les échantillons d'eau ont été filtrés à travers des capsules filtrantes Sterivex fermées (PVDF, taille des pores de 0,45 µm) à l'aide d'une pompe péristaltique, et un tampon Longmire a été ajouté aux capsules pour la préservation de l'ADN. Afin d'éviter toute contamination, seuls des matériaux neufs ou javellisés ont été utilisés, et un échantillon témoin négatif d'eau de rinçage et un échantillon témoin négatif d'air ont été prélevés chaque jour d'échantillonnage.

L'ADN a été extrait dans une station de travail dédié à la manipulation de faibles concentrations d'ADN. L'extraction a été réalisé l'aide d'un protocole que j'ai adapté pour les filtres Sterivex conservés dans le tampon Longmire. Des témoins négatifs d'extraction ont été inclus dans chaque lot d'extraction. Sept paires d'amorces ont été utilisées pour amplifier les régions ITS2, rbcL et trnL pour les plantes, le marqueur COI pour les invertébrés, deux marqueurs 16S pour les mollusques et la région 12S pour les vertébrés.

Dans le cadre d'une stratégie de multiplexage des métabarcodes, chaque paire d'amorces pour plantes a été combinée avec une paire d'amorces pour invertébrés (y compris les mollusques) dans la même réaction d'amplification en chaîne par polymérase (ACP). Des témoins négatifs et positifs de ACP ont été inclus sur chaque plaque et la ACP a été réalisée en quatre réplicas. En outre, certaines combinaisons de tags ont été laissées inutilisées comme témoins négatifs de tagging. Les ACP amplifiant l'ADN provenant d'échantillons d'autres campagnes d'échantillonnage (dans la région de Neu-Woerr) ont été cyclées sur les mêmes plaques de ACP et les produits ont été mis dans la même librairie de séquençage. Le pipetage a été effectué par un robot et le séquençage a été réalisé par des machines Illumina Novaseq. Des bases de données de référence ont été créées avec CRABS. Elles comprenaient des espèces de tous les genres de plantes et de vertébrés, y compris les espèces envahissantes, connues en Europe, ainsi que toutes les espèces de macroinvertébrés sur Terre pour lesquelles des séquences de référence étaient disponibles. Un pipeline OBITools4 a été utilisé pour la bio-informatique.

L'un des marqueurs 16S était spécifique aux Unionidae et n'avait pas amplifié d'ADN de ce groupe, probablement parce ce groupe était absent des mares échantillonnées. Pour cinq des

paires d'amorces, le nombre de reads et de variantes de séquence d'amplicon ne différait pas entre les échantillons et les témoins négatifs. Comme cela incluait les témoins négatifs de tagging, il est probable que quelque chose ait mal tourné avec les tags. Je n'ai pas analysé les résultats de ces cinq paires d'amorces. Seulement le jeu de données ITS2 sur les plantes possédait des échantillons présentant un nombre de reads et d'ASV significativement plus élevé que les témoins négatifs. J'ai donc analysé ce jeu de données. Cependant, comme il y avait quand même des reads et des ASV dans les témoins négatifs, j'ai nettoyé les données à l'aide de deux méthodes alternatives : les méthodes de nettoyage « Romahn » et « MetabaR ».

Pour les deux ensembles de données ITS2 résultant des deux méthodes de nettoyage, la dissimilarité de Dice-Sørensen entre les communautés d'unités taxonomiques opérationnelles a été calculée et une analyse PERMANOVA a été effectuée afin de tester si les échantillons différaient entre les mares et entre les mésohabitats au sein des mares. Des diagrammes de Venn ont été construits afin de visualiser toutes les espèces, les espèces de macrophytes, et les genres qui n'ont été détectés qu'à l'aide des relevés conventionnels, du métabarcoding de l'ADN environnemental et des deux méthodes.

Résultats et discussion

Il est possible que dans les cinq jeux de données qui n'ont pas été analysés, le changement de tags entre les amplicons provenant de différentes réactions ACP (« tagjumps ») se soit produit très fréquemment. Ce problème était probablement moins présent dans le jeu de données ITS2, qui a été nettoyé à l'aide de deux méthodes permettant de corriger ce problème et la contamination. La méthode de nettoyage Romahn a entraîné une perte de données plus importante que la méthode MetabaR. Cependant, la méthode Romahn a peut-être été plus efficace pour éliminer les détections faussement positives. La méthode Romahn a retenu les données de 36 échantillons et comprenait 5,7 millions de reads, regroupées en 257 unités taxonomiques opérationnelles attribuées à 77 taxons. L'ensemble de données MetabaR comprenait 52 échantillons et consistait en 12,1 millions de reads regroupées en 853 unités taxonomiques opérationnelles attribuées à 183 taxons.

Pour les deux jeux de données issus des deux méthodes de nettoyage, les échantillons provenant de la même mare présentaient des compositions d'unités taxonomiques opérationnelles plus similaires que les échantillons provenant de différentes mares. Dans le jeu de données Romahn uniquement, les deux réplicas provenant du même mésohabitat étaient plus similaires que les échantillons provenant de mésohabitats différents au sein de la même mare. La plupart des espèces détectées par métabarcoding de l'ADN environnemental étaient terrestres et seulement 35% des espèces dans le jeu de données Romahn et 20% des espèces dans le jeu de données MetabaR étaient des macrophytes. Dans les neuf mares, les relevés conventionnels avaient détecté 58 espèces de macrophytes, et seule une petite fraction d'entre elles a également été détectée par métabarcoding de l'ADN environnemental : cinq dans le jeu de données Romahn et 7 dans le jeu de données MetabaR. Les espèces de macrophytes détectées par métabarcoding de l'ADN environnemental n'étaient souvent pas détectées dans les deux réplicas provenant du même mésohabitat. Ceci pourrait indiquer que l'hétérogénéité de l'ADN environnemental dans les mares était élevée, que les volumes d'eau prélevés n'était pas suffisant ou que le nettoyage des données était trop strict.

De modestes niveaux de contamination sont probablement inévitables dans les études de métabarcoding. Afin d'évaluer la qualité des données et de permettre leur correction, il est indispensable d'inclure systématiquement des témoins négatifs à toutes les étapes du métabarcoding. Les réplicas ACP sont également importants. Les futures études de métabarcoding devraient également toujours utiliser des protocoles de préparation de librairies qui évitent les tagjumps. Je ne recommande pas le multiplexage de plusieurs paires d'amorces

dans une seule réaction ACP. Les recherches futures pourraient porter sur l'optimisation des protocoles de nettoyage des données. Concernant les macrophytes, les recherches futures sur le métabarcoding de l'ADN environnemental pourraient viser à améliorer la concordance avec les relevés conventionnels, par exemple en filtrant de plus grands volumes d'eau, en utilisant plusieurs marqueurs et en optimisant la stratégie d'échantillonnage ainsi que le choix des marqueurs. Les échantillons de sédiments et la collecte passive d'ADN environnemental sont également prometteurs pour l'échantillonnage de l'ADN dans les mares. Dans un manuscrit en cours de révision (Werner et al., en cours de révision), nous avons comparé la collecte passive d'ADN environnemental au filtrage actif décrit dans cette thèse, et les résultats sont prometteurs. Toutefois, les méthodes doivent encore être affinées.

VI. Discussion générale

La création de réseaux de mares peut être très bénéfique pour le renforcement de la biodiversité aquatique et la conservation des espèces. Des projets de création de mares ont été mis en œuvre en Europe, et leur nombre est censé augmenter grâce à des initiatives comme le règlement européen sur la restauration de la nature, qui est entré en vigueur l'année dernière. Cependant, des lacunes existent encore sur les connaissances d'une conception efficace de réseaux de mares pour atteindre les objectifs en matière de biodiversité et de conservation, ainsi que sur la manière de surveiller et d'évaluer le succès de réseaux de mares artificielles à atteindre leurs objectifs. Cette thèse visait à combler ces lacunes en étudiant les variables environnementales et spatiales qui peuvent influencer les communautés de macrophytes et de macroinvertébrés dans les réseaux de mares artificielles, ainsi que les techniques de suivi des mares. Dans la discussion générale, je présente une synthèse des résultats et des limites de l'étude. Je tire également des implications pour la création, le suivi, l'évaluation et la gestion de réseaux de mares, et je propose des perspectives pour de futures pistes de recherche intéressantes.

Synthèse

Sur la base des résultats des chapitres 3 et 4 et des théories décrites au chapitre 1, j'ai proposé des modèles hypothétiques des principaux facteurs influençant les communautés de macrophytes et de macroinvertébrés dans les mares de Neu-Woerr et de Silene. Dans ces modèles hypothétiques, les facteurs importants qui peuvent influencer les communautés sont l'écrevisse calicot à Neu-Woerr, le degré d'entourage des mares par des arbres, les concentrations de nutriments et peut-être l'âge des mares à Silene. Je pose l'hypothèse que les communautés de macrophytes influencent directement les communautés de macroinvertébrés en leur fournissant un habitat physique, et indirectement en fournissant des surfaces sur lesquelles peut se développer le périphyton, qui sert de nourriture aux macroinvertébrés. De plus, certaines variables, telles que le pH, pourraient avoir influencé à la fois les macrophytes et les macroinvertébrés.

Plusieurs variables qui pourraient avoir influencé les communautés de macrophytes et de macroinvertébrés n'ont pas été mesurées ou n'ont pas été mesurées correctement. L'alcalinité, la structure des sédiments, les fluctuations du niveau d'eau et les concentrations de pesticides n'ont pas été mesurées. Les concentrations en nutriments auraient pu être mesurées en hiver, les concentrations en oxygène le matin et l'abondance des écrevisses à l'aide de pièges artificiels. Il serait intéressant d'étudier les effets des castors et des élans présents à Silene sur les macrophytes des mares. Il serait également intéressant de continuer à étudier la succession écologique des mares au fil du temps. Il serait préférable de suivre les mêmes mares au fil du temps plutôt que d'adopter une approche chronoséquentielle. Dans les réseaux de mares étudiés, toutes les mares étaient situées à moins de 3 km les unes des autres et la distance entre les mares n'était pas un facteur important dans la structuration des communautés de

macrophytes. Il serait intéressant d'étudier des mares plus éloignées les unes des autres. En outre, d'autres réseaux de mares artificielles pourraient être étudiés afin de comprendre comment le contexte dans lequel les réseaux de mares sont créés peut influencer les résultats.

Dans le cadre de cette thèse, deux techniques conventionnelles adaptées au suivi des mares ont été utilisées : les relevés de macrophytes et l'échantillonnage des macroinvertébrés selon le protocole S_3i. De plus, le métabarcoding de l'ADN environnemental, qui doit encore être optimisé pour les mares, a été testé. En raison de probable problèmes de tagjump, cinq des jeux de données d'ADN environnemental n'ont pas pu être analysés. Seul le jeu de données ITS2 sur les plantes a pu être analysé après nettoyage des données. La concordance entre les relevés conventionnels de macrophytes et le métabarcoding de l'ADN environnemental était faible.

Implications

Certaines des variables environnementales qui ont probablement influencé les communautés de macrophytes et de macroinvertébrés dans les réseaux de mares étudiés, telles que le degré d'ombrage des arbres environnants, pourraient être relativement faciles à influencer par la conception et la gestion des réseaux de mares, tandis que d'autres, telles que les niveaux de nutriments, nécessitent de prendre en compte la dynamique des bassins versants, et d'autres encore, telles que l'abondance des écrevisses bioturbatrices, sont notoirement difficiles à contrôler. Pour le réseau de mares à Silene, la diversité bêta des macrophytes pourrait être maintenue en veillant à ce que les mares du réseau présentent toujours différents niveaux de transparence de l'eau et d'ombrage provenant des arbres environnants. Cela nécessiterait une gestion périodique toutes les quelques années.

La conception, le suivi, l'évaluation et la gestion des réseaux de mares doivent toujours être adaptés aux objectifs et cibles des activités restauratives clairement définis. Avant de créer des réseaux de mares, il est nécessaire d'évaluer les causes de la dégradation et les potentiels effets négatifs des activités restauratives proposées. Les réseaux de mares créés pour la biodiversité et la conservation doivent faire l'objet d'un suivi. J'ai proposé un protocole de suivi minimal pour les réseaux de mares créés avec pour objectif une augmentation générale du recouvrement macrophytique.

Perspectives

Les perspectives prometteuses pour la recherche sur les réseaux de mares artificielles que je voudrais souligner ici sont l'utilisation des nouvelles technologies dans le suivi des mares et les approches paysagères pour leur emplacement. La collecte passive d'ADN environnemental ne nécessite pas de filtrage de l'eau et est prometteuse pour les mares, car elle pourrait permettre un échantillonnage rapide à de nombreux endroits dans une mare et évite les contraintes liées au colmatage des filtres Dans un manuscrit en cours de révision (Werner et al., en cours de révision), nous avons comparé la collecte passive d'ADN environnemental au filtrage actif décrit dans cette thèse, et les résultats sont prometteurs. Toutefois, les méthodes doivent encore être affinées. En outre, des études récentes montrent que les techniques de capture-incubation-libération sont prometteuses pour la détection non invasive des macroinvertébrés dans les cours d'eau. Elles pourraient être adaptées aux mares, et pour des groupes spécifiques comme les coléoptères, qui constituent un groupe indicateur très intéressant. Le métabarcoding de l'ADN environnemental pourrait permettre de détecter des groupes bioindicateurs intéressants pour le biosurveillance, mais qui ne sont pas utilisés actuellement parce qu'ils sont difficiles à identifier, comme les hydracariens, les diptères ou des groupes de micro-organismes.

Pour évaluer correctement les avantages potentiels des mares artificielles pour la biodiversité régionale d'eau douce, il faudrait étudier leurs communautés par rapport à celles d'autres types de milieux d'eau douce. De plus, des études de connectivité pourraient être menées afin d'évaluer

comment la création de mares pourrait modifier la connectivité des paysages aquatiques, ce qui est intéressant dans le contexte de l'adaptation au changement climatique, et afin d'évaluer où les mares pourraient être placées pour renforcer les populations en déclin d'espèces rares et protégées. Je pense que les macrophytes et les macroinvertébrés pourraient en eux-mêmes faire l'objet de projets de conservation. En ce qui concerne la conservation des macroinvertébrés, l'un des principaux enjeux est que le statut de conservation et la répartition de nombreuses espèces sont inconnus, et des études futures pourraient améliorer ces connaissances.

Table of contents

Glossary

*This Glossary provides the definitions of terms as they are used in this PhD. In some cases, synonyms are listed together, separated by a semicolon in the term column. Words shown in **bold** are other terms that are defined in this Glossary.*

Term	Definition
Alien species; Non-native species; Introduced species	A species whose presence in a region is attributable to human actions that enabled them to overcome fundamental biogeographical barriers (Richardson, 2011).
Amplicon	A section of DNA that has been amplified through a reaction such as **polymerase chain reaction** (Bruce et al., 2021).
Amplicon sequence variant; Exact sequence variant	Inferred unique DNA sequence that is obtained by a process called denoising which accounts for sequencing errors. Unlike **operational taxonomic units**, Amplicon sequence variants are not obtained from clustering (Callahan et al., 2017).
Barcoding	Taxonomic identification of a species based on DNA **sequencing** of a short gene region that shows variation at the species level (Bruce et al., 2021).
Biodiversity	The variability among living organisms from all sources including, among others, terrestrial, marine and other aquatic ecosystems, and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems (adapted from Gann et al., 2019)
Bioinformatics	Computational processing of sequence data, in which **next generation sequencing** data are quality filtered, summarised and compared against reference databases for taxonomic assignment (Bruce, et al., 2021).
Biotic interactions	Interactions between species such as mutualism, commensalism, facilitation, symbiosis, partnership, competition, predation, and parasitism (Silknetter et al., 2020).
Bioturbation	All transport processes carried out by animals that directly or indirectly affect sediments. These processes include both particle reworking and burrow ventilation (Kristensen et al., 2012).
Catchment; Watershed; Drainage basin	A land area that channels rainfall and snowmelt to creeks, streams, and rivers, and eventually to outflow points such as reservoirs, bays, and the ocean (National Oceanic and Atmospheric Administration, 2024)
Chaotic	When future states are only predictable on the short but not on the long term, are very sensitive to initial conditions and exhibit bounded **deterministic** aperiodic fluctuations (Munch et al., 2022).
Coinertia	For two datasets that have been measured on the same sites (for example environmental variables and species abundances), coinertia is a global measure of the co-structure of sites in the two datasets: it is high when the structures of the two datasets vary simultaneously (and also when they vary inversely), and low when they vary independently, or when they do not vary (Based on Dray et al., 2003).
Community assembly	The process by which the species composition of a community is determined (Mittelbach & Schemske, 2015).
Community; Local community	The individuals of all species that potentially interact within a single **patch** (Leibold et al., 2004).
Connectedness; Landscape structural connectivity	Measure of how spatially connected the elements in a **landscape** are, without reference to any particular ecological process (Wu, 2013).

Connectivity; Landscape connectivity; Landscape functional connectivity	The degree to which a **landscape** facilitates or impedes the exchange of organisms, energy, material, and information among elements (Wu, 2013).
Created wetland	A human-made **wetland** that is engineered to optimize a specific function such as water quality improvement, often with impervious liner and planted (Vymazal, 2005).
Degradation; Ecosystem degradation	A level of deleterious human impact to ecosystems that results in the loss of **biodiversity** and simplification or disruption in their composition, structure, and functioning (Gann et al., 2019).
Deterministic	When future states are exactly predictable given exact knowledge of the starting state (Munch et al., 2022).
Dispersal	Permanent movement away from an origin and long-term settlement at a new location (Lowe & McPeek, 2014).
Dispersal limitation	A condition when species cannot reach all suitable sites in a region because of large spatial distances or physical obstacles (Heino et al., 2021).
Disturbance	A relative discrete event in time that disrupts ecosystem, community, or population structure and changes resources, substrate availability or the physical environment (Pickett et al., 1986).
Ecological restoration	The process of assisting the recovery of an ecosystem that has been **degraded**, damaged, or destroyed (Gann et al., 2019).
Ecological theory	Explanation of an ecological phenomenon (pattern or process). Can take form of conceptual description or mathematical model (Grainger et al., 2022; Palmer, 2009).
Ecological trap	An outcome where organisms select habitats based on environmental cues, but the cues provide an inaccurate depiction of the suitability of the habitats for reproduction and survival (Brand & Snodgrass, 2010).
Ecosystem functions	Biological, physical, and geochemical processes that take place within ecosystems including productivity, decomposition, nutrient recycling, and energy flow-related functions (Cuenca-Cambronero et al., 2023).
Ecosystem services	The direct and indirect contributions of ecosystems to human wellbeing. These can be divided into provisioning, regulating, cultural and supporting services (Cuenca-Cambronero et al., 2023).
Ecosystem structure, ecosystem form	The biological, physical and geochemical parts of the ecosystem at a point in time, such as biomass and species composition (Grayson et al., 1999).
Effect trait	A **trait** that affects ecosystem processes (Engelhardt, 2006)
Environmental DNA	A complex mixture of genomic DNA from many different organisms found in an environmental sample (Taberlet et al., 2018)
Environmental heterogeneity	The diversity of abiotic conditions and biotic interactions among habitat **patches** (Based on Leibold et al. (2004) and Logue et al. (2011).
Evaluation	An assessment of an activity, or set of activities, in relation to the previously stated goals and objectives (adapted from Conservation Measures Partnership, 2013).
Flagship species	A species that has become a symbol and leading element of an entire conservation campaign (Barua, 2011).
Functional diversity	The extent of **functional trait** variation among the species in a community (Petchey & Gaston, 2002).
Functional trait	Any **trait** which impacts fitness indirectly via its effects on growth, reproduction and survival (Violle et al., 2007).
Goal	Formal statement of the medium and long-term desired ecological or social condition (Gann et al., 2019).

Habitat	*The term habitat has two meanings:* *1) Organism specific*: An area containing the particular combination of resources and environmental conditions that are required by individuals of a given species or group of species to carry out life processes. *2) Environment based*: A terrestrial or aquatic area distinguished by geographic, abiotic and biotic features (Miller & Hobbs, 2007).
Habitat type	Habitat type listed in Annex I of the EU Habitats Directive (European Union, 1992) This is the environment based meaning of the word **habitat** (Miller & Hobbs, 2007).
Habitat complexity	Aspects of a **habitat** that include at least the scale, diversity, spatial arrangement, size and abundance of **habitat structural** elements (Tokeshi & Arakaki, 2012).
Habitat heterogeneity	The diversity of **habitat structural** elements (Tews et al., 2004).
Habitat structure	The geometry of the physical **habitat**; this includes the bare substrate itself (e.g. rock, soil, soft sediments) and the structure provided by the species that characterise that habitat (e.g. macrophytes, trees, oysters, corals) (Loke & Chisholm, 2022).
Hydroseral succession	**Succession** that starts from freshwater.
Indicator; Ecological indicator	A component or a measure of environmentally relevant phenomena used to depict or evaluate environmental conditions or changes or to set environmental goals (Prach et al., 2019).
Invasive species; Invasive alien species	**Alien species** that sustain self-replacing populations over several life cycles (Richardson, 2011).
Landscape	A geographic area in which variables of interest are spatially heterogeneous. The boundary of a landscape may be delineated based on geographic, ecological, or administrative units which are relevant to the research questions and objectives (Wu, 2013).
Library; Sequencing library	A mixture of labelled **amplicons** from different PCR reactions that has been prepared for **sequencing** (Taberlet et al., 2018).
Local	The scale at which individuals move and interact with each other in the course of their routine feeding and breeding activities (Hanski & Gilpin, 1991).
Macrophyte	Large aquatic plants that are not planktonic or filamentous algae. Macrophytes include aquatic angiosperms, bryophytes, pteridophytes, some species of encrusting lichens and charophytes (Bornette & Puijalon, 2011).
Mass-effect perspective	A perspective that focuses on the effect of immigration and emigration. **Habitat patches** are **environmentally heterogeneous,** but **dispersal** is high and source–sink dynamics enable species to exist at sites normally considered marginal or outside of their environmental range (Leibold et al., 2004; Logue et al., 2011; Winegardner et al., 2012)
Matrix	All non-habitat areas of the **landscape** (Vasudev et al., 2015).
Mesohabitat	A visually distinct and easily identifiable **habitat** within the freshwater body (Della Bella et al., 2005).
Metabarcoding	Taxonomic identification of multiple species simultaneously from a complex (multi-species) sample, using **next generation sequencing** of a standardized DNA fragment (Bruce et al., 2021).
Metacommunity	A set of **local communities** connected by the **dispersal** of multiple interacting species (Leibold et al., 2004).
Mitigation, compensatory mitigation	The **restoration**, **rehabilitation**, enhancement or creation of a habitat to compensate for damages in another habitat (Zedler, 2000).
Monitoring	The collection of data that can be used for the **evaluation** of an activity or set of activities (adapted from Conservation Measures Partnership, 2013).

Neutral perspective	A perspective in which all species are equivalent in their competitive ability, movement and fitness. **Local** dynamics are determined by **stochastic** emigration, immigration, extinction and speciation (Based on Leibold et al., 2004; Winegardner et al., 2012).
Next generation sequencing; High throughput sequencing; Second generation sequencing	DNA sequencing technology that produces millions of DNA sequence reads in parallel. Enables thousands of different organisms from a mixture of species to be sequenced at once. Illumina technology is most common (Bruce et al., 2021).
Objective	Formal statement of the desired outcome of an activity or set of activities (adapted from Conservation Measures Partnership, 2013).
Operational taxonomic unit; Molecular operational taxonomic unit	Proxy for species obtained using clustering algorithms to bioinformatically process **sequencing** data obtained from **metabarcoding**. **Reads** are clustered using a sequence similarity threshold (Bruce et al., 2021).
Patch; Habitat patch; Locality	A discrete area of habitat that can hold a local community (Leibold et al., 2004).
Patch dynamics perspective	A perspective that assumes that **patches** are environmentally homogeneous. Species differ in their competitive and dispersal abilities and local dynamics are influenced by competition and colonization (Based on Leibold et al., 2004; Logue et al., 2011).
Pioneer	Species with **traits** that allow them **to** grow after **disturbance**
Polymerase chain reaction	A method that uses cyclical variations in temperature in the presence of a polymerase enzyme to rapidly create millions of copies of a predefined DNA fragment (Bruce et al., 2021).
Pond	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 (Richardson et al., 2022).
Pond creation	Creating a new **pond** on a site where there was formerly no water body (Cuenca-Cambronero et al., 2023).
Pond restoration	Restoring an existing **pond** or resurrecting a **pond** formerly present, but currently in-filled (Cuenca-Cambronero et al., 2023).
Pondscape	Network of **ponds** spatially distributed in a terrestrial **matrix** and their **connectedness** (Boothby, 1997).
Primer pair	Two short, single-stranded nucleic acid molecules consisting of a sequence of DNA bases that are designed to match the target DNA at either end of the **(meta)barcode** region to be amplified (Bruce et al., 2021).
Read; Sequence read	An inferred sequence of base pairs corresponding to a DNA fragment (Wikipedia, 2025).
Reference database	A collection of DNA sequences derived from specimens of known identity (Bruce et al., 2021).
Regional	The scale at which individuals or propagules infrequently **disperse** from one local **habitat patch** to another, typically across areas that are not suitable for their feeding and breeding activities, and often with substantial risk of failing to locate another suitable habitat patch in which to settle. (Adapted from Hanski & Gilpin, 1991).
Rehabilitation	Management actions that aim to reinstate a level of **ecosystem functioning** on **degraded** sites, where the goal is renewed and ongoing provision of **ecosystem services** rather than the **biodiversity** and integrity of a designated native reference ecosystem (Gann et al., 2019).
Remediation	A management activity, such as the removal or detoxification of contaminates or excess nutrients from soil and water, that aims to remove sources of **degradation** (Gann et al., 2019)**.**
Response trait	A **trait** that predicts how species respond to environmental factors (Engelhardt, 2006).

Restoration ecology	The branch of ecological science that provides concepts, models, methodologies and tools for the practice of **ecological restoration**. It also benefits from direct observation of and participation in restoration practice (Gann et al., 2019).
Restorative activities	Activities (including **ecological restoration**) that reduce **degradation** or improve conditions for the partial or full recovery of ecosystems (Gann et al., 2019).
Sequencing	The process of determining the nucleotide sequence of a given DNA fragment (Bruce et al., 2021).
Sequencing coverage; Sequencing depth	The total number of reads per sample or **library** in **next generation sequencing** (McMurdie & Holmes, 2014).
Species pool; Regional species pool	The set of species at a larger geographic scale that includes the **community** of interest; often believed to represent the pool of potential colonists that could reach a specific **habitat** (Cadotte & Tucker, 2017)**.**
Species-sorting perspective	A perspective that emphasizes the **environmental heterogeneity** of **habitat patches**. **Dispersal** is important because it allows compositional changes to track changes in **local** environmental conditions (Based on Leibold et al., 2004; Logue et al., 2011).
Stochastic	When future states, given past states, are not exactly predictable, even with exact knowledge of the starting state (noisy or random) (Munch et al., 2022).
Succession	Temporal change in species composition or ecosystem state following **disturbance** (Pickett et al., 1987).
Succession pathway	Temporal pathway in community type or ecosystem state (Adapted from Pickett et al., 1987).
Tag	A small oligonucleotide label that is attached to an **amplicon**. This label allows association of the resulting **sequence read** to the polymerase chain reaction (and therefore the sample or control) the amplicon came from.
Tagjump; Tagswitch	The generation of an artefactual sequences in which an amplicon carries different **tags** than originally applied (Schnell et al., 2015).
Target; Conservation target	An element of biodiversity at a project site, which can be a species, habitat, or ecological system that a project has chosen to focus on (Conservation Measures Partnership, 2013).
Taxonomic distinctness	The taxonomic relatedness of species in a community (Clarke & Warwick, 1998).
Trait	Any morphological, physiological or phenological feature measurable at the individual level, from the cell to the whole-organism level, without reference to the environment or any other level of organization (Violle et al., 2007).
Umbrella species	A species with such demanding **habitat** requirements and large area requirements that saving it will automatically save many other species (Barua, 2011; Simberloff, 1998).
Wetlands	Areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six metres (Ramsar Convention, 1971).

Chapter 1

Utricularia flowering in Neuburg

Chapter 1: General introduction

*Terms defined in the Glossary are presented in **bold** at their first occurrence in the text.*

1.1. Freshwater biodiversity in need of science-based restoration

Freshwater **biodiversity** is in severe decline (Tickner et al., 2020). While lakes, rivers, reservoirs, floodplains, mires and ephemeral freshwaters only cover 5.1% of the Earth's surface (Lehner & Döll, 2004), they host a very rich biodiversity, including, for example, 9.5% of described animal species (Balian et al., 2008; Reid et al., 2019). However, this diversity is decreasing with an alarming pace. Of all freshwater vertebrates included in the International Union for Conservation of Nature (IUCN) Red List, 32% is threatened, which is higher than for marine and terrestrial vertebrates (Collen et al., 2014). Population declines are also more severe for freshwater vertebrates than for terrestrial and marine vertebrates (WWF, 2024).

Major threats to freshwater biodiversity include **habitat** destruction and degradation (*Fig. 1.1*, Dudgeon et al., 2006). Between 1940 and 2014, the loss of **wetlands** was three times higher than that of forests (Ramsar Convention on Wetlands, 2018). Wetlands were for example lost because of drainage for agriculture or diking of rivers. In Europe, most wetlands were destroyed from the medieval period to the middle of the last century. During the Soviet era, wetland drainage for agricultural practice was enforced. All large European rivers have been diked, which cut off the rivers from their floodplains. As a result many floodplains were destroyed or heavily modified (Junk et al., 2013).

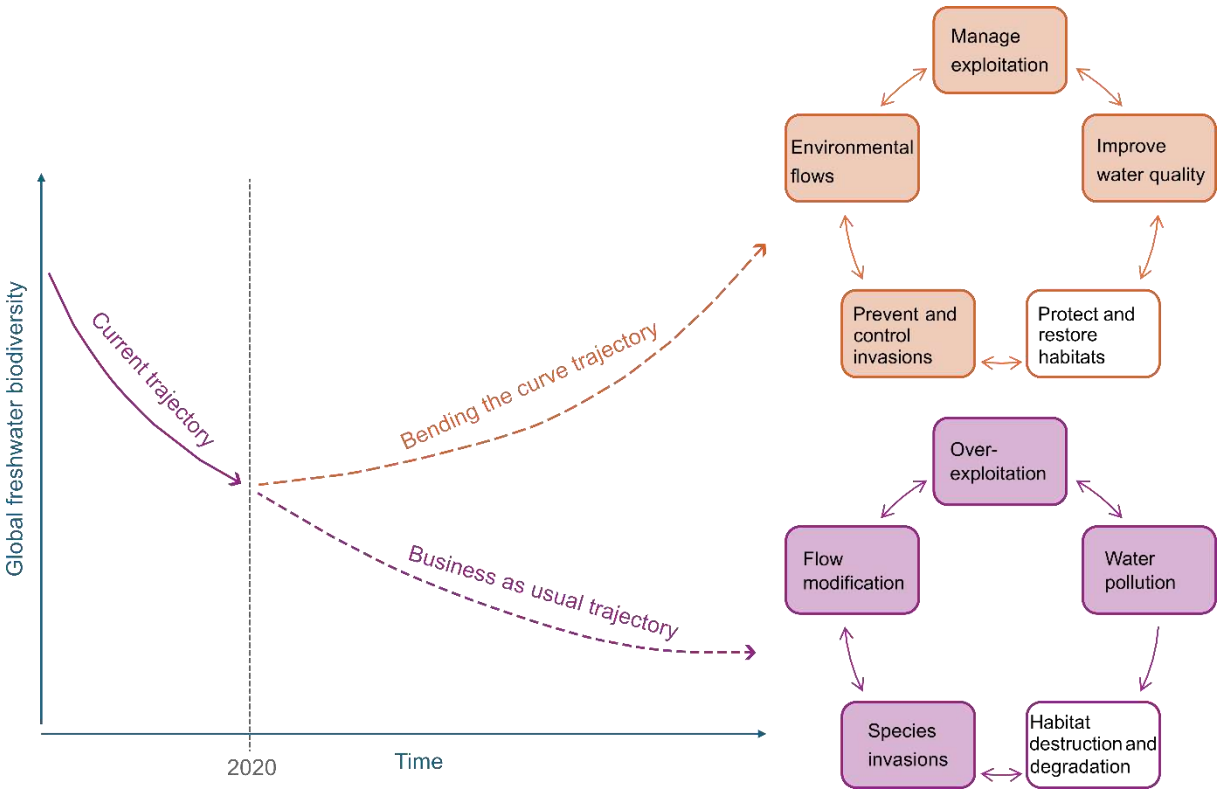


Fig. 1.1. Major threats to freshwater biodiversity (purple boxes) and proposed responses (orange boxes). Threats and responses of importance for this PhD in white boxes. Figure adapted from Dudgeon et al., 2006 and Tickner et al., 2020

Given that habitat destruction and degradation are major causes of freshwater biodiversity decline, the protection and restoration of freshwater habitats should be included in mitigation efforts (*Fig. 1.1*, Tickner et al., 2020). The need to protect and restore is recognized in recent global initiatives (Piczak et al., 2024). We are, for example, currently in the United Nations (UN) Decade on Ecosystem Restoration (2021-2030) (United Nations, 2019). Furthermore, the Kunming-Montreal Global Biodiversity Framework includes a target for at least 30% of degraded ecosystems to be under effective restoration by 2030 (*Table 1.1,* Convention on Biological Diversity, 2022; Piczak et al., 2024). The European Union (EU) Nature Restoration Regulation (European Union, 2024) even sets legally binding restoration targets and has the ambitious goal to implement restoration measures in 90% of **habitat types** that need them by 2050.

Table 1.1. Relevant targets and goals for 2030 and 2050 of the Kunming-Montreal Global Biodiversity Framework and the EU Nature Restoration Regulation. Habitat type refers to the habitat types listed in Annex I of the EU Habitats Directive (European Union, 1992).

	Kunming-Montreal Global Biodiversity Framework	**EU Nature Restoration Regulation**
Adopted by	Conference of the Parties to the Convention on Biological Diversity	European Parliament and Council of the European Union
Identifier	CBD/COP/DEC/15/4	Regulation (EU) 2024/1991
Date adopted	19 December 2022	24 June 2024
Legally binding	No	Yes
Target or goal for 2030	Have at least 30% of degraded ecosystems under effective restoration (Target 2)	Put in place restoration measures on at least 30% of the total area of all habitat types that is not in good condition (Article 4.1.a)
Target or goal for 2050	The integrity, connectivity and resilience of all ecosystems are maintained, enhanced, or restored (Goal A)	Put in place restoration measures on at least 90% of the area of each group of habitat types that is not in good condition (Article 4.1.b)

The number of freshwater restoration projects has been increasing (Piczak et al., 2024), and the implementation of the EU Nature Restoration Regulation will amplify this increase. To seize the growing momentum for restoration, it is critically important that **restorative activities** are based on ecological principles (Prach et al., 2025). Current wetland restoration projects mostly do not achieve the desired degree of recovery (Moreno-Mateos et al., 2012, 2020; Palmer, 2009). This non-achievement can be due to insufficient application of ecological principles, and to gaps in the scientific knowledge needed for effective restoration (Moreno-Mateos et al., 2012; Palmer, 2009). The science of **restoration ecology** still has a key role to play in translating ecological principles to practical restoration strategies, in testing the effectiveness of restoration methods, and in responding to knowledge needs of practitioners (*Fig. 1.2*, Palmer, 2009; Prach et al., 2025).

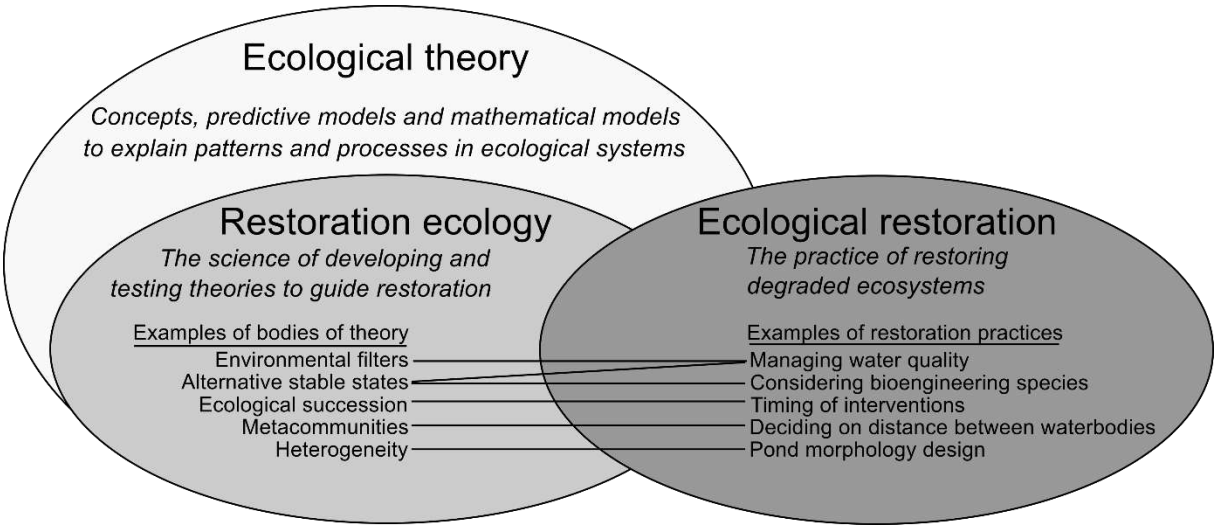


Fig. 1.2. The role of restoration ecology in translating ecological theory to practical applications and responding to needs of restoration practitioners. Figure adapted from Palmer 2009, with different examples.

1.2. Pond creation and restoration as restorative activities

1.2.1. The restorative continuum

The question of what exactly constitutes ecological restoration has been hotly debated, and the most accepted definition was provided by the Society for Ecological Restoration (SER) in 2004 (Martin, 2017). This definition is:

> "Ecological restoration is the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed" (Gann et al., 2019; SER, 2004).

In some cases, however, ecosystem recovery is not possible, not relevant in face of climate change, or not desirable (Gann et al., 2019; Geist & Hawkins, 2016). For example, full recovery of a floodplain ecosystem that was cut off from its river by a dike would be highly undesirable if this would lead to periodical flooding of the houses nearby. In those cases where recovery is not possible, relevant or desirable, it may still be possible to perform restorative activities that improve biodiversity or bring back some **ecosystem services** (Gann et al., 2019; Geist & Hawkins, 2016). The Society for Ecological Restoration considers these restorative activities as part of a continuum that can contribute to ecosystem recovery (*Fig. 1.3*, Gann et al., 2019).

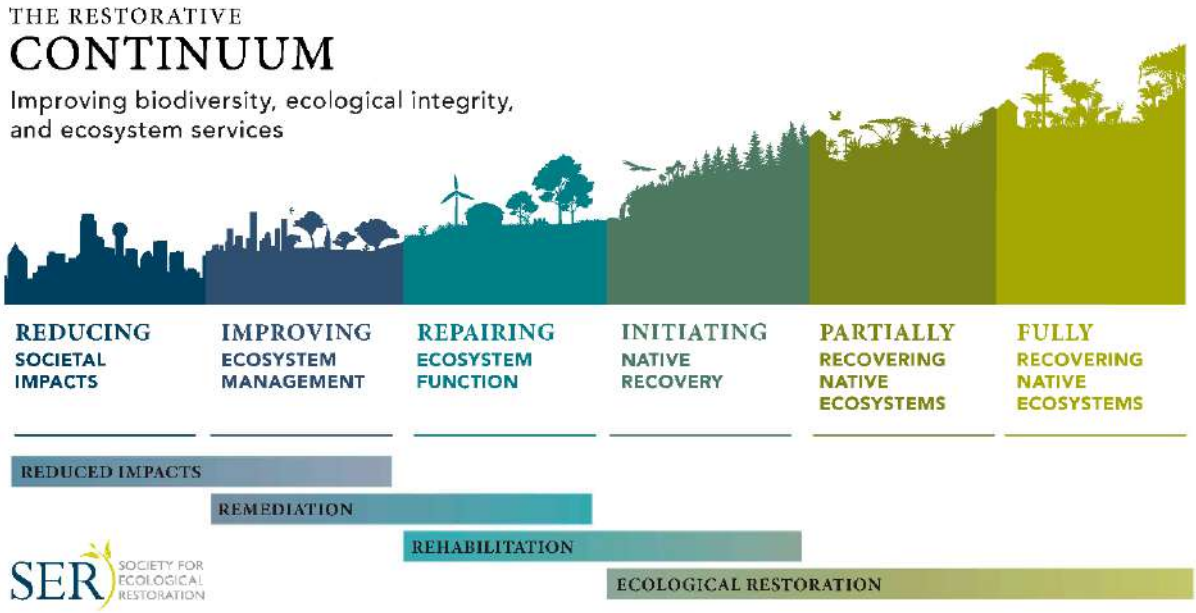


Fig. 1.3. The restorative continuum. Figure from the Society of Ecological Restoration (Gann et al., 2019)

Definitions are necessary for effective communication, and in this PhD thesis it is aimed to apply the term "ecological restoration" according to the definition by the Society of Ecological restoration, and to use the term "restorative activity" for the wider range of activities that span the restorative continuum (see Glossary). In the literature, however, the word "restoration" is often applied to the entire range of actions on the restorative continuum, as well as to practices that could be called "mitigation" or "creation" (Kentula, 2000). In the Kunming-Montreal Global Biodiversity Framework, "restoration" is not clearly defined and could encompass all the activities on the restorative continuum (Convention on Biological Diversity, 2022; Hughes & Grumbine, 2023). The restoration measures that are required by the EU Nature Restoration Regulation may also be interpreted in the large sense. Furthermore, the designation of the object of the restoration is subtle. When a project is not exactly assisting the recovery of an ecosystem, one could still argue that it restores a habitat or a population of a specific species, and when an intervention entails the creation and not really the restoration of a waterbody, it may still be described as restoring the aquatic **connectivity** or the presence of standing water in a **landscape.**

1.2.2. Definition and biodiversity value of ponds

Recently there has been a surge in research on ponds, as exemplified by a rapid rise in the number of pond publications between 2000 and 2019 (Hill et al., 2021). It has become evident that ponds can host high numbers of taxa, including rare and endangered species, and make an important contribution to regional freshwater biodiversity (Davies et al., 2008; Hill et al., 2021; Scheffer et al., 2006; Williams et al., 2004). Ponds can be natural, created or restored, and may be permanent or temporary (Biggs & Williams, 2024). These pond types may differ in their species richness and community composition, but all types can be valuable for the conservation of aquatic invertebrates, amphibians and **macrophytes** (Coccia et al., 2016; Della Bella et al., 2008; Hill et al., 2025; Minot et al., 2021; Rannap et al., 2009; Ruhí et al., 2009; Scheffer et al., 2006; Williams et al., 2008).

Ponds also contribute to the functioning of surrounding terrestrial ecosystems, for example, through the supply of emerging aquatic insects (Fehlinger et al., 2023). Besides, ponds are important because of the ecosystem services they provide to people. They can provide water for livestock, irrigation and fire protection and can be used for the production of fish and crayfish.

Ponds can reduce the input of nutrients, sediment and other pollutants to surface waters and contribute to flood alleviation. They are also used for recreation, education, religious expression and celebration, and research (Hill et al., 2021; Oertli et al., 2005a).

Ponds differ from lakes in their ecological structure and functioning (Richardson et al., 2022; Søndergaard et al., 2005). Based on these differences, functional boundaries distinguishing ponds from lakes have been established: ponds have a surface area smaller than 5 ha and a depth of less than 5 m (Glossary, *Fig. 1.4*, Richardson et al., 2022). Due to their shallow depth, the entire surface area of a pond can be covered by macrophytes, provided the water is clear (Biggs & Williams, 2024; Oertli et al., 2005a; Søndergaard et al., 2005). Furthermore, the small size of ponds results in a high perimeter-to-area ratio, giving them a relatively large littoral zone and making them strongly influenced by terrestrial inputs (Palik et al., 2001; Søndergaard et al., 2005). Because of their low water volume, sediment chemistry has a greater influence on water quality in ponds than in lakes (Søndergaard et al., 2005). This influence is further enhanced by the high diel temperature fluctuations in ponds, which can promote nutrient release from sediments (Richardson et al., 2022). Ponds often lack inflows and outflows, and this isolation allows their communities to differ from those in nearby waterbodies, including other ponds (De Meester et al., 2005; Scheffer et al., 2006; Søndergaard et al., 2005). Fish are commonly absent from ponds, which can give rise to invertebrate top predators and increased amphibian richness (Scheffer et al., 2006; Søndergaard et al., 2005). Lastly, ponds are often sheltered from wind, which results in low gas exchange with the air above the water, and permits free-floating macrophytes to form mats under suitable conditions (Richardson et al., 2022; Scheffer et al., 2003; Søndergaard et al., 2005).

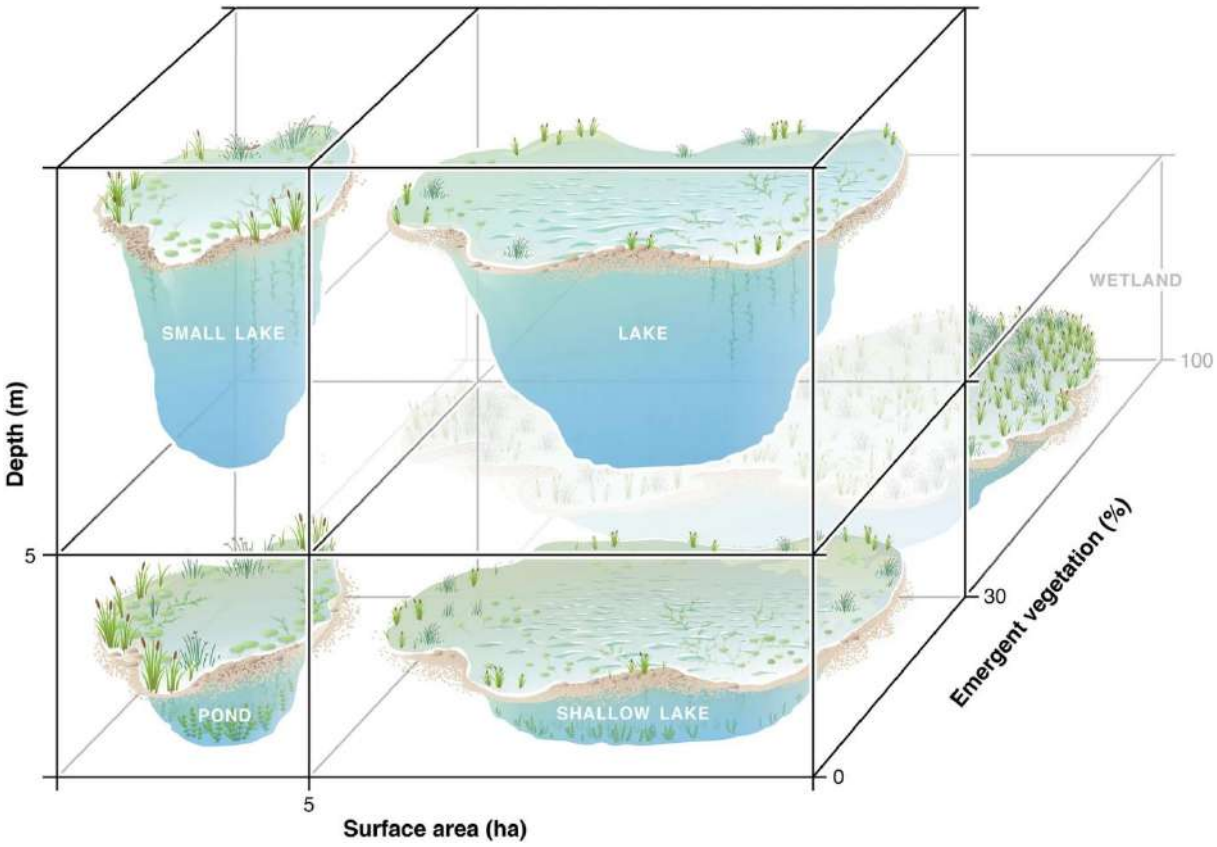


Fig. 1.4. Distinction between pond and lake. Figure copied from Richardson et al., 2022. Note that in this figure the North American definition of wetland is used, which is narrower than the Ramsar definition (Ramsar Convention on Wetlands, 2018, see Glossary).

1.2.3. The increasing relevance of pond creation and restoration

Natural processes that create ponds, such as erosion and floodplain dynamics, have been limited by changes in land use, and cultural practices such as farm and fishpond creation have largely been discontinued (Fehlinger et al., 2023; Hill et al., 2025; Jeffries, 2005; Oertli, 2018). At the same time, ponds have disappeared from the landscape in consequence of drainage, infilling and abandonment related to agricultural intensification and urbanization (Fehlinger et al., 2023; Hill et al., 2025). In the United Kingdom, where much of the pond research is concentrated, pond numbers have been declining since the nineteenth century, but seem to have stabilized since the 1990's (Biggs & Williams, 2024). Remaining ponds are often degraded because of nutrient and pesticide pollution, species invasions, and the cessation of traditional grazing and management practices like shrub and reed removal (Biggs & Williams, 2024; Hill et al., 2025; Ulrich et al., 2022). Climate change is a more recent threat that could change the hydrology of ponds (De Necker et al., 2025; Fehlinger et al., 2023; Hill et al., 2025).

Given their important contributions to regional freshwater biodiversity and their ecosystem services, the protection of good quality ponds should be a conservation priority, and degraded ponds should be restored (Céréghino et al., 2008; Fehlinger et al., 2023; Hill et al., 2021, 2025; Oertli, 2018). In addition, the creation of new ponds is a promising strategy that could enhance freshwater biodiversity at the landscape scale (Hill et al., 2025; Oertli, 2018; Williams et al., 2008). According to the Society for Ecological Restoration's definition's (Glossary, Gann et al., 2019), the restoration of a degraded or formerly existing pond is a form of ecological restoration, whereas the creation of a new pond for biodiversity conservation should be seen as **rehabilitation**. Both practices are part of the restorative continuum (*Fig. 1.3*), and could be interpreted as restoration under the Kunming-Montreal Global Biodiversity Framework and EU Nature Restoration Regulation (Convention on Biological Diversity, 2022; European Union, 2024).

Pond restoration and creation projects are widely implemented in Europe, and most projects combine the two approaches (De Necker et al., 2025; Hill et al., 2025). At present, the largest funder of pond projects in Europe is the EU LIFE programme, and most projects are carried out in Natura 2000 areas (De Necker et al., 2025). De Necker et al. (2025) sent out a questionnaire regarding the goals, methods and **monitoring** of pond restoration and creation projects started between 2013 and 2023 in Europe to the project managers. Most projects had a primary goal of specific amphibian species conservation. The restorative activity most performed was the digging of new ponds, followed by the dredging of existing ponds (*Table 1.2*, De Necker et al., 2025). In most cases, no actions such as application of clay or plastic foil or pond liner were performed to make new ponds less permeable to water, and no plants or seeds were introduced. Descriptions of implemented monitoring were highly unclear and focussed on amphibians (De Necker et al., 2025).

Table 1.2. Pond restorative activities undertaken in Europe by respondents to the questionnaire of De Necker et al. (2025) and the percentage of projects in which the activities were undertaken

Restorative activity and percentage of projects in which it was undertaken		Type
Digging new ponds	76%	Pond creation
Dredging ponds (removing sediment)	62%	Pond restoration
Removal of trees/bushes surrounding ponds	52%	Pond restoration
Excavating ponds that existed in the past	52%	Pond restoration
Establishing buffer zones surrounding the ponds	43%	Pond restoration
Removal of invasive species	38%	Pond restoration
Removal of littoral vegetation (reeds)	38%	Pond restoration
Land restoration in the surrounding area	33%	Pond restoration
Modification of the bank profile	33%	Pond restoration
Fencing off ponds (e.g. to avoid livestock access)	29%	Pond restoration
Enlarging existing ponds	29%	Pond restoration
Removing anthropogenic waste	24%	Pond restoration
Changes in land-use	19%	Pond restoration
Re-introduction of animals	19%	Pond restoration
Re-introduction of seeds or plants	19%	Pond restoration
Increasing water level (raising groundwater, diverting river flow)	19%	Pond restoration
Other	14%	Pond restoration
Removal of fish	5%	Pond restoration

1.2.4. The need for more research on pond creation for freshwater biodiversity

As far as I am aware, there are only two studies, from France by Minot et al. (2021) and from the UK by Hill et al. (2025), that compare pond restoration with pond creation in Europe. Both studies found that pond creation and restoration can be equally beneficial for freshwater diversity. No difference was detected in the species richness of larval dragonflies and damselflies between restored and created ponds in the first three years after the restoration or creation (Minot et al., 2021). Macrophyte colonization was faster in the restored than in the created ponds studied by Hill et all. (2025). Between one to six years after the restoration or creation, macrophyte richness was higher in restored ponds than in created ponds, but eleven or more years after the intervention this difference had disappeared (Hill et al., 2025). Regarding Minot et al. (2021), it may be that the absence of difference between restored and created ponds was due to the fact that damselflies and especially dragonflies are efficient dispersers, or was due to the pond restoration method, which entailed digging out the pond entirely after complete and prolonged drying (Minot et al., 2021). The methods applied to the restored ponds studied by Hill et al. (2025), namely major woody vegetation and sediment removal, were probably less intrusive and did allow some seedbanks to remain, which could explain the faster increase in macrophyte richness in the restored ponds (Hill et al., 2025).

The studies by Minot et al. (2021) and Hill et al. (2025), as well as other studies (Kadoya et al., 2004; Rannap et al., 2009; Williams et al., 2008) show very promising results of pond created for biodiversity and species conservation. However, these are case studies, and knowledge obtained from case studies is often only locally applicable (Oertli, 2018). Ponds that were created for reasons other than biodiversity and species conservation, such as fish, stormwater and garden ponds, show more ambiguous results (Fehlinger et al., 2023). There are even studies that found that ponds with inadequate designs can act as **ecological traps**, when they attract for instance amphibians for breeding, but do not provide the required conditions for the larvae to metamorphose (Brand & Snodgrass, 2010). Such ecological traps can, for example, occur when

the ponds are polluted or attract predators of the species or groups they were meant to conserve (Calhoun et al., 2014; Clevenot et al., 2018; Fehlinger et al., 2023).

With the implementation of the EU Nature Restoration Regulation, it can be expected that the number of pond creation projects will increase in Europe. To seize this opportunity and ensure that these projects will be beneficial for freshwater biodiversity, it is crucial that the projects employ evidence-based methods. However, more knowledge is needed on how to most effectively perform pond creation (Hill et al., 2021). Important knowledge gaps remain, for example regarding the role of landscape connectivity on pond communities, and regarding the monitoring of ponds, which is very challenging because of their abundance and heterogeneity (Hill et al., 2021).

1.3. Ecological concepts relevant to pond creation for biodiversity

To provide a background for studies on pond creation for biodiversity and species conservation, and for integrating these results into knowledge, **ecological theory** is needed. Ecological theories, the explanations of ecological phenomena, can take form of either mathematical models or conceptual descriptions (Grainger et al., 2022; Palmer, 2009). Unfortunately, restoration ecology still needs to better understand mechanisms leading to restoration outcomes and formalize them into mathematical models (Brudvig, 2017). Mathematical models are not used in this thesis. However, general concepts from community ecology can already be very useful for restoration practice (Palmer, 2009).

Several bodies of theory are relevant for pond creation. The benefit of pond creation is often framed using the concept of **ecological succession**; as adding early-successional habitats with their distinctive biota to the landscape. Although succession still requires a modern formulation for ponds, is therefore very relevant. Furthermore, changes in pond communities and ecosystem properties over time are often described using the concept of ecological succession (Hassall et al., 2012; Hill et al., 2021; Ruhí et al., 2012). Like succession, the **environmental filtering** concept can be used to explain patterns in community composition, and is seen as pertinent to restoration practice (Hulvey & Aigner, 2014).

On the ecosystem level, the **shallow lakes theory of alternative stable states** explains how ponds can shift between states characterized by dominance of different groups of primary producers, for example submerged macrophytes and phytoplankton (Scheffer et al., 1993; Scheffer & Van Nes, 2007). Macrophyte cover is a main determinant for macroinvertebrate richness in ponds (Hassall et al., 2011). It has been hypothesized that it is especially the **habitat complexity** provided by macrophytes that allows for high macroinvertebrate richness. However, the mechanisms by which habitat complexity result in diversity still have to be elucidated (Meerhoff & De Los Ángeles González-Sagrario, 2021; St. Pierre & Kovalenko, 2014).

Ponds cannot be understood by only studying them individually on a local scale, and the concept of **metacommunity** can be used to include regional processes shaping pond communities as well (Fehlinger et al., 2023; Hassall et al., 2012). Furthermore, the still debated importance of **stochastic** and **deterministic** processes in shaping pond communities (Villsen et al., 2025) and the causes and impacts of species **invasions** (Daly et al., 2023) also have direct consequences for the interpretation of pond creation outcomes. I will briefly explain the abovementioned theories as interpreted in this thesis, and describe their relevance for pond creation.

1.3.1. Ecological succession

Succession takes place when a new site is available, species are differentially available to the site and perform differentially at the site (Pickett et al., 1987). When a new pond is available, aquatic macrophytes arrive quickly, and usually several species establish already within a year

(Barnes, 1983; Fleury & Strehler Perrin, 2004). Among these early colonizers there are often **pioneers**, species with traits that allow them to grow after **disturbance**, for example stonewort (charophyte) species (Bornette & Arens, 2002; Wade, 1990). When more resources become available, other species can establish, and more competitive species may become dominant (Bornette & Puijalon, 2011; Fleury & Strehler Perrin, 2004). Although succession is mainly a plant related concept, it has also been used to describe changes in macroinvertebrate communities over time. Some macroinvertebrates colonize new ponds quickly, with many species arriving within a year as well (Barnes, 1983; Minot et al., 2021; Ruhí et al., 2009). Species that cannot fly (e.g. snails), or require specific macrophyte life forms are believed to need more time to establish (Barnes, 1983; Fairchild et al., 2000; Minot et al., 2021; Ruhí et al., 2009).

Succession is a key concept in pond creation. Pond creation is often perceived as a way to add early succession ponds to a landscape that lacks disturbance. Natural disturbances, such as beaver damming, flooding and large herbivore grazing, may create ponds and delay succession. Ponds in landscapes with natural disturbances are therefore thought to occur at different successional stages. Since there is turnover in pond communities during succession, these ponds at different stages should exhibit high beta and gamma diversity (Hassall et al., 2012; Hill et al., 2021). In landscapes that lack disturbance, human made pond creation is therefore seen as a method to add early succession ponds to the landscape, and to thereby increase biodiversity (Hassall et al., 2012; Sayer et al., 2012).

Ecological succession therefore provides therefore a key rationale for how pond creation can increase biodiversity. However, there is no comprehensive modern theory on succession in ponds (Hill et al., 2021; Poorter et al., 2023). There is the classic model of **hydroseral succession**, which suggests that over time a permanent pond fills in with organic sediments, becomes temporary, and eventually ends up as a forest. However, this climax model is inadequate because alternative succession pathways exist, for example bogs may form or temporary ponds may remain stable features for millennia (Collinson et al., 1995; Klinger, 1996; Williams, 1997). Succession theories based on terrestrial plants include many simultaneously or consecutively operating mechanisms that can result in a wide array of **succession pathways** (Pickett et al., 1987; Poorter et al., 2023). Frameworks for different succession pathways that may occur after pond creation and the influence of succession on pond diversity still need to be developed (Hill et al., 2021).

1.3.2. The environmental filtering metaphor

Like succession, the environmental filtering metaphor (Keddy, 1992) can be used to explain **community assembly** in newly created ponds. Compared to succession, environmental filtering focusses less on changes over time and more on the final community (Young et al., 2025). The metaphor describes a set of filters that select out of the regional **species pool** a subset of species that establish in a site and form a community (Cadotte & Tucker, 2017; Keddy, 1992). The filters often included represent **dispersal**, selection by the abiotic environment and **biotic interactions** (*Fig. 1.5*, Belyea, 2004; Cadotte & Tucker, 2017; Lortie et al., 2004).

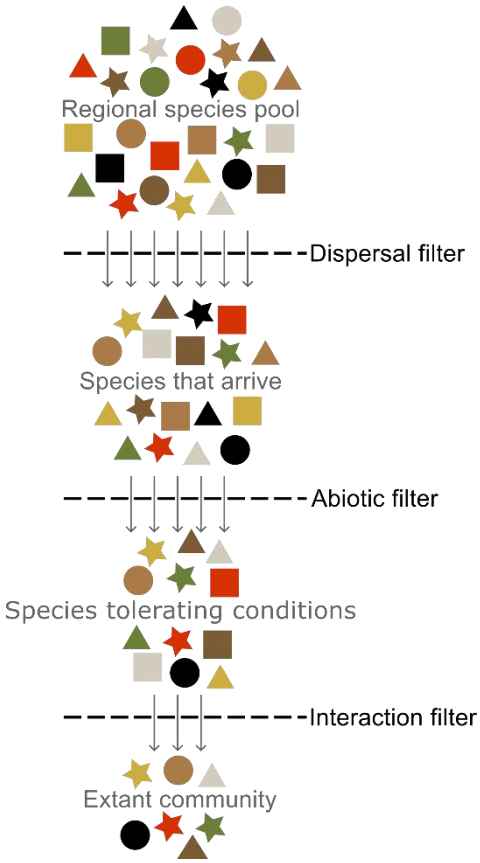


Fig. 1.5. The environmental filtering metaphor with at the top the regional species pool and at the bottom the assembled community. Figure based on Cadotte & Tucker (2017).

The use of the environmental filtering concept has been criticized because it is not possible to assign observed patterns of community composition to the individual filters. Furthermore, the metaphor simplifies processes that in reality interact in complex ways (Cadotte & Tucker, 2017). Although the metaphor is flawed, dispersal, abiotic factors and biotic interactions are principal determinants for community assembly, and the metaphor is useful to conceptualise restoration alternatives (Cadotte & Tucker, 2017; Funk et al., 2023; Hulvey & Aigner, 2014). The metaphor also exists in more nuanced forms that are more explicit about biotic interactions and consider additional assembly processes and interactions between processes as well (e.g. Belyea, 2004; Kraft et al., 2015; Lortie et al., 2004).

1.3.3. Shallow lakes theory of alternative stable states

The original **shallow lakes theory of alternative stable states** (Scheffer et al., 1993) was developed to explain the observation that increasing nutrient levels can cause shallow lakes to shift abruptly from a clear water macrophyte dominated to a turbid water phytoplankton dominated state. Furthermore, it was observed that restoration attempts to return a turbid lake to the preferred clear water state through nutrient reductions were resisted by the system. This resistance was explained by positive feedback mechanisms that keep a lake in one of the alternative stable states. For example, a clear water macrophyte dominated lake is kept in this state because macrophytes enhance water clarity by preventing sediment resuspension and supressing phytoplankton growth. With increasing nutrient levels, phytoplankton growth and therefore water turbidity increase (*Fig. 1.6a*). Once the turbidity is past a critical threshold, macrophyte growth is prevented by light limitation. Without vegetation, algal growth is not controlled and this stabilizes the turbid state (Scheffer et al., 1993).

Shallow lakes theory also explains how a turbid water state can be induced and stabilised by sediment resuspending fish (Scheffer et al., 1993), and by extension by sediment resuspending crayfish (*Fig. 1.6b*, Twardochleb et al., 2013). Furthermore, the theory has been extended to shifts between submerged and free floating macrophyte dominated states (*Fig. 1.6c*), and to shifts between stonewort (charophyte) and angiosperm dominated states (*Fig. 1.6d*, Scheffer & Van Nes, 2007).

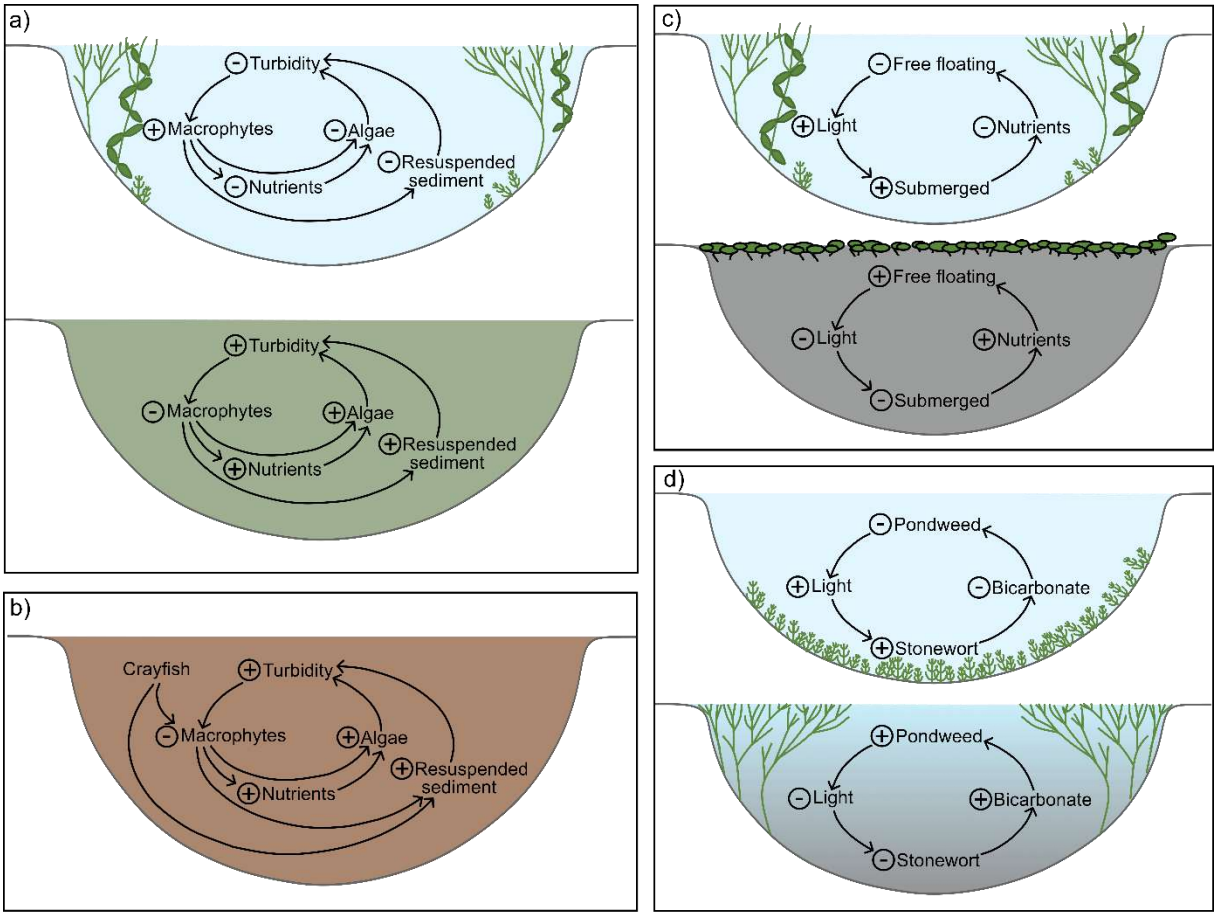


Fig. 1.6. Alternative states in ponds stabilized by positive feedback loops described for shallow lakes in Scheffer & Van Nes (2007). a) Clear water macrophyte dominated and turbid water algae dominated states that can exist at similar levels of nutrient input. b) Turbid water state induced by crayfish. Note that the bioturbating organisms in Scheffer & Van Nes (2007), fish, have been replaced by crayfish in this figure. c) Submerged and free-floating macrophyte dominated states that can exist at similar levels of nutrient input. Light is short for under water light conditions. d) Stonewort and Pondweed (Potamogetonacea) dominated states that can occur at similar levels of bicarbonate input.

The composition and diversity of pond communities differ between the alternative stable states (Cottenie et al., 2001). Waterbodies in clear water submerged macrophyte dominated states exhibit higher alpha diversity than those in turbid or free floating macrophyte dominated states (Scheffer & Van Nes, 2007). Since clear water is often a desired pond creation outcome, the shallow lake theory can help to understand mechanisms that could lead to this result, making it a very relevant concept regarding pond creation for biodiversity conservation.

1.3.4. Habitat complexity and diversity

The reason why submerged macrophyte dominated states are more diverse than phytoplankton or free-floating macrophyte dominated stages may lie in the higher **habitat complexity** provided by the submerged macrophytes (Meerhoff & De Los Ángeles González-Sagrario, 2021). The habitat complexity-diversity hypothesis poses that more complex habitats host more diverse communities (Palmer et al., 2010; Tokeshi & Arakaki, 2012). Indeed, in ponds, habitat complexity provided by macrophytes has been related to macroinvertebrate taxonomic richness (St. Pierre & Kovalenko, 2014). Habitat complexity exists at different spatial scales and should at least be characterized by the diversity, spatial arrangement, sizes and abundance or density of **habitat structural elements** (Tokeshi & Arakaki, 2012). In ponds, specifically the number of different size categories of interstitial spaces in macrophytes has been related to macroinvertebrate richness (St. Pierre & Kovalenko, 2014).

Habitat heterogeneity, the component of habitat complexity that relates to the diversity of structural elements, was believed to be very important for ecological restoration. As a result, many stream restoration projects focussed on increasing habitat heterogeneity, for example by adding meanders or physical structures such as boulders. However, in general these actions did not increase stream biodiversity (Palmer et al., 2010). In systems where reduced habitat complexity caused biodiversity loss, addressing the origin of the reduced complexity is still important for freshwater restoration, but various aspects of habitat complexity, not only the diversity of structural element, should be included (St. Pierre & Kovalenko, 2014).

1.3.5. Pondscapes and metacommunities

Ponds are not independent from other ponds in the landscape, nor from their terrestrial surroundings. They function as habitat networks, or **pondscapes** (Boothby, 1997; Hassall et al., 2012). Pondscapes are delineated by their **connectedness**, meaning that the ponds consisting a pondscape are spatially close. When these ponds host communities that can disperse between them, the pondscape host a **metacommunity** (*Fig. 1.7*, Boothby, 1997; Fehlinger et al., 2023).

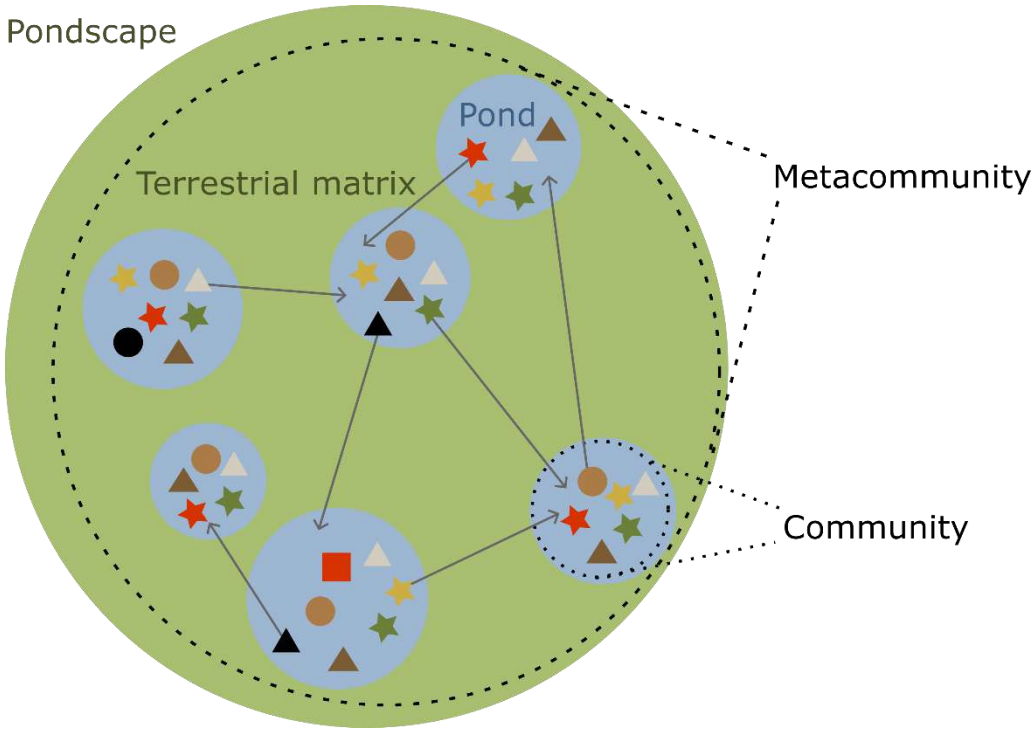


Fig. 1.7. The pondscape consisting of ponds (blue) in a terrestrial matrix (green) and hosting a metacommunity. Figure inspired by Chase et al. (2020)

The metacommunity concept is essential for studying pond communities, as it incorporates the effects of **local** and of **regiona**l processes, and their interactions (Fehlinger et al., 2023; Leibold et al., 2004; Warfe et al., 2013). The theoretical framework describing metacommunity processes was originally presented as four paradigms: **species sorting**, **mass effects**, **patch dynamics**, and **neutral processes** (Leibold et al., 2004). These paradigms are not mutually exclusive, and empirical studies show that community assembly often exhibits features of multiple paradigms (Logue et al., 2011). For example, it has been shown that both species sorting and mass effects influence plant community assembly in ponds (Lozada-Gobilard et al., 2019). For the empirical study of community assembly and for biodiversity conservation, the separation of metacommunity processes in four paradigms may not be useful (Chase et al., 2020; Logue et al., 2011; Winegardner et al., 2012). The four paradigms can be seen as special cases in a continuum of metacommunity assembly mechanisms that spans a range of degrees to which equivalence among species regarding niche and fitness, **environmental heterogeneity** and dispersal are important (*Fig. 1.8*, Logue et al., 2011).

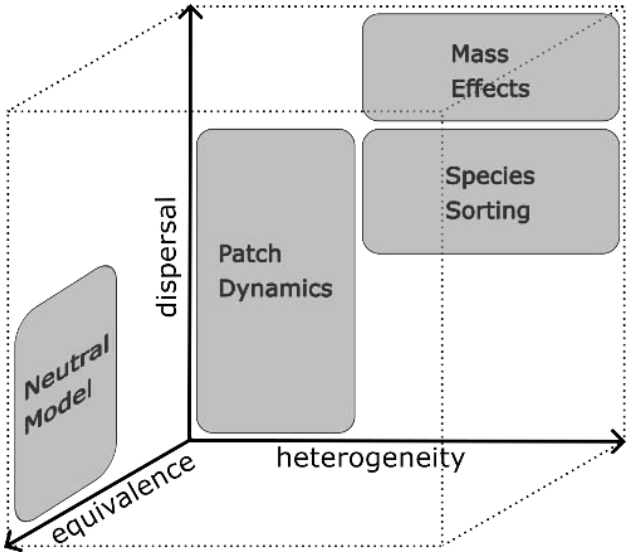


Fig. 1.8. Metacommunity assembly continuum with the mass effects, species sorting, patch dynamics and the neutral model perspectives positioned according to the rate of dispersal, environmental heterogeneity and the equivalence of species regarding niche and fitness. Figure based on Logue et al. (2011). Note that environmental heterogeneity is not equivalent to habitat heterogeneity.

Dispersal rates of individuals and propagules between habitat patches may depend on the distance between the **patches**, the permeability of the **matrix** between the patches and the dispersal capacities of the organisms (Lowe & McPeek, 2014; Soininen et al., 2007). Within a metacommunity, dispersal rates can therefore be sufficient, high or limited (Heino et al., 2021; Leibold et al., 2004; Winegardner et al., 2012). When dispersal is sufficient and environmental conditions differ between habitat patches, species are expected to sort themselves among patches according to their niche requirements. However, in case dispersal rates are very high, immigration could supplement individuals to patches that have marginal or unfavourable conditions, and emigration can lower the population density of favourable patches (Winegardner et al., 2012). Furthermore, in case dispersal is limited, species cannot reach all patches and may as a result be absent from local communities in patches with suitable environmental conditions (Heino et al., 2017). Both dispersal limitation and decreases in environmental similarity with geographic distance can lead to the often observed pattern that community similarity decreases with geographic distance between habitat patches (Diniz-Filho et al., 2012; Morlon et al., 2008; Soininen et al., 2007).

1.3.6. Deterministic and stochastic community assembly

Ponds close to each other with similar age and (seemingly) similar environmental conditions often develop divergent communities (Friday, 1987; Jeffries, 2008). Furthermore, environmental conditions generally only explain a limited proportion of the variation in pond communities, and responses to environmental variables are often not consistent among studies (Batzer, 2013; Capers et al., 2010; Friday, 1987; Jeffries, 2008; Reindl et al., 2023). The **deterministic** variables included in pond studies and wetland studies are clearly inadequate in explaining invertebrate and macrophyte community assembly (Jeffries, 2008; Reindl et al., 2023). A range of suggestions has been made on the nature of the unexplained variance in pond community composition.

Firstly, it has been proposed that **stochasticity**, or chance, influences community assembly (Batzer, 2013; Edvardsen & Økland, 2006a; Jeffries, 2008; Villsen et al., 2025). Indeed, a combination of deterministic and stochastic processes is thought to structure communities (Lortie et al., 2004; Villsen et al., 2025). Deterministic processes thereby include species interactions and responses to environmental variables, whereas dispersal is partly stochastic (Lowe & McPeek, 2014; Villsen et al., 2025). A second proposition is that **priority effects**, which arise from the interaction between the partly stochastic order of arrival and deterministic processes, influence pond community assembly (Belyea & Lancaster, 1999; Hoverman & Relyea, 2008; Jeffries, 2008). Thirdly, it may be that community assembly is deterministic, but that important factors are not taken into account, especially when studying communities at one location or a single point in time (Batzer, 2013; Jeffries, 2008; Reindl et al., 2023). For instance, patterns in pond communities that would probably be attributed to stochasticity if studied at one point in time, could be attributed to subtle variations in hydrology over a ten-year study period (Jeffries, 2008). Lastly, community assembly may be **chaotic**, meaning that it is deterministic but highly sensitive to initial conditions and can only be predicted in the short, but not in the long term (Batzer, 2013; Hastings et al., 1993). Chaos may, for example, render the outcomes of interspecies competition unpredictable, and is probably common in ecosystems, but empirical evidence of chaos in real ecosystems is rare (Benincà et al., 2008; Huisman & Weissing, 2001; Munch et al., 2022; Rogers et al., 2022).

1.3.7. Drivers and impacts of biological invasions

Invasions of **alien species** can have strong adverse effects on aquatic ecosystems and are seen as one of the most important threats to freshwater biodiversity (*Fig. 1.1*, Dudgeon et al., 2006; Gallardo et al., 2016). Moreover, restored and newly available habitats may be especially susceptible to invasions (D'Antonio & Meyerson, 2002). On the other hand, because of their isolation, ponds may be less vulnerable to invasions than other freshwater systems (Hill et al., 2021). Although the impact of invasive species on ponds is not well understood (Hill et al., 2021), it is crucial to understand drivers and impacts of biological invasions to avoid detrimental consequences of invasions in newly created ponds.

Biological invasion is seen as a staged process where many alien species get introduced, some become established, and few become widespread or abundant (Inderjit 2005). There are numerous hypotheses on factors that promote or inhibit transitions to the next invasion stage (Daly et al., 2023; Enders et al., 2020; Inderjit, 2005). More than thirty hypotheses are generally accepted to be relevant (Enders et al., 2020). These hypotheses concern the invasiveness of the species, and the invasibility of the communities and ecosystems (Daly et al., 2023; Inderjit, 2005). One of the species-level hypotheses, for example, states that the invasiveness of a species may be influenced by the performance, originality and plasticity of its traits. Community level hypotheses include the idea that communities are susceptible to invasion when they have empty niches, and that more diverse communities are more resistant to invasion because more niches are filled. Enemy release, or the absence or reduction of regulation by natural enemies in the

recipient community, may also allow alien species to increase in abundance. On the ecosystem level, invasions may be more successful in highly disturbed systems (Daly et al., 2023; Enders et al., 2020; Inderjit, 2005).

Invasive species can impact resident communities through direct biotic interactions and through indirect changes in abiotic conditions. The impacts thereby differ between trophic levels of invasive species. In aquatic ecosystems for example, primary producers and filter collectors can have positive influences on macrophytes, but the impact of omnivores can be negative (Gallardo et al., 2016). Among the most influential invasive species are the "invasive engineers", invasive species that modify their environment (Cuddington & Hastings, 2004; Gallardo et al., 2016). These may have especially large impacts when occurring at high densities (Daly et al., 2023; Galib et al., 2022; Matsuzaki et al., 2009). Sediment resuspending crayfish can, for example, cause an entire freshwater system to shift from a clear water to a turbid state (Gallardo et al., 2016; Gherardi et al., 2011; Twardochleb et al., 2013). Invasive species can cause **ecosystem degradation**, but in some degraded systems, it may be hard to discern whether populations of invasive species are "drivers", causing the degradation, or "passengers", benefiting from degraded conditions. Invasive populations could also be "backseat drivers", which benefit from degraded conditions and make them worse (Daly et al., 2023; Dercksen et al., 2025; MacDougall & Turkington, 2005).

1.3.8. Spatial scales and level of organisations

The theories described above are formulated to explain phenomena at different spatial scales and levels of organisation. I mapped the theories according to their relevance for local and regional phenomena and whether they apply to ecosystems, communities or both (*Fig. 1.9*). As described in section 1.3.5, both local and regional processes can influence the outcomes of pond network creation for biodiversity. The level of organisation most relevant for ponds created for biodiversity conservation is the community level. However, since the composition and diversity of communities establishing in ponds is related to ecosystem processes, ecosystem level theories are useful as well.

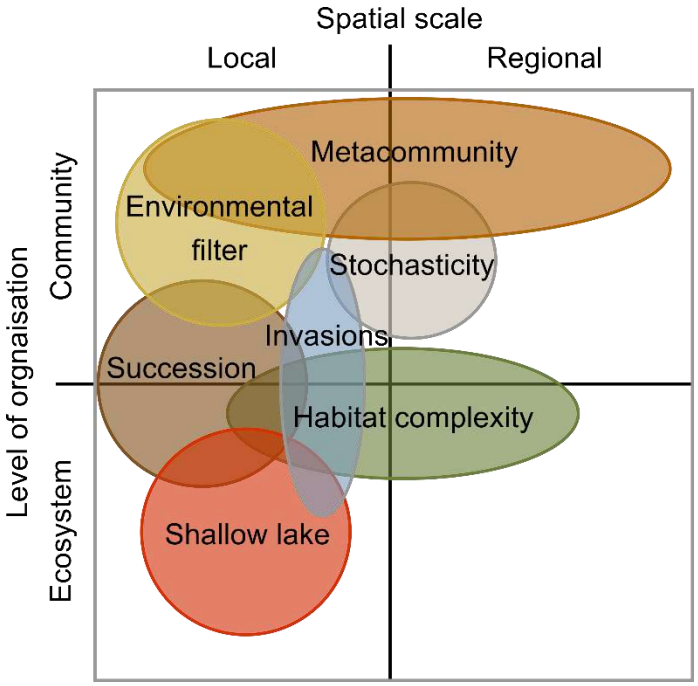


Fig. 1.9. Theories discussed in this section according to their spatial scale and level of organisation.

1.4. The evaluation of pond creation success

Evaluating the success of restorative activities is essential (De Necker et al., 2025; Grayson et al., 1999; Prach et al., 2019). Without evaluation, it cannot be known if the resources spent on a restorative activity were used effectively, or could have better been used for other conservation, restoration or management purposes. Furthermore, if restorative activities are not evaluated, future projects may use the same untested restoration methods (De Necker et al., 2025; Grayson et al., 1999). It is therefore problematic that pond creation and restoration projects are often not evaluated or only based on a limited set of **indicators** (De Necker et al., 2025).

The question of how to evaluate restoration success has been the topic of considerable debate in the field of restoration ecology (Prach et al., 2019). Success can be defined as the degree to which stated **objectives** are achieved (Adapted from Kentula, 2000). Objectives are desired outcomes of a restorative activity. They should relate to the **goal**, which is the desired condition for the system where the restorative activity is implemented (Gann et al., 2019). The setting of realistic goals and objectives for restorative projects is, however, not evident and a wide variety of objectives exist (Ehrenfeld, 2000; Grayson et al., 1999; Moreno-Mateos & Comin, 2010; Perring et al., 2015; Prach et al., 2019). Objectives can be classified as relating to the form, function and stability of the ecosystem, as well as to socio-economic outcomes (Hallett et al., 2013). The formulation of objectives for pond creation and restoration projects in specific has received little attention, but a variety of objectives can be found in the literature (*Table 1.3*).

Table 1.3. Examples of objectives for pond creation and restoration with references

Objectives relating to form	
Increase occupation/abundance of target species	De Necker et al., 2025; Rannap et al., 2009
Increase richness/abundance of rare/protected/threatened species	Williams et al., 2008
Increase alpha/beta/gamma diversity	Thiere et al., 2009
Reduce abundance invasive species	De Necker et al., 2025
Objectives relating to functioning	
Improve water quality/nutrient retention	Cuenca-Cambronero et al., 2023; De Necker et al., 2025; Thiere et al., 2009
Provide more habitat to target species	Cuenca-Cambronero et al., 2023; De Necker et al., 2025; Rannap et al., 2009
Improve flood protection	Cuenca-Cambronero et al., 2023; De Necker et al., 2025
Improve fire protection	De Necker et al., 2025
Increase water storage in the landscape	De Necker et al., 2025
Enhance connectivity	Maynou et al., 2017; Rannap et al., 2009
Increase pollination	Cuenca-Cambronero et al., 2023
Socio-economical objectives	
Increase opportunities for recreation	Cuenca-Cambronero et al., 2023
Increase opportunities for education	Primarck et al., 2000
Increase opportunities for culture/art/inspiration	Cuenca-Cambronero et al., 2023; Primarck et al., 2000

In the planning phase of a restorative project, the overall goal and the more specific objectives of the project should be stated explicitly (*Fig. 1.10*). They should be based on a diagnosis of the causes of the degradation of the ecosystem and the degree to which it can recover. Furthermore, appropriate indicators, variables that can be measured and relate the objectives and goal should be selected, and a monitoring plan should be developed. Monitoring is the collection of data on

the indicators (Gann et al., 2019; Prach et al., 2019). Furthermore, an evaluation strategy should be selected that will allow to assess the success of the restorative activity in relation to the stated goals and objectives. It is not entirely clear what type of evaluation strategy should be recommended for pond creation and restoration projects, nor which indicators to use. Furthermore, the monitoring of numerous and heterogeneous ponds remains challenging (Hill et al., 2021). Subsection 1.4.1 describes evaluation strategies for restorative activities and subsection 1.4.2 discusses ecological indicators that could be applicable to ponds. Subsections 1.4.3 and 1.4.4 introduce conventional and molecular techniques to monitor biological indicator groups important for ponds.

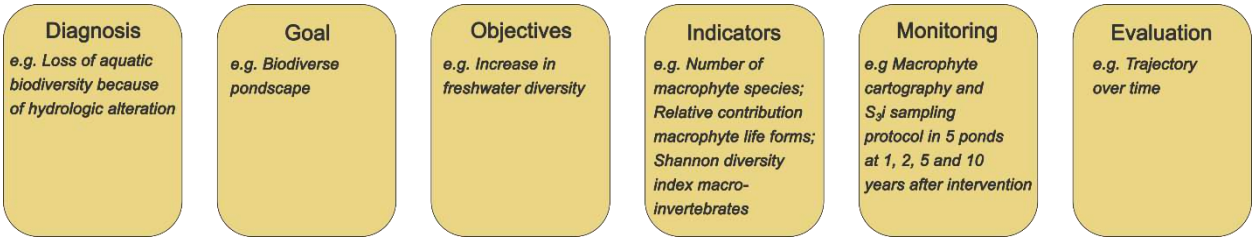


Fig. 1.10. Elements required for success evaluation of restorative activities, which should be defined during the planning phase of activities, and examples. Figure inspired by Prach et al. (2019) and Gann et al. (2019).

1.4.1. Types of evaluation strategies

Several strategies to evaluate the progress towards stated objectives can be distinguished (*Table 1.4).* First, compliance with a contract or permit can be evaluated (Kentula, 2000). However, this evaluation of compliance success does not necessarily reflect whether ecological functions are restored, or whether the restoration outcomes are sustainable in the long term (Kentula, 2000; Van Den Bosch & Matthews, 2017). A second strategy entails documentation of the trajectory of ecosystem after the restorative activity (Kentula, 2000). In case the ecosystem is also monitored before the restorative activity, the evaluation strategy can be labelled “Before-After” (Mahlum et al., 2018). Instead of comparing a restored site with its condition before the restorative activity, the site may also be compared with nearby unrestored degraded control sites, in what is called a “Control-Impact” evaluation (Mahlum, 2017, Januschke 2021).

Table 1.4. Different types of evaluation strategies for restorative activities

Type of evaluation strategy	Reference
1) Compliance with agreement	Kentula, 2000; Van Den Bosch & Matthews, 2017
2) Trajectory since intervention	Kentula, 2000
3) Before-After	Mahlum et al., 2018
4) Control-Impact	Januschke et al., 2011; Mahlum et al., 2018
5) Before-After-Control-Impact (BACI)	Mahlum et al., 2018
6) Before-After-Control-Impact with undegraded reference	Grayson et al., 1999
7) Comparison with undegraded reference	Gann et al., 2019; Kentula, 2000; Wortley et al., 2013
8) Contribution to landscape or catchment	Kentula, 2000; Moreno-Mateos & Comin, 2010; Thiere et al., 2009
9) Capacity, opportunity and realised function	Kentula, 2000

Both Before-After and Control-Impact may be suboptimal evaluation strategies. This is because Before-After evaluation assumes that any measured change is attributable to the restoration activity, while other factors may be responsible, and Control-Impact assumes that control sites are comparable to restored sites before the restorative activity, which may not be true either. In a preferable situation one would therefore monitor both the site of restoration and an undegraded control site, before and after the restorative activity, in what is termed a Before-After-Control-Impact (BACI) evaluation (Mahlum et al., 2018; Smokorowski & Randall, 2017). The optimal scenario would be to include degraded unrestored control sites as well as undegraded reference sites, and monitor them along with the restoration site at multiple points in time before and after the restorative activity (Grayson et al., 1999).

The Society for Ecological Restoration recommends the evaluation of restored sites against a reference model. The reference model describes the conditions of undegraded reference sites, and may also include historical information, as long as background environmental change is considered as well. According to the Society for Ecological Restoration, reference models should include indicators relating to the absence of threats, species composition, community structure, physical conditions, **ecosystem function**, and external exchanges (Gann et al., 2019).

The type of evaluation strategy that is appropriate for a restorative activity depends on the objectives of the activity and the data that can be obtained. For example, when the objective of pond creation or restoration is not to replace a specific type of freshwater that has been lost, but to increase freshwater biodiversity in general, evaluation strategies using reference models may not be suitable. Furthermore, BACI evaluations are not applicable to pond creation, because comparisons with terrestrial control sites (which would be the “Before”) would be little informative. Of course, it is very important to perform baseline inventories of the site before pond creation to ensure no terrestrial biodiversity is impaired by the activity. Furthermore, when restorative activities are aimed to improve conditions at the landscape or catchment scale, evaluation should also be performed at this scale as well (Kentula, 2000; Moreno-Mateos & Comin, 2010). Lastly, in case of habitat restoration, not only the presence of the target species, but the capacity of the habitat to sustain viable populations, the opportunity for the target species to access and use the restored habitat, and the realised function, that is the actual use of the habitat by the target species, could be evaluated (Kentula, 2000).

1.4.2. Indicators relevant to pond creation and restoration for biodiversity

It has been advised to select a few indicators, neither only one nor too many, to evaluate the success of a restorative activity (Prach et al., 2019). Socio-economic objectives and indicators of restoration success are beyond the scope of this PhD thesis. Ecological indicators relate to the form or function of the ecosystem. They should be easy to measure and interpret, respond to ecological stressors in a predictable manner, and exhibit a demonstrated relationship to the **ecosystem structure** or process they are intended to measure (Prach et al., 2019).

When biodiversity improvement is the main objective of a restorative measure, an appropriate indicator may be species richness (Prach et al., 2019). Furthermore, other biodiversity metrics, such as the Shannon diversity index and measures of taxonomic distinctness, may provide complementary information (Gallardo et al., 2011; Ruhí et al., 2009). Metrics of **functional diversity** have also been proposed as indicators for biodiversity and restoration success (Cadotte et al., 2011; Coccia et al., 2021; Gallardo et al., 2011). However, functional diversity is often redundant to species richness, and can be difficult to interpret, because it depends on the number and identity of the traits included, as well as the source of trait information (Gallardo et al., 2011; O’Brien et al., 2022; Ruhí et al., 2009).

Pond multimetric indices

A few multimetric indices for the ecological status of ponds have been developed in Europe (*Table 1.5*). These indices describe the status of ecosystems using a combination of individual metrics. The existing indices describe various aspects of pond ecosystems. The IBEM and PLOCH indices describe, for example, the quality of the species richness of a pond. They consist of metrics for multiple taxonomic groups that describe the ratio of the predicted species richness for the pond compared to the actual estimated species richness in the pond (Angélibert et al., 2010; Oertli et al., 2005b). The CIEPT index, on the other hand, describes the ecological condition of a pond by the means of three macroinvertebrate metrics, that have been shown to relate to conditions that differ between reference and degraded ponds (the macrophyte richness, trophic level and degree of exposure to five anthropogenic stressors linked to land use) (Menetrey et al., 2011).

The listed multimetric indices may be useful for the evaluation of pond creation and restoration, as long as an index relates to the objective of the restorative activity to evaluate, and the restorative activity is performed in the region where the index was calibrated. Many regions in Europe still lack a calibrated multimetric index for the ecological status of ponds. This may be because most EU member states only monitor standing waters larger than 50 ha for the Water Framework Directive, and did not develop pond specific indices (Kristensen & Globevnik, 2019; Labat & Usseglio-Polatera, 2023). Indices designed for lakes are not directly applicable to ponds because of their differences in ecological functioning (Menetrey et al., 2011, see also section 1.2.2).

Table 1.5. Multimetric indices for ponds in Europe with the aspect of the pond ecosystem they describe, the region they apply to, the indicator groups and the number of metrics included, and whether the index calculation accounts for natural variations in environmental variables that are not stressors.

Acronym	Index for	Region	Indicator groups	Metrics	Natural variation?	Reference
PSYM	Ecological quality	England and Wales	Macrophytes, Macroinvertebrates	6	Yes	Howard, 2002
PLOCH	Quality of species richness	Switzerland	Macrophytes, Gastropoda, Coleoptera, adult Odonata and Amphibia	5	Yes	Oertli et al., 2005b
PMII	Eutrophication	Italian mountains	Macroinvertebrates	7	No	Solimini et al., 2008
IBEM	Quality of species richness	Switzerland	Macrophytes, Gastropoda, Coleoptera, adult Odonata and Amphibia	5	Yes	Angélibert et al., 2010; Indermuehle et al., 2010
CIEPT	Ecological condition	Swiss lowlands	Macroinvertebrates	3	No	Menetrey et al., 2011
BECOME	Anthropogenic stressors (13)	France	Macrophytes, Macroinvertebrates	11	Yes	Labat & Usseglio-Polatera, 2023

Indicator groups for ponds

The indicator groups macrophytes, macroinvertebrates and amphibians are used in the existing pond multimetric indices (*Table 1.5*). These groups are suitable indicators for ponds because they 1) are reasonably diverse but with a complete taxonomy, 2) include species with different functions in the ecosystem, 3) are geographically widespread, 4) are accessible for standardized sampling, 5) have substantial ecological knowledge, and 6) are likely to attract political and public support (Oertli Auderset Joye, et al., 2005).

Fish, mammals and birds not suitable indicators because are often absent from ponds (Labat & Usseglio-Polatera, 2023; Oertli et al., 2005b). Short lived phytobenthos, phytoplankton and zooplankton are useful to detect short term, e.g. daily or weekly, environmental changes, but are not suitable to indicate changes on yearly timescales (Labat & Usseglio-Polatera, 2023; Stamou et al., 2022). They may also generate less political and public support (Oertli et al., 2005b). Other microorganisms living in ponds are subject to the same limitations as phytobenthos, phytoplankton and zooplankton. Furthermore their taxonomy, identification methods and ecology are less well developed (Rohwer et al., 2018; Weisse, 2006).

This PhD thesis focusses on the indicator groups macrophytes, aquatic photosynthetic organisms large enough to see with the naked eye *(Fig. 1.11*), and macroinvertebrates, animals without a backbone and large enough to see with the naked eye (*Fig. 1.12*). Both groups are of key importance to pond ecosystems. Macrophytes maintain a clear water state by controlling phytoplankton growth and preventing sediment resuspension (see also section 1.3.3). Furthermore, they provide shelter, food, sites for egg-laying and emergence, and materials for case building to animals, whereby macrophytes of different life forms (*Fig. 1.11*) are important for different taxa (Biggs & Williams, 2024; Capers et al., 2010; Carpenter & Lodge, 1986; Chambers et al., 2008). Adult and larval stages of the diverse group of macroinvertebrates inhabit various pond **mesohabitats** and perform a large range of functions. Macroinvertebrates contribute, for example, to leaf litter breakdown, nutrient cycling and maintenance of the clear water state. They can also act as top predators and provide food for animals as well as outside the pond (Fehlinger et al., 2023; Labat & Usseglio-Polatera, 2023; Macadam & Stockan, 2015; Tachet et al., 2010) .

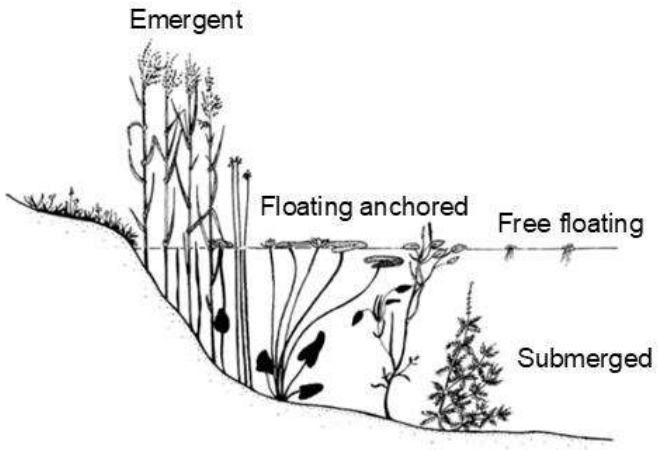


Fig. 1.11. Macrophyte life forms. Figure adapted from Brönmark & Hansson, 2008

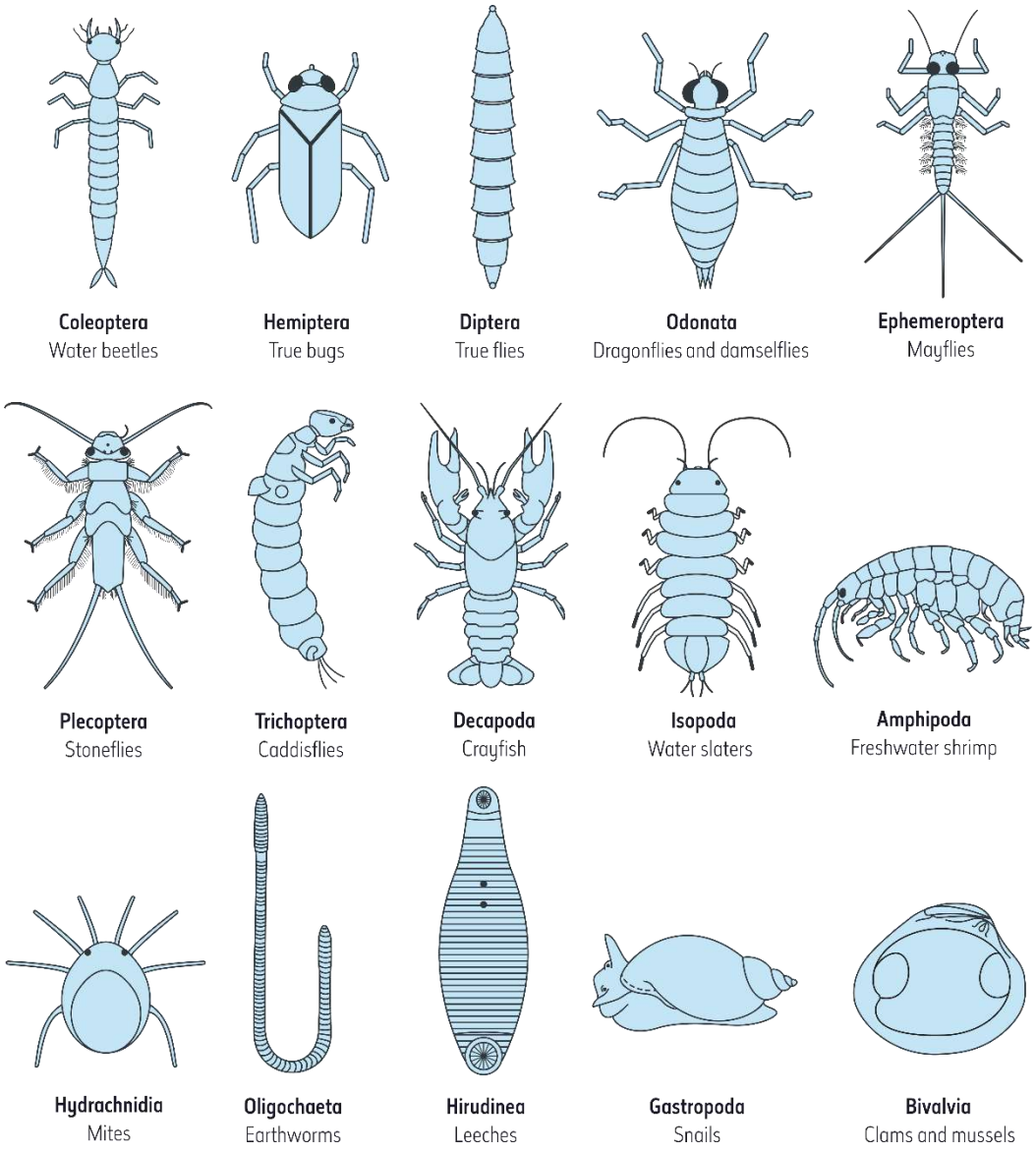


Fig. 1.12. Diagrams representing forms of common freshwater macroinvertebrate groups. Images from Pieterse et al. (z.d.)

1.4.3. Traditional monitoring of macrophytes and macroinvertebrates

Pond macrophytes are generally identified by observation in the field and only for some species a small amount of plant material is brought to the laboratory for identification (Howard, 2002; Labat & Usseglio-Polatera, 2023). Some methods survey macrophytes in plots along transects (e.g. Indermuehle et al., 2010; Oertli et al., 2005b) while others survey the entire pond area (e.g. Friday, 1987; Howard, 2002; Labat & Usseglio-Polatera, 2023). Surveys are generally performed by walking around the pond, wading in the pond, and using a boat if necessary. In deeper areas a grapnel or rake can be used to collect submerged macrophytes (Howard, 2002; Labat & Usseglio-Polatera, 2023). Some methods only record the presence of observed species (e.g. Indermuehle et al., 2010; Oertli et al., 2005b) while others estimate their cover as well (e.g. Friday, 1987; Labat & Usseglio-Polatera, 2023).

Macrophytes are not used in all pond multimetric indices listed in *Table 1.5*, but macroinvertebrates are. All the listed indices employ macroinvertebrate sampling protocols based on hand netting. Since the distribution of macroinvertebrates in ponds is heterogeneous, different mesohabitats are sampled (Howard, 2002; Indermuehle et al., 2010; Labat & Usseglio-Polatera,

2023; Menetrey et al., 2011; Oertli et al., 2005b; Solimini et al., 2008). Samples are stored in ethanol and brought to the laboratory for sorting and identification, which are time consuming, and therefore expensive (Angélibert et al., 2010; Hering et al., 2018; Indermuehle et al., 2010; Labat et al., 2022). The Sampling of Small Shallow lake invertebrates (S_3i) sampling and identification protocol, which is suitable for calculating the BECOME index, was designed to be more rapid than the sampling and identification protocol for the IBEM index, which was in turn designed to be less time consuming, and requiring less taxonomic expertise than the protocol for the PLOCH index (Angélibert et al., 2010; Labat et al., 2022; Labat & Usseglio-Polatera, 2023; Oertli et al., 2005b).

Besides being time consuming and requiring taxonomic expertise, the existing pond macroinvertebrate sampling protocols are also invasive: habitats are trampled, sediment is resuspended, and the sampled animals are placed in ethanol. Since ponds portray high beta diversity, it is important to sample a substantial proportion of them to represent a pondscape. Furthermore, ponds are numerous and sometimes difficult to access. All these factors make pond monitoring challenging (Hill et al., 2021). A promising non-invasive method that could overcome these challenges and enable upscaling of pond monitoring is **environmental DNA** metabarcoding. This method may enable the detection of a wide range of taxa from a single sample. It could also allow inclusion of early larval stages and taxonomic groups that are difficult to identify, such as dipterans, in pond monitoring protocols (Harper et al., 2019; Hill et al., 2021; Robertson, 2024; Thomsen & Willerslev, 2015; Zizka et al., 2025).

1.4.4. Environmental DNA metabarcoding

Environmental DNA (eDNA) is “a complex mixture of genomic DNA from many different organisms found in an environmental sample”, for example a water sample (Taberlet et al., 2018). Macroorganismal eDNA may originate from excreted cells or tissues from living or dead organisms. It may be released, or “shed”, from sources including skin, hairs, mucus, faeces, urine, saliva, blood, gametes, pollen and decaying tissues (Taberlet et al., 2018; Thomsen & Willerslev, 2015). Outside of the organism eDNA may exist in tissues, cells or organelles, as well as dissolved in water or adsorbed to particles (Mauvisseau et al., 2022). Depending on the water chemistry, and likely on the microbial activity as well, after shedding macroorganismal eDNA may remain detectable between one day and two weeks in temperate waters (Barnes et al. , 2014; Mauvisseau et al., 2022).

There has been a rapid development in eDNA research since the term was coined in 1987 by Ogram and colleagues (*Fig. 1.13*, Ogram et al., 1987; Taberlet et al., 2018). In 1990, the first study using **metabarcoding**, the simultaneous detection of multiple species by **sequencing** a specific DNA section, was published (Giovannoni et al., 1990; Taberlet et al., 2018). Environmental DNA was initially only used to study microorganisms, and the first macroorganismal eDNA studies (Ficetola et al., 2008; Martellini et al., 2005; Willerslev et al., 2003) appeared in the 2000’s (Taberlet et al., 2018; Thomsen & Willerslev, 2015). Since **Next Generating Sequencing** (NGS) became commercially available in 2005, DNA **amplicons** can be sequenced on large scales (Margulies et al., 2005; Taberlet et al., 2018). The last decade has seen a large expansion of eDNA research, with a strong focus on applications for biomonitoring (*Fig. 1.14*, Blackman et al., 2024a).

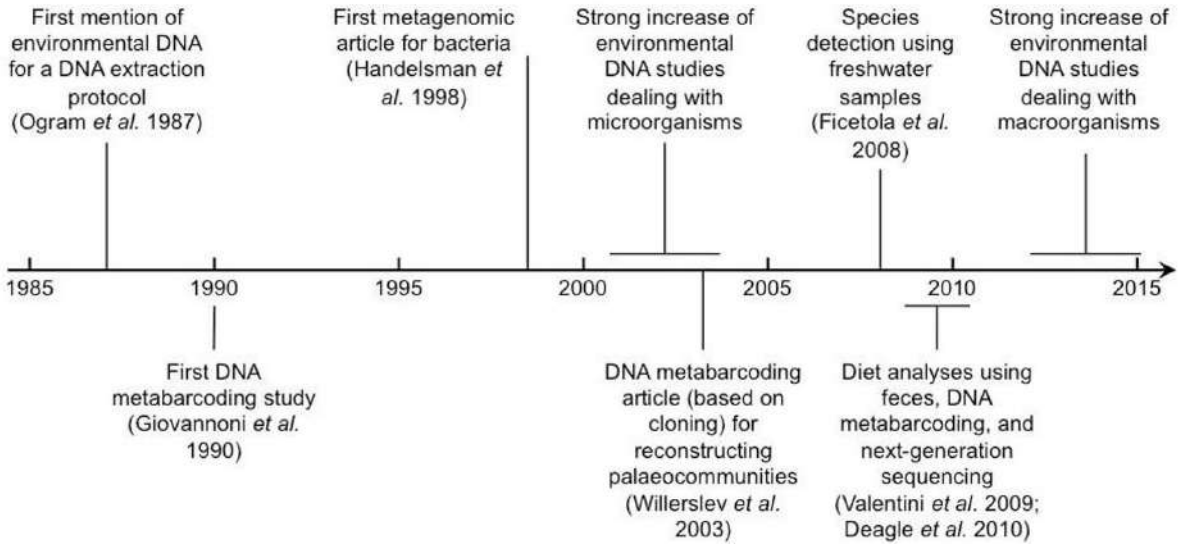


Fig. 1.13. The emergence of eDNA studies. Figure from Taberlet et al., 2018.

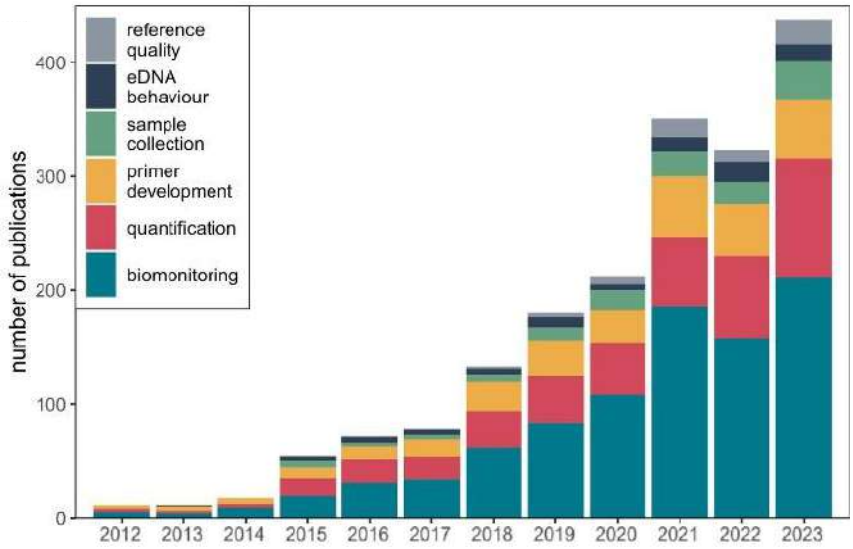


Fig. 1.14. The number of publications using environmental DNA per year since 2012 with colours indicating research areas. Figure reproduced from Blackman et al., 2024a.

Environmental DNA approaches for biomonitoring can be divided into single-species methods, which for example aim to detect the presence of a specific threatened or **invasive species**, and multi-species approaches (Schenekar, 2023; Taberlet et al., 2018; Takahashi et al., 2023). Currently, almost all multi-species macroorganismal studies employ **Polymerase Chain Reaction** (PCR) based metabarcoding (*Fig. 1.15*), with methods for each step in the workflow differing between studies (Schenekar, 2023; Takahashi et al., 2023).

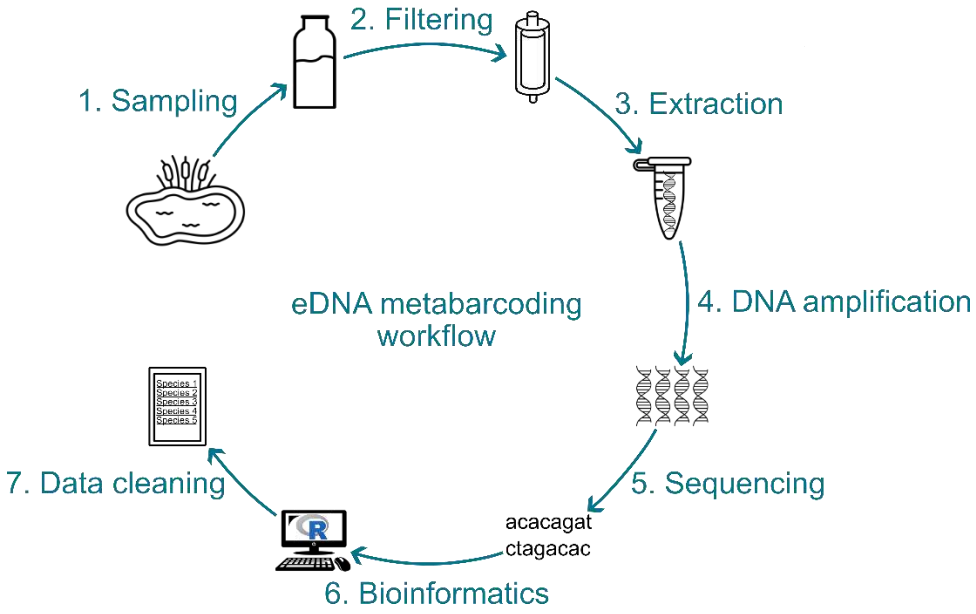


Fig. 1.15. A generalized aquatic eDNA metabarcoding workflow. Steps 1 to 7 describe actions and the symbols objects (pond, sample, solid parts of the sample on a filter, extracted DNA, amplicons, sequencing data, sequences organised according to the sample or control they came from and assigned to taxa, a species list per sample).

The choice of **primer pair**, which amplifies a specific metabarcode in the PCR (step 4 in *Fig. 1.15*) is crucial (Schenekar, 2023; Taberlet et al., 2018). The metabarcode (*Fig 1.16*) is a short DNA region that varies between the target species, so that it can be used to identify the species from which the DNA originates. This region must lie between two regions that are (almost) identical for all species of the target group, to which the forward and reverse primer can anneal. In the PCR, the metabarcode is amplified for all target species, which, after the sequencing and initial **bioinformatics** steps, can be identified by comparing the amplified variable region to sequences stored in **reference databases** (Taberlet et al., 2018).

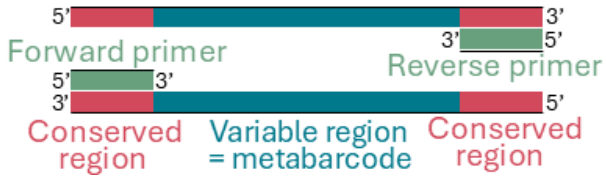


Fig. 1.16. Metabarcode (blue) with the conserved flanking regions (red) to which primers (green) can bind.

Environmental metabarcoding methods are still developing and are not yet implemented in biomonitoring schemes (Blackman et al., 2024a). Techniques are most advanced for fish, and are equally reliable as conventional electrofishing and netting methods in generating species lists (Blackman et al., 2024a; Keck et al., 2022; Schenekar, 2023; Takahashi et al., 2023). Also for amphibians, metabarcoding is as efficient in species detection as conventional visual, netting and acoustic detection methods (Moss et al., 2022; Schwesig et al., 2025; Valentini et al., 2016). For other taxa metabarcoding is less developed, but still holds potential to complement conventional biomonitoring (Blackman et al., 2024a; Schenekar, 2023; Takahashi et al., 2023).

Macroorganismal metabarcoding studies of pond communities other than vertebrates are still rare (Harper et al., 2019; Robertson, 2024). Only very recently a couple of pond invertebrate and plant studies were published (Robertson, 2024; Zizka et al., 2025; Harper et al., 2021). Ponds have their peculiarities, implying that metabarcoding protocols optimized for other waterbodies like rivers or lakes are not directly applicable. Due to limited mixing of water in ponds, eDNA in may be heterogeneously distributed. Furthermore, pond waters can be turbid, which complicates filtering as this causes filters to clog quickly. Ponds are also often rich in organic matter which can complicate DNA extraction and inhibit PCRs (Harper et al., 2019). Therefore, although promising, development, optimization and validation studies are still needed before eDNA metabarcoding can be applied to the monitoring of pond communities (Harper et al., 2019; Hill et al., 2021).

1.5. PhD objectives and outline

As described above, habitat loss and degradation are among the leading causes of freshwater biodiversity decline (Revenga et al., 2005). The protection and restoration of freshwaters are therefore priority actions to safeguard freshwater biodiversity (Tickner et al., 2020). The need to restore ecosystems is recognized in the Kunming-Montreal Global Biodiversity Framework and EU Nature Restoration Regulation (Convention on Biological Diversity, 2022; European Union, 2024).To fully make use of the current momentum for restoration, restorative activities should be based on ecological principles. My PhD focusses on a restorative activity that is increasingly implemented in Europe and will be further promoted under the EU Nature Restoration Regulation: the creation of pond networks.

Case studies have shown that human-made pond networks can host rich plant and invertebrate communities, thereby contributing to regional freshwater biodiversity (e.g. Williams et al., 2008). However, these studies were only performed in some, mainly western European, countries and cannot be generalized to Europe as a whole. Community assembly in human-made ponds may depend on a wide range of variables, including environmental variables at the scale of individual ponds and the spatial distance between ponds. My PhD research investigates environmental and spatial variables that could influence communities of the indicator groups macrophytes and macroinvertebrates in human-made pond networks, with the aim to provide advice on the creation and management of pond networks.

Pond creation projects are usually not monitored, or only monitored based on a limited set of taxa, and the impact of such projects remains therefore largely unknown (De Necker et al., 2025). In general, the monitoring of natural and human-made ponds is rare and national scale surveys have been limited to a few countries such as the UK, France and Switzerland (Biggs et al., 2005; Labat et al., 2022; Oertli et al., 2005b). Knowledge on pond biota is minimal for many parts of Europe, including the Baltic countries. In this PhD project, the indicator groups macrophytes and macroinvertebrates are studied in French, German and Latvian human-made ponds. For the macroinvertebrates, the S_3i macroinvertebrate sampling protocol, which was recently developed in France (Labat et al., 2022) is tested. Furthermore, to start investigating the contribution of human-made ponds to regional freshwater diversity, in this PhD macroinvertebrate communities of human-made ponds and other nearby freshwater habitats are compared.

As detailed above, pond biodiversity and ecological quality assessments are generally based on the indicator groups macrophytes, macroinvertebrates and amphibians. However, conventional monitoring of these groups is time consuming and requires taxonomic expertise. Macroinvertebrate sampling is also invasive. Environmental DNA metabarcoding has been proposed as a non-invasive tool that would allow upscaling of pond monitoring in space and time (Harper et al., 2019; Hill et al., 2021). However, the use of eDNA metabarcoding for the monitoring

of macrophytes and macrophytes in ponds still needs to be developed and validated. To fill in this knowledge gap, this PhD aims to benchmark eDNA metabarcoding of macroorganisms in ponds.

1.5.1. Project EMYS-R

This PhD is part of project EMYS-R, which aims to define "the most effective, socially supported, ecological methods to restore wetlands in favour of the European pond turtle reintroduction, and associated biodiversity throughout Europe." (https://emysr.cnrs.fr/). This European project involves a wide range of academic and non-academic partners from France, Germany, Poland and Latvia. The project consists of five work packages (WP) that each contain multiple tasks (T) (*Fig. 1.17*).

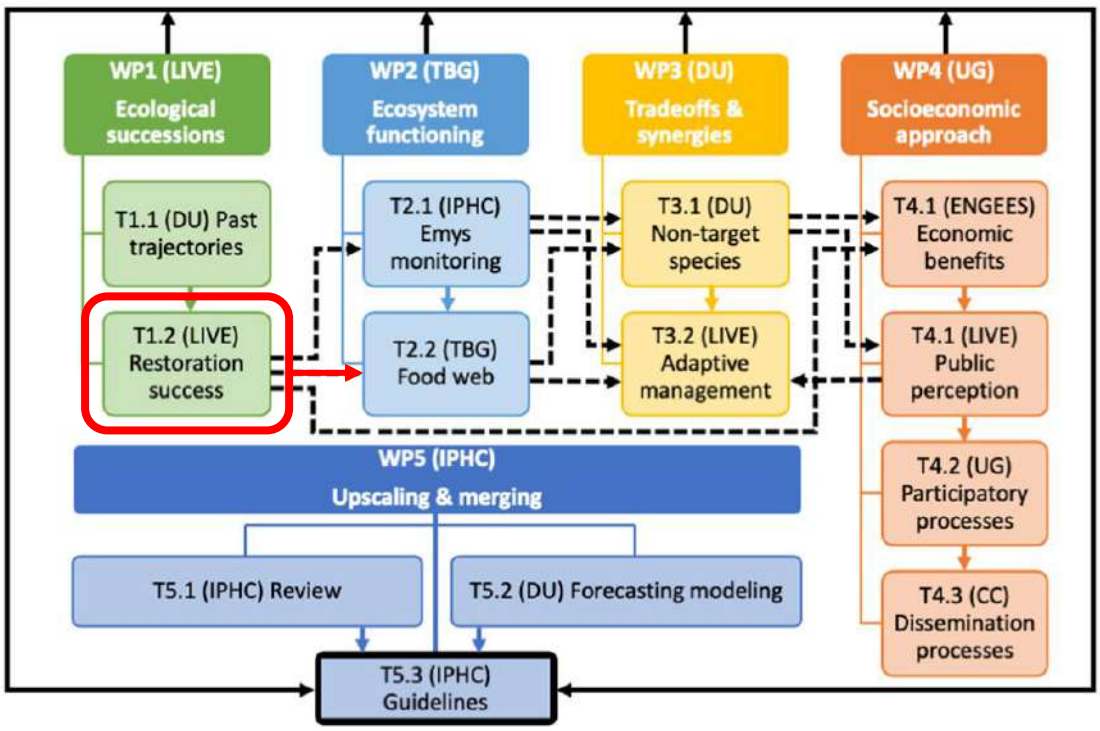


Fig.1.17. Work packages (WP) and tasks (T) of the EMYS-R project with the abbreviations of the partners leading each task between brackets: Daugavpils University (DU), Laboratoire Image, Ville, Environnement (LIVE), Institut Pluridisciplinaire Hubert Curien (IPHC), LOEWE Centre for Translational Biodiversity Genomics (TBG), École Nationale du Génie et de l'Eau et de l'Environnement de Strasbourg (ENGEES), Collegium Civitas (CC), Uniwersytet Gdański (UG). My task in the project is outlined in red.

My PhD is part of work package 1 "ecological successions" and forms task 1.2 "restoration success". The sites studied in this PhD, described in Chapter 2, are also investigated in other work packages. The original goals for task 1.2 were to a) characterise ecosystem dynamics over time and to b) evaluate the success of restorative activities. To characterise the ecosystems over time, despite the constraints of the three-year timeframe of the project, it was planned to compare ponds created at different points in time, but this was not possible because the studied ponds were similarly aged. Furthermore, the assessment of the success of restorative activities has not been finalized yet. As explained above, success can only be evaluated with respect to stated goals and objectives. Objectives of the restorative activities were to provide habitat for reintroduced European pond turtles (*Emys orbicularis* L., 1758) and associated biodiversity. To assess the success of the restorative activities, information is therefore needed on the survival and reproduction of reintroduced *E. orbicularis*, and on the presence of required habitat elements

for this species, including food resources. PhD candidate Johannes Meka of work package 2 is still working on the population monitoring and diet analysis of *E. orbicularis* in the study sites. A full evaluation of the restoration success is therefore not yet possible.

My PhD focusses only on outcomes for the "associated biodiversity", for which I use macrophytes and macroinvertebrates as indicator groups. The data I collected to answer the research questions described in the following section, as well as other eDNA data I collected specifically for this reason (Appendix 5.C) are used in WP2 as inventories of potential food items for *E. orbicularis.*

1.5.2. Dissertation outline

The general objectives of this PhD are to create knowledge on how to best design, monitor, evaluate and manage pond networks for freshwater biodiversity conservation in Europe. The PhD aims to provide answers to the following overarching research questions:

A. How do environmental and spatial variables influence macrophyte and macroinvertebrate communities in human-made pond networks?
B. What monitoring techniques are suitable for human-made pond networks?

In the general methods, **Chapter 2**, the two studied networks of human-made ponds and their history are described. Furthermore, a brief overview of the collected data is given. However, details of the field, laboratory and analysis methods are described in Chapters 3, 4 and 5.

Chapter 3 examines the structuring of macrophyte communities by environmental and spatial factors in the studied pondscapes, and draws lessons for the design and management of created pond networks to most effectively enhance macrophyte diversity. This chapter has been published in Ecological Engineering (Van Der Zon et al., 2026).

In **Chapter 4** the recently developed S_3i macroinvertebrate sampling protocol is used to study macroinvertebrate communities in Baltic ponds, systems for which very little data exist. As we hypothesised that human-made ponds host other macroinvertebrate communities than other types of freshwater habitats, the macroinvertebrate communities are compared to those in nearby large lakes. The influence of macrophytes and environmental variables on the macroinvertebrate communities are examined, from which implications for pond creation and management are derived. Furthermore, pond macroinvertebrate sampling and evaluation methods are discussed.

Chapter 5 describes the application of environmental DNA metabarcoding to study invertebrate, macrophyte, fish and amphibian communities in ponds. The spatial heterogeneity of the captured eDNA is examined and macrophyte communities detected with eDNA metabarcoding and conventional surveys are compared.

Lastly, the general discussion, **Chapter 6**, provides a synthesis of the study results and limitations, advice for pond creation and research prospects.

Chapter 2

Corinne and Marion caught a lot of crayfish in this pond on the Woerr

Chapter 2: General methods

2.1. Study sites

Study sites Neu-Woerr and Silene are located in the catchments of the Rhine and Daugava rivers *(Fig. 2.1*), respectively. Neu-Woerr, on the French German border, is located in the former floodplain of the Rhine and is separated from the river by a dyke. Silene, Latvia, is only located about 160 m above sea level, but is nonetheless in the upland catchment of the Daugava. Concise descriptions of climate and land cover in the study sites can be found in **Chapter 3**. The study sites consist of pond networks that reside partially or entirely within protected nature areas (*Table 2.1*). Neu-Woerr is in part located in the Neuburger Altrhein (Neu), Germany, north of the Old Lauter river, and the Woerr, in France, south of the river (*Fig. 2.2a*). Silene is located within Silene Nature Park and mostly within Ilgas Nature Reserve as well (*Fig. 2.2b*). In the following subsections the history of the study locations and the restoration projects that gave rise to the ponds studied in this thesis are described.

Table 2.1. Protected nature areas in which the study sites (partially) reside. Table constructed with data from Bundesamt für Naturschutz (2023); Conseil Général Bas-Rhin (2015); European Environment Agency (2023); Nature Conservation Agency Republic of Latvia, (2025); Office National des Forêts, (z.d.) and Ramsar (z.d.).

Site	Protected area name	Established	Protected under	Area (ha)
Neu-Woerr	Naturschutzgebiet (NSG) Neuburger Altrhein, südlicher Teil	1983	German “Naturschutzgebiet“ (CDDA 82229)	15
	Naturschutzgebiet (NSG) Neuburger Altrhein, westlicher Teil	1983	German “Naturschutzgebiet“ (CDDA 82230)	18
	Neuburger Althrein	2004	European Natura 2000 area - Birds (DE7015405)	108
	Rheinniederung Neuburg-Wörth	2004	European Natura 2000 area - Habitats (DE6915301)	1148
	Oberrhein	2008	International Ramsar Listed wetland (1809)	25117
	Espace Naturel Sensible (ENS) Woerr	2001	French “Espace Naturel Sensible"	30
	Réserve Biologique Dirigée (RBD) de Lauterbourg	1998	French “Réserve Biologique Dirigée“ (FR2300163)	57
	Vallée du Rhin de Lauterbourg à Strasbourg	2005	European Natura 2000 area - Birds (FR4211811)	8816
	Rhin supérieur	2008	International Ramsar Listed wetland (1810)	22410
Silene	Silene Nature Park	1977	Latvian “Nature Park“, European Natura 2000 area - Birds and Habitats (LV0300400)	3825
	Ilgas Nature Reserve	1999	Latvian “Nature Reserve“	157

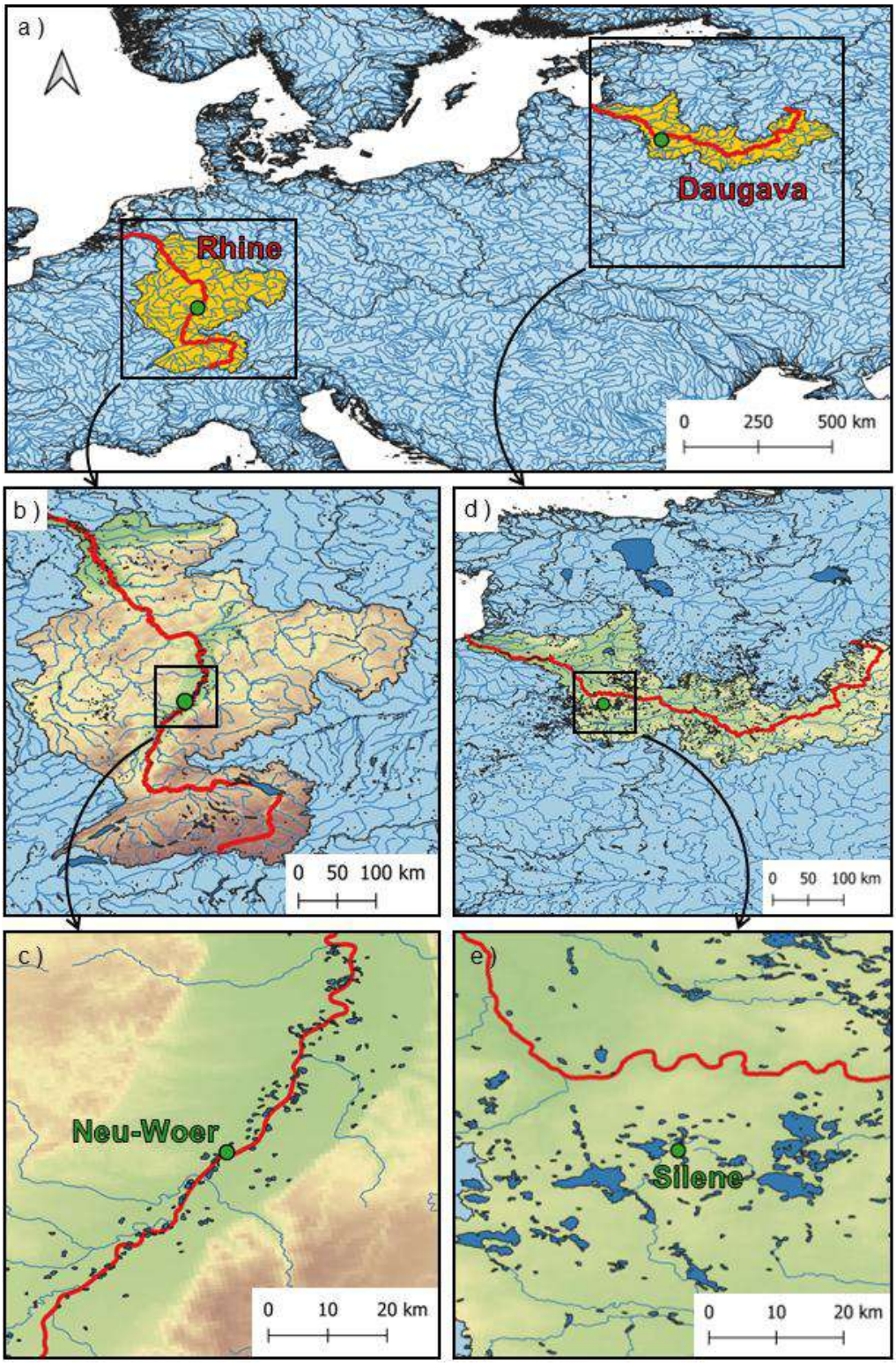


Fig. 2.1. Locations of study sites Neu-Woerr and Silene (green dots) in the catchments of the Rhine and Daugava rivers. The Rhine and Daugava rivers are in red and other rivers in blue. a) Location of the study sites and catchments (yellow). b,c) Location of Neu-Woerr on an elevation map of the Rhine catchment, with tributaries and lakes in blue. d,e) Location of Silene on an elevation map of the Daugava catchment, with tributaries and lakes in blue. Elevation maps range from green (sea level), to brown (mountain) but do not represent actual elevation data. Figure made in QGIS 3.28 with maps from the European Commision Joint Research Centre (2007a); European Commission Joint Research Centre (2007b) and European Environment Agency (2016) in coordinate system EPSG:4326.

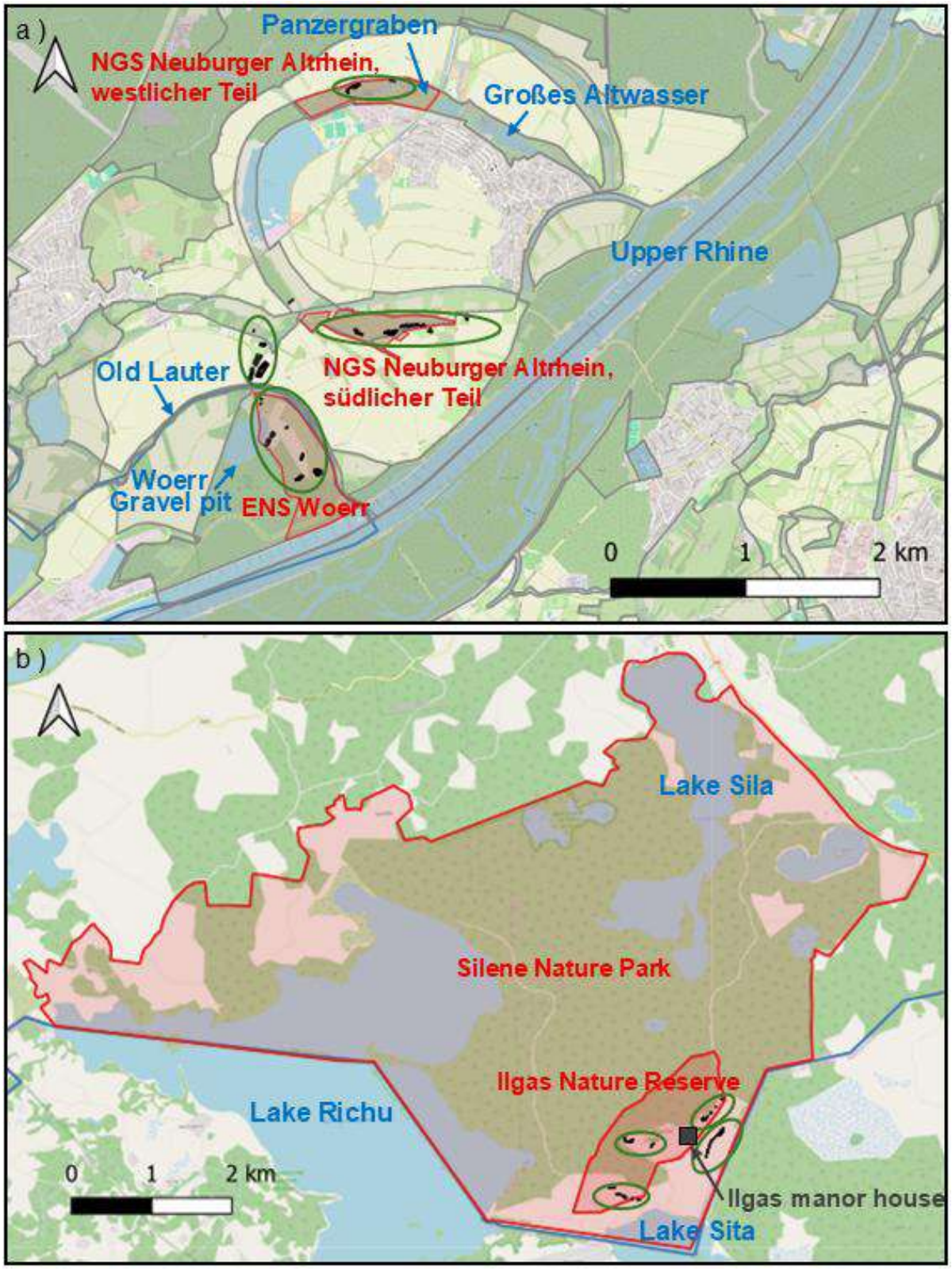


Fig. 2.2. Locations of the studied ponds (in black) on the study sites (green ellipses) with selected protected areas in red. Neu-Woerr with the ENS Woerr and NGS Neuburger Althrein in red. b) Silene within Silene Nature Park and mostly within Ilgas Nature Reserve. Map created in QGIS 3.28 with maps from the European Environment Agency (2022) and OpenStreetMap (z.d.)

2.2.1. History

Knowledge of the history of a site and its degradation are important for effective implementation and evaluation of restorative activities (Higgs, 2003). This applies also when, as is often the case, full restoration of historic conditions is unrealistic or undesirable, for example in light of climate change, species invasions or flood protection (Hallett et al., 2013; Higgs, 2003). It is beyond the scope of this thesis to provide an exhaustive account of the history of the study sites. However, based on information from site management plans and orthophotos that were easily available, I will briefly outline the history of the degradation of the studied sites. Furthermore, I will describe the first establishment of nature protection areas on the sites.

Neu-Woerr

Neu-Woerr is located on the left bank of the Upper Rhine River, which was originally a meandering and braiding river with a floodplain up to four kilometres wide. Already in the 14th century meanders were artificially cut, but the most important engineering works were performed in the 19th and 20th century. River regulation since 1817 completely altered the river's morphology. About 2000 islands disappeared and 100 square kilometres of floodplain area was reclaimed between Basel and Worms (Uehlinger et al., 2009). The section of the Upper Rhine between Strasbourg and Lauterbourg, where the Woerr is located, was regulated between 1907 and 1924 (*Fig. 2.3*, Drapier, 2010). Upstream of Neu-Woerr, not only regulation works, but also the construction of the Grand Canal d'Alsace (1928-1959) and eleven hydropower dams (1928-1974) greatly impacted the Upper Rhine river basin (EDF, 2013; Uehlinger et al., 2009).

Neuburger Althrein is a former meander of the Rhine, which was already naturally cut off from the main river before the regulation works started (perhaps around 1600 or 1800). Some oxbow lakes (Großes Altwasser, Kleines Altwasser) remain. In 1939, anti-tank ditches (Panzergraben) were constructed in the meander, and at this time agricultural drainage ditches were already present. Reeds in Neuburger Altrhein used to be harvested for roof and mat making, a practice that was discontinued. Furthermore, the oxbow lakes used to be weeded as part of fishing activities, which ceased in the 1970's. At the moment, there is no more commercial fishing in the oxbow lakes, and willows are taking over the reedbeds (Höllgärtner, 2010; IUS Kandel, 1998). In order to protect the former Rhine meander with its freshwaters, reeds and marshes, all habitats to rare species, in 1983 two nature reserves were created: NGS Neuburger Althrein, südlicher Teil, and NGS Neuburger Althrein, westlicher Teil (*Fig. 2.2a*).

The Woerr is located in Alsace, which was part of the German Empire from 1871 to 1918, French from 1918 to 1940, German during the second world war, and has been part of France since (Collins, 1998). After the second world war, land in the Woerr area belonging to German owners was confiscated by the French state. Since 1984, France has been in a process of returning this land to the German owners, although for most of the land the owners are unknown (Drapier, 2010). Between the end of the second world war and 2000, corn was cultivated on the Woerr. Furthermore, from the 1960's till 1994, gravel, sand and granulates were extracted (Drapier, 2010; Rainette Grand Est, 2019). Since 1994 the extraction pit has been rehabilitated by the creation of lagunas and softening of the bank slopes (*Fig. 2.4*). In 1998, the Réserve Biologique Dirigée de Lauterbourg was created to protect most of the gravel pit and the forest south of it. Furthermore, in 2001, the Espace Naturel Sensible Woerr was founded in the rest of the gravel pit and the grasslands, reeds and forests west of the Réserve Biologique Dirigée. Since then the grasslands are maintained by mowing once per year (Drapier, 2010).

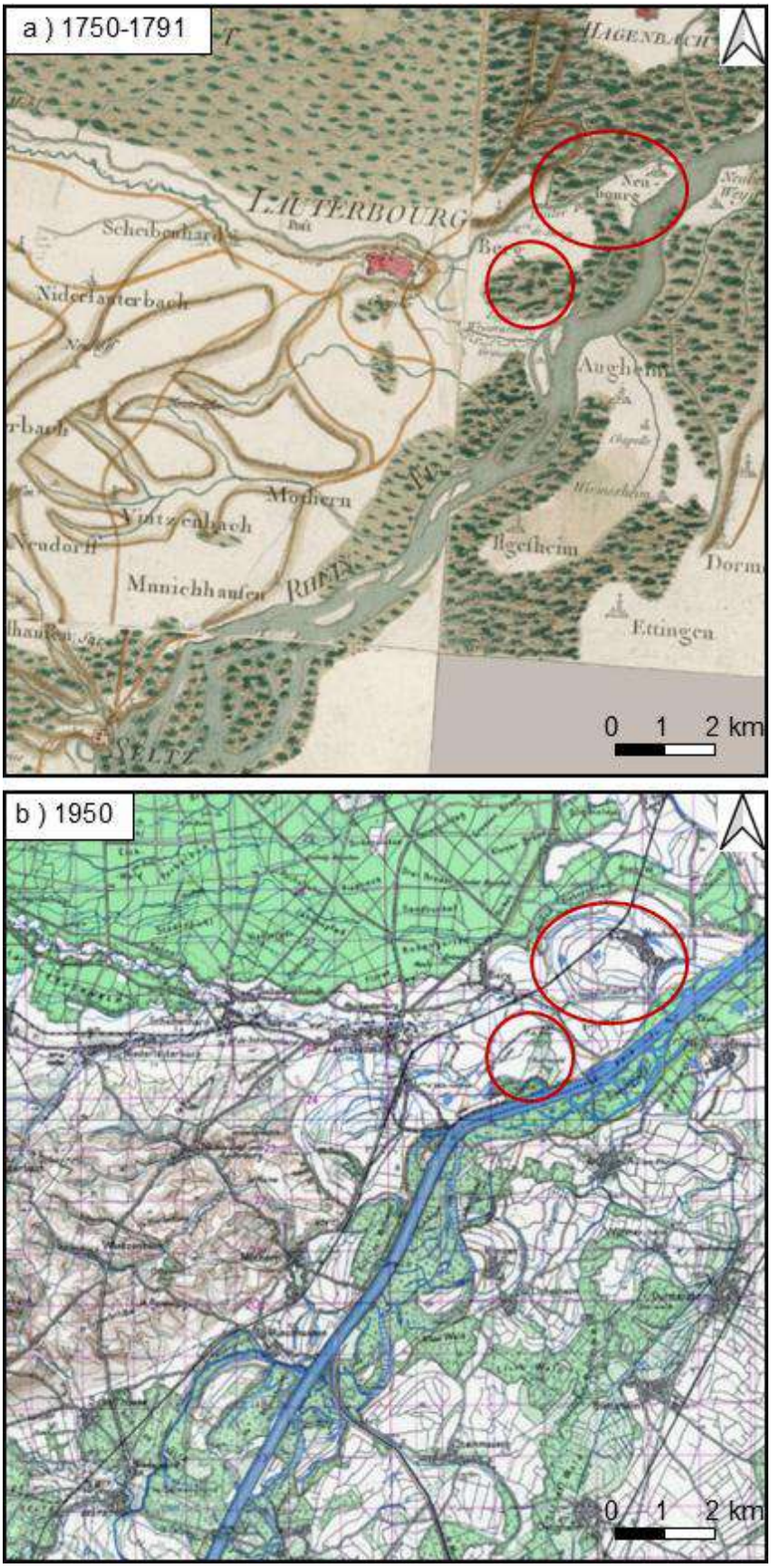


Fig. 2.3. Historic French maps of the Neu-Woerr area (red circles) before and after regularisation. a) 1750-1791 Cassini map. b) 1950 map. Maps retrieved from IGN-France (2025).


a) 1950-1965
Rhine
500 m
b) 2000-2005
Rhine
500 m

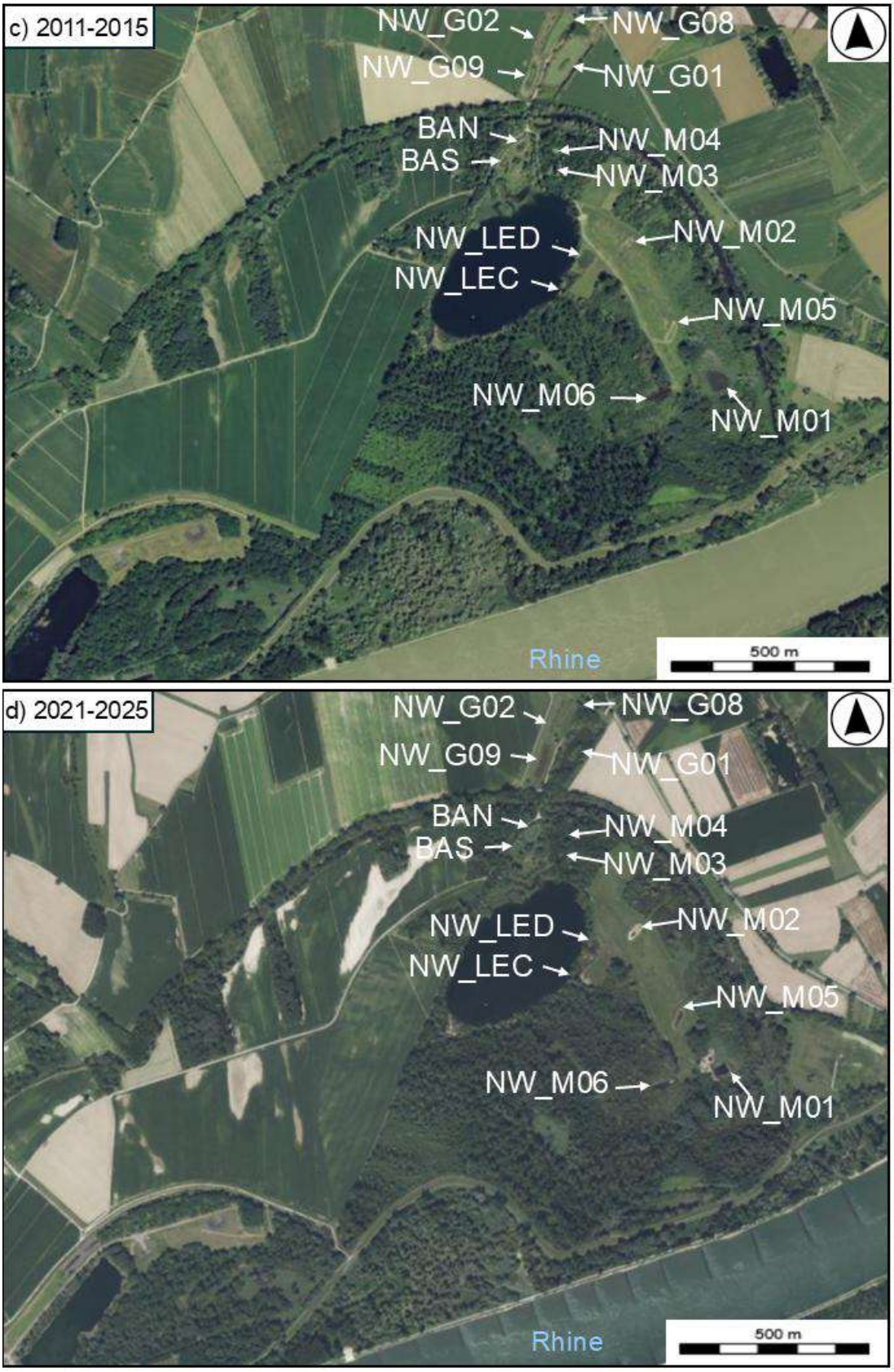


Fig. 2.4. Orthophotos of the Woerr study site bordering the Rhine. In white the lagunas NW_LED and NW_LEC, the acclimatisation basins BAN and BAS, and the created ponds. a) 1950-1965, b) 2000-2005, c) 2011-2015, d) 2021-2025. Ponds created in 2015 are visible on map c, and adaptations to ponds M02 and M01 performed in 2023 and 2024 are visible on map d. Orthophotos retrieved from IGN-France (2025).

Silene

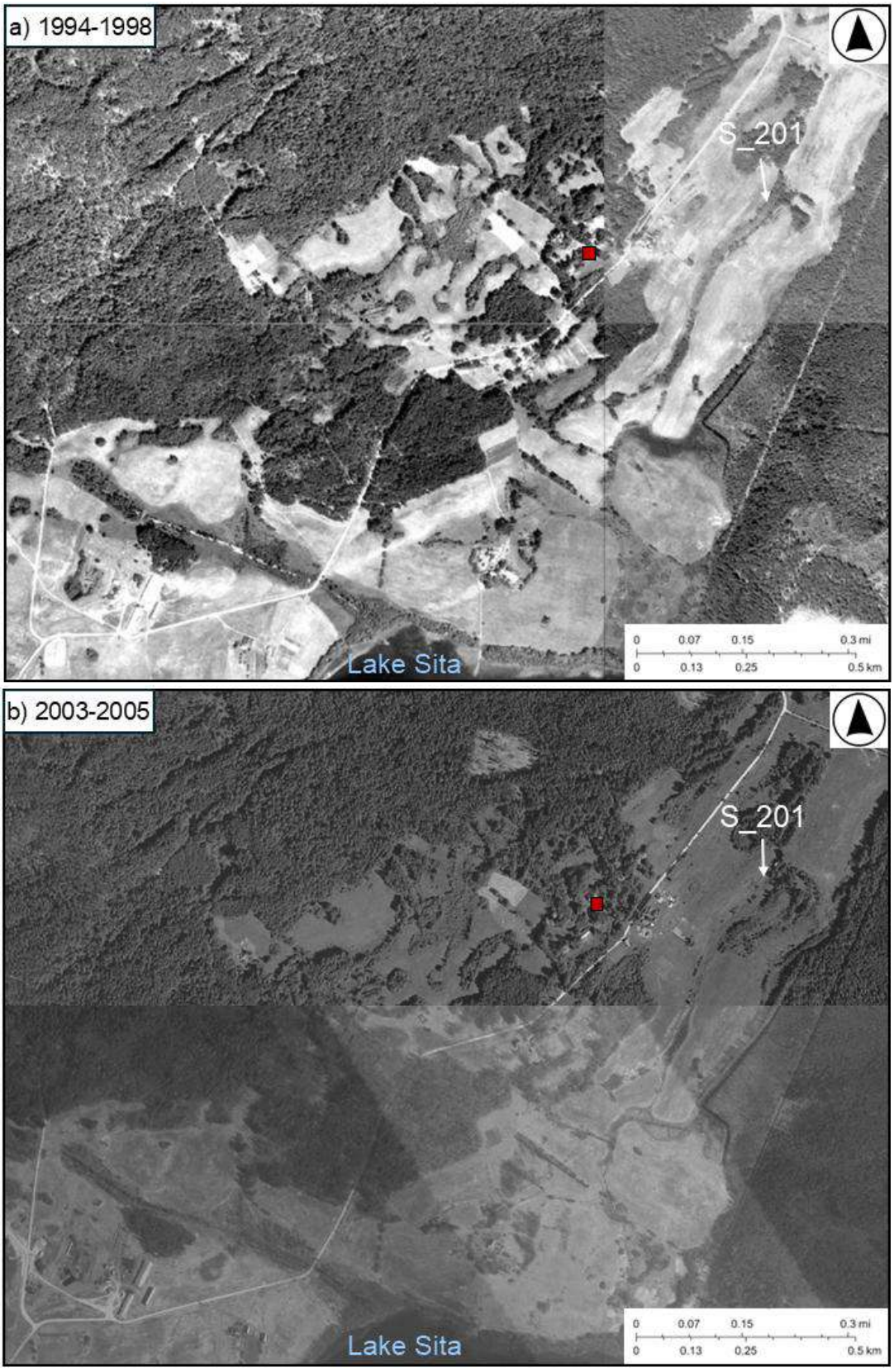
Study site Silene is located in Latvia, which was part of the Soviet Union from 1944 to 1991. The site surrounds Ilgas manor house, a hunting castle built in the 1890's, which has been a study and research centre of Daugavpils University since the 1950's (*Fig. 2.5*). The site lies within Silene Nature Park, which was founded in 1977 to protect valuable lakes, including Lakes Richu and Sita, and bogs. Lakes Richu and Sitas are mesotropic clear water lakes, and their condition is mainly threatened by anthropogenic eutrophication. Eutrophication of the lakes was first observed in the 1930's, and intensified with increased land drainage, agriculture, forestry, housing and recreation in the 1950's. The first drainage channels in the Nature Park were dug in the last quarter of the 19th century. These channels not only contributed to eutrophication of the lakes, but also caused degradation of the bogs and transitional marshes in the area. Since the Nature park was founded in 1977, no more drainage channels were constructed (Estonian, Latvia & Lithuanian Environment, 2007; Vides Konsultāciju Birojs, 2019).

Silene Nature Park also contains EU Habitats Directive forest and grassland habitat types. Forestry, which is still practiced in the park, as well as drainage have impacted the forests. The grasslands were formed by agricultural activity, which is declining in the area. Many of the grasslands are becoming overgrown with woody shrubs. A recent increase in recreation and tourism, which declined after the end of the Soviet Union, may increase anthropogenic pressure on Silene Nature Park. However, the area is sparsely populated. The entire site studied in this PhD thesis is located in the two kilometre wide buffer zone around the Belarusian border that visitors older than 16 years are only allowed to access with a special permit (Estonian, Latvia & Lithuanian Environment, 2007; Vides Konsultāciju Birojs, 2019).

2.2.2. Herpetofauna in need of habitat

The ponds studied in this PhD thesis all result from restoration projects with amphibians as well as the European pond turtle *Emys orbicularis* (L., 1758) as target species. On both study sites declining populations of amphibian species were present. (*Table 2.2*, Höllgärtner, 2010; Pupina & Pupins, 2008). In 2010, on the German and French parts of the Neu-Woerr, separate inventories of EU Habitats Directive habitat types, birds, amphibians, reptiles, grasshoppers and plants, and in the French part also butterflies, dragonflies and spiders, were performed (Drapier, 2010; Höllgärtner, 2010). In the German surveys, among other amphibians, small populations of the common spadefoot *Pelobates fuscus* (Laurenti, 1768) (three reproductive records), the European tree frog *Hyla arborea* (L., 1758) (six reproductive records), and the great crested newt *Triturus cristatus* (Laurenti 1768) (four reproductive records) were detected (Höllgärtner, 2010). These species, also observed in the French part of the site, are listed in Annexes II "Species requiring designation of Special Areas of Conservation" and IV "Species in need of strict protection" of the EU Habitats Directive (European Union, 1992, *Table 2.2*) and in appendix II "Strictly protected fauna species" of the Bern Convention on the Conservation of European Wildlife and Natural Habitats (Council of Europe, 1982).

On the German site, at the time of the 2010 inventory, the breeding habitats required by *P. fuscus*, *H. arborea* and *T. cristatus* were degrading: ponds were drying up, and both ponds and reedbeds were becoming overgrown with willows. Therefore, the creation of ponds was seen as an important measure to avoid disappearance of these, as well as other amphibians and wetland birds, from the area (Höllgärtner, 2010). The ENS Woerr is seen as an important site for the conservation of *P. fuscus* in Alsace, where only a few populations of this toad occur. The creation of ponds on the Woerr was included in the measures for the conservation of *P. fuscus* described in its regional action plan (Michel, 2012).


a) 1994-1998
S_201
Lake Sita
0 0.07 0.15 0.3 mi
0 0.13 0.25 0.5 km
b) 2003-2005
S_201
Lake Sita
0 0.07 0.15 0.3 mi
0 0.13 0.25 0.5 km

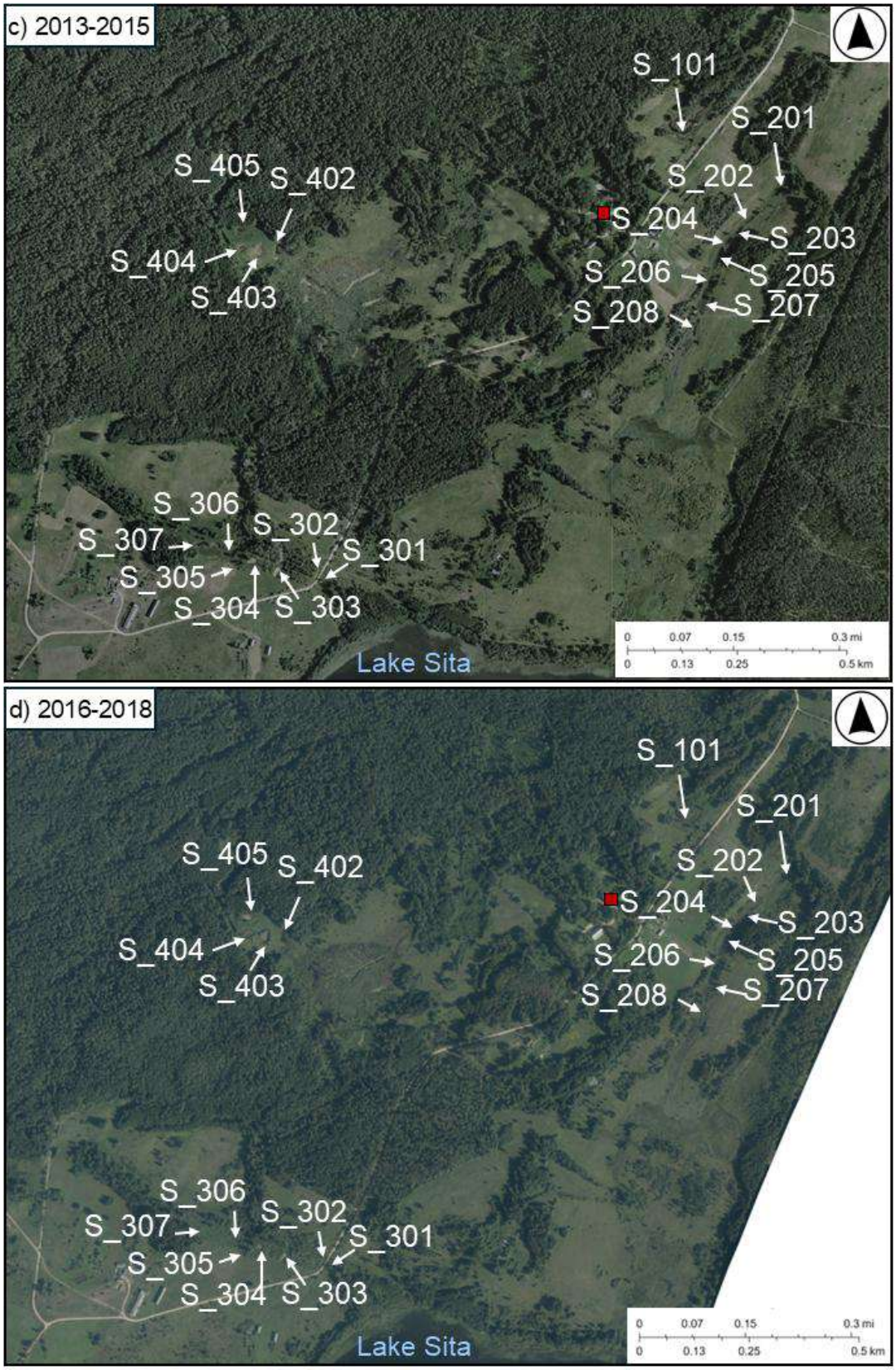


Fig. 2.5. Orthophotos of the Silene study site with lake Sita and Ilgas manor house (red square). In white natural pond S_201 and the created ponds. a) 1994-1998, b) 2003-2005, c) 2013-2015, d) 2016-2018. The ponds created in 2018 are not visible on the map. Orthophotos retrieved from (Latvijas Ģeotelpiskās informācijas aģentūra, z.d.)

Table 2.2. Legal status and occurrence of amphibians present on the study sites according to the EU Habitats Directive (European Union, 1992) and Bern convention (Council of Europe, 1982). Data from Drapier (2010), Höllgärtner (2010), Raintette Grand Est (2019) and SIA "Vides Konsultāciju Birojs" (2019).

	Habitats Directive	**Bern Convention**	**Neuburg 2010**	**Woerr 2010**	**Silene 2013-2018**
Pelobates fuscus	Annex II, IV	Annex II	Small breeding population	Present	Breeding population
Triturus cristatus	Annex II, IV	Annex II	Small breeding population	Present	Breeding population
Bombina bombina	Annex II, IV	Annex II			Reinforced breeding population
Lissotriton vulgaris	Annex II, IV	Annex II	Breeding population	Present	
Epidalea calamita	Annex IV	Annex II	Occasional observation	Observed nearby	
Hyla arborea	Annex IV	Annex II	Small breeding population	Present	
Bufotes viridis	Annex IV	Annex II			Occasional observation
Rana arvalis	Annex IV	Annex II			Breeding population
Rana dalmatina	Annex IV	Annex II	Breeding population	Present	
Pelophylax lessonae	Annex IV, V	Annex III	Occasional observation	Present	Breeding population
Rana temporaria	Annex V	Annex III		Present	Breeding population
Pelophylax kl. esculentus	Annex V	Annex III	Breeding population	Present	Possible
Pelophylax ridibundus	Annex V	Annex III	Breeding population	Present	
Bufo bufo		Annex III	Breeding population	Present	
Lissotriton heleveticus		Annex III	Occasional observation	Present	

In Silene, a population of fire-bellied toads *Bombina bombina* (L., 1761) has been known since the 1970's (Pupina & Pupins, 2008). This species is also listed in Annexes II and IV of the EU Habitats Directive and in Appendix II of the Bern Convention (*Table 2.2*). The *B. bombina* population in Silene was stable until the 1980's but had declined to a few individuals by 2006. At this point in time, ponds where *B. bombina* used to occur had become overgrown with trees and shrubs and had dried up. Furthermore, a pond that was known to host *B. bombina* had become invaded by the invasive fish *Perccottus glenii* (Dybowski, 1877), which was the likely cause of the disappearance of *B. bombina* from this pond (Pupina & Pupins, 2008). In 2006, population management of *B. bombina* started in Ilgas Nature Reserve (part of Silene Nature Park, *Fig. 2.2b*). This included the creation of ponds and population reinforcement with captive-bred individuals (Drews & Meier, 2011; Pupina & Pupins, 2014).

Both study sites are also reintroduction locations for *Emys orbicularis,* a freshwater turtle also listed in Annexes II and IV of the EU Habitats Directive and in Appendix II of the Bern Convention (Meyer et al., 2025; Pupins & Pupina, 2014b). The species has experienced the most severe decline of all reptiles in Europe, mainly due to habitat loss and degradation (Liuzzo et al., 2024; Meyer et al., 2025). *Emys orbicularis* probably disappeared from Alsace in the 19th century (Philippot & Georges, 2023). In current Latvia, records of *E. orbicularis* exist since 1820. However, observations of the species are very rare. In Silene Nature Park, only a few individuals have been observed since the 1960's (Pupins & Pupina, 2014b). Both in the Neu-Woerr and in the Silene study sites, the creation of pond networks was part of the actions performed in light of the *E. orbicularis* reintroductions (Meyer et al., 2025; Pupins & Pupina, 2014a).

2.2.3. Ponds created as part of restorative projects

Except for two ponds, the Neu-Woerr ponds studied in this PhD thesis were created during the EU co-financed INTERREG Upper Rhine IV project "pond turtles without borders" (*Table 2.3*). The remaining two ponds, both located on the Woerr, were created after the INTERREG project by the French Department Council Bas-Rhin to complete pond network initiated by the project. The INTERREG project had the aims to reintroduce *E. orbicularis*, rehabilitate the Woerr gravel pit and improve the conservation status of habitats and species listed in the annexes of the EU Habitats Directive that were present on the site. In this project, *E. orbicularis* was both seen as a flagship species, expected to increase conservation support due to its charisma, and an umbrella species, whose conservation would lead to the conservation of a whole range of other species (European Union, 1992; Höllgärtner, 2010; Levresse, 2012).

Table 2.3. Restoration projects that gave rise to the ponds studied in this PhD (Höllgärtner, 2012; Pupiņš, 2019; Pupins & Pupina, 2014a, 2014b).

Project	Target species	Duration and location	Ponds restored and created	Other restorative actions on study site
INTERREG Upper Rhine IV C12 "Pond turtles without borders"	*Emys orbicularis, Pelobates fuscus, Triturus cristatus, Hyla arborea*, and birds	2009-2014 France and Germany (Neu-Woerr)	18 (all in Neu-Woerr)	Gravel pit bank softening, creation lagunas and acclimatisation basins, creation egg laying sites, release E. orbicularis
LIFE-HerpetoLatvia LIFE09 NAT/LV/000239	*E. orbicularis, Bombina bombina, Coronella austriaca*	2010-2014 Latvia (Silene nature park, Kemeri national park and Demene district)	43 (of which 16 in Silene)	Tree cutting and shrub removal, breeding and release of *E. orbicularis*
LVAFA 1-08/263/2018	*E.orbicularis, B. bombina, Epiladea calamita,*	2018 Latvia (Kateri and Ilgas nature reserve)	10 (of which 7 in Silene)	Shrub removal, creation turtle enclosure

As part of the INTERREG project, 18 ponds were created, of which one is connected to a channel and therefore not studied in this PhD. Each of the ponds created on the German part of the site was explicitly designed for a set of target species. This set included *E. orbicularis* for all ponds, and depending on the pond, several amphibians and birds. The target species were chosen because they had nearby declining remnant populations and were presumed to act as umbrella species (Höllgärtner, 2010, 2012, 2014). Ponds created on the French part of the site were all designed for *E. orbicularis* as well as *P. fuscus* (Rainette Grand Est, 2019). Furthermore, two experimental lagunas were made that border the Woerr gravel pit (*Fig. 2.4c,d*, Höllgärtner, 2014; Rainette Grand Est, 2019). The lagunas are not studied in this thesis because their functioning is influenced by the gravel pit, which is 39 m deep and contains large fish (J.-Y. Georges, pers. com.). The two acclimatisation basins for *E. orbicularis* that were established during the project (Levresse, 2012) are also not studied in this thesis. In contrast to the studied ponds, the basins are lined with an impermeable material and have a pumping system to keep their water level constant. Furthermore, the basins are surrounded by a low metal wall that in the first years of the project kept pond turtles in and calico crayfish out (L. Razafindralay, pers. com.).

In Silene Nature Park, three ponds were already created in 2006 during the LIFE-Bombina project “Management of fire-bellied toad (*Bombina bombina*) populations in the Baltic region” LIFE04NAT/D/000028 (Drews & Meier, 2011). These are not studied in this PhD because they were too difficult to access. The Silene ponds studied in this thesis result from the LIFE-HerpetoLatvia project “Conservation of rare reptiles and amphibians in Latvia” co-funded by the EU, and from the Latvian environmental protection fund (Latvijas vides aizsardzības fonda administrācija, LVAFA) financed project “Implementation of habitat management measures for endangered amphibian and reptile species in nature reserves "Karateri" and "Ilgas" (Pupina & Pupins, 2014; Pupins & Pupina, 2014a, M. Pupins, unpub. data). As part of the LIFE-HerpetoLatvia project, which focussed on *E. orbicularis* in the Silene study site, 16 ponds were created in 2013, in which one year later *E. orbicularis* were released. Conservation measures for other target species were implemented at different locations (Pupins & Pupina, 2014a, 2014b). In Ilgas Nature Reserve (Silene site), the LVAFA 1-08/263/2018 project was implemented for the conservation of the *E. orbicularis* and *B. bombina* populations. In 2018, during this project, seven ponds were excavated, some of which on locations where remnants of ponds were still present (Pupiņš, 2019).

2.2. Surveying and sampling overview

In total 26 ponds were studied in this PhD. These ponds were permanent, created during the projects listed in Table 2.4 and not attached to large (>5 ha) waterbodies. Based on initial field observations, a selection of 13 ponds in Neu-Woerr and 13 ponds in Silene was made with the aim to cover the largest macrophyte diversity possible. Information about these ponds can be found in **Chapter 3,** *Table 3.1*. Macrophytes surveys were performed in all ponds, but invertebrate and environmental DNA (eDNA) sampling was only performed in a subset (*Table 2.4*). During the sampling campaign also other nearby waterbodies were surveyed and sampled with the aim to provide data for other work packages of the EMYS-R project (**Chapter 1**). The methods for the macrophyte surveys are described in **Chapter 3**, for the macroinvertebrate sampling in **Chapter 4** and eDNA sampling in **Chapter 5**. Environmental parameters were also recorded, as described in these chapters.

Table 2.4. Overview surveying and sampling events for macrophytes (MP), macroinvertebrates (MI) and eDNA in the Neu-Woerr (NW) and Silene (S) ponds in the years 2022 and 2023. Data used in Chapters 3, 4 and 5 of this thesis are indicated in yellow, green and pink, respectively.

	Macrophytes		**Macroinvertebrates**		**Environmental DNA**	
Code	**MP 2022**	**MP 2023**	**MI 2022**	**MI 2023**	**eDNA 2022**	**eDNA 2023**
NW_G01	24-06	13-06	29-07	08-06	13-09	20-04, 19-06
NW_G03	24-06	13-06				
NW_G04	22-06	13-06	28-07	08-06		20-04
NW_G05	23-06	13-06				
NW_G06	23-06	13-06	28-07			
NW_G07	22-06	13-06	28-07	07-06	16-09	17-04
NW_G08	22-06	13-06	28-07	08-06		20-04, 19-06
NW_G11	22-06	13-06	28-07	07-06		20-04, 21-06
NW_G14	23-06	14-06	28-07	07-06	14-09	18-04, 22-06
NW_M01	23-06	07-06	29-07	06-06		19-04, 20-06
NW_M04	04-07	08-06		06-06		
NW_M05	06-07	08-06		06-06		21-04, 20-06
NW_M06	07-07	07-06	29-07	06-06		21-04, 20-06
S_101	22-07	10-07	22-07	03-07	22-07	
S_102	22-07	10-07	22-07	04-07		
S_103	22-07	11-07				
S_104	22-07	11-07	22-07	04-07	21-07	
S_202	21-07	10-07	21-07	05-07	22-07	
S_204	24-07	10-07	24-07		21-07	
S_206	24-07	10-07	22-07	05-07	21-07	
S_302	19-07	08-07	19-07	05-07	21-07	
S_303	19-07	08-07	19-07		19-07	
S_308	21-07	08-07	19-07	04-07		
S_309	20-07	11-07				
S_310	20-07	11-07				
S_401	20-07	11-07	20-07	04-07		

Chapter 3

Isabelle actually found many plants other than willows in this Silene pond

Chapter 3: Environmental and spatial processes structuring macrophyte metacommunities in restored pondscapes

This chapter has been published as:

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3.1. Introduction

Wetland biodiversity decline is most importantly caused by habitat degradation and loss (Dudgeon et al., 2006; Kingsford et al., 2016), which can be considerable, as in Europe, where 71% of wetland surface area *(sensu lato)* has been lost since 1900 (Davidson, 2014). To help mitigate biodiversity decline, wetland habitat restoration and creation have been recommended (Verhoeven, 2014). Emerging evidence shows that even the creation of small aquatic habitats can significantly enhance freshwater biodiversity (Williams et al., 2008). However, more research is needed to understand how ponds can contribute to biodiversity decline mitigation (Cuenca-Cambronero et al., 2023; Hill et al., 2021).

The term 'pond' refers to small (1 m^2 to 2-5 ha surface area), shallow (maximum depth of 5 m) standing waters that can be permanent or temporary (with water at for least 4 months per year) and natural or human-made (Biggs & Williams, 2024; Richardson et al., 2022). We reserve the term "restored pond" for ponds where a restorative intervention has been performed on the location of an existing or formerly existing pond, and the terms "man-made" and "created pond" for ponds that were made on locations where no known waterbody existed before (as in Cuenca-Cambronero et al., 2023). It should be noted that these terms are not synonymous with "constructed wetlands", which refers to systems in which macrophytes are planted during construction with the specific aim of water quality improvement (Vymazal, 2005).

Although individual natural, restored and man-made ponds can be exceptionally biodiverse (Biggs et al., 2005; Williams et al., 2004, 2008), their contribution to regional biodiversity should be studied at the scale of the "pondscape" (Hassall et al., 2012), which refers to a network of ponds and their surrounding terrestrial matrix (Boothby, 1997; Hill, et al., 2021). Communities of different ponds within a pondscape are linked by dispersal of individuals and/or propagules, and thus function as a metacommunity (Céréghino et al., 2008; Fehlinger et al., 2023; Leibold et al., 2004). Following the metacommunity perspective (Heino et al., 2021; Leibold et al., 2004; Logue et al., 2011; Winegardner et al., 2012), both environmental processes at the local pond scale and spatial processes between ponds can interact and influence the composition and diversity of local pond communities withing a pondscape.

For effective design of pond networks it is important to understand how local environmental as well as spatial processes structure macrophyte metacommunities. Macrophytes are central to the functioning of pondscapes as they enhance water transparency, cycle nutrients, regulate sediment dynamics, and provide shelter, food and breeding habitat for other organism groups such as invertebrates, herpetofauna and birds (Capers et al., 2010; Carpenter & Lodge, 1986; Chambers et al., 2008). Locally, abiotic environmental filters (*sensu* Keddy, 1992), such as the availability of light, nutrients and bicarbonate, are known to structure macrophyte communities (Bornette & Puijalon, 2011; Lacoul & Freedman, 2006; Scheffer & Van Nes, 2007). They are therefore expected to influence the community composition, diversity and life forms of

macrophytes in man-made ponds. Local biotic factors could also shape pond macrophyte communities by acting as filters, or by influencing abiotic filters. For example, algal growth or sediment resuspension by animals can increase water turbidity (Bakker et al., 2013). Depending on the species and abundance, crayfish can be a major biotic factor reducing macrophyte biomass and altering community composition, both by grazing and by resuspending sediment (Herrmann et al., 2022; Nyström et al., 1996; Twardochleb et al., 2013). Spatially, both the degree of isolation and the distance between ponds could influence macrophyte communities through dispersal limitation. Dispersal of sexual and vegetative macrophyte propagules was formerly assumed to be efficient, especially by water birds, but recent studies reported evidence suggesting that macrophyte dispersal can be limited, meaning that species present in the metacommunity do not reach suitable habitat patches often enough to establish a population, which influences macrophyte community composition and diversity (Capers et al., 2010; Gledhill et al., 2008; Lozada-Gobilard et al., 2019).

Despite the importance of macrophytes in pond network creation and restoration, for example to contribute to the mitigation of freshwater biodiversity decline, still few studies examined the processes shaping macrophyte communities in networks of ponds made for conservation purposes (Fleury & Strehler Perrin, 2004; Williams et al., 2008). Moreover, spatial factors that could influence the macrophyte metacommunities are seldom taken into account. To address this knowledge gap, we studied both environmental and spatial factors that could influence macrophyte metacommunities in two networks of permanent man-made ponds. Our study aimed to answer the following questions: 1*) How do abiotic and biotic conditions of man-made permanent ponds relate to macrophyte community composition, and to structural and functional macrophyte community metrics? 2) How does the land cover surrounding man-made permanent ponds relate to environmental variables in the ponds? 3) How do distances between man-made permanent ponds in a pond network, and differences in environmental conditions between the ponds, influence the dissimilarity of their macrophyte communities?*

We performed the same analyses for a western (France-Germany) and a northeastern (Latvia) European pondscape, to see if the responses to the research questions were similar for the two sites. To assess the influence of spatial factors, we included the distance to the nearest water body in the assessment of the correlation between environmental variables and macrophyte community composition and metrics. We also examined the influence of spatial distance, as well as differences in environmental conditions, on the macrophyte community dissimilarity between ponds. Accordingly, our study applies a metacommunity perspective to man-made pond networks and investigates factors known to shape macrophyte communities in this novel context, with the aim of informing pondscape design for freshwater biodiversity conservation.

3.2. Methods

3.2.1. Study sites

The two studied pond networks were made in already existing protected nature areas, with the aim to restore habitat for the European pond turtle *Emys orbicularis* (L., 1758), amphibians and other wetland dependent species. The ponds were colonised naturally and had not been planted. Study site Neu-Woerr is located in the continental biogeographical region (European Environment Agency, 2016) (*Fig. 3.1a*). The climate is semi-continental with an annual average (1981 – 2010) air temperature of 10.6 °C (1.8 °C in January, 19.4 °C in July) and precipitation of 883 mm (Météo-France, 2022). Potential evapotranspiration in the region can exceed annual precipitation (Météo-France, 2024).

Study site Neu-Woerr includes protected areas Naturschutzgebiet (NSG) Neuburger Altrhein in Germany, and Espace Naturel Sensible (ENS) Woerr in France (European Environment Agency,

2022) (*Fig. 3.1b,c*). The site is located in the floodplain of the Upper Rhine, which has been disconnected from the river by channelization works that started in the mid-19th century (Staentzel et al., 2018; Wantzen et al., 2022). The protected areas contain former agricultural fields, a former extraction pit, an anti-tank ditch from the second world war, oxbow lakes, woods and reedbeds that used to be harvested but are now becoming overgrown with willows. The site is bordered by corn fields and a gravel pit. Between 2011 and 2015, 19 ponds and three shallow extensions of larger water bodies were made in and adjacent to the protected areas. The ponds are connected to the groundwater of the Upper Rhine aquifer and can show large water level fluctuations (> 1 m, L. Razafindralay, unpub. data). Parts of the area get flooded when the water level in the Rhine is high. Since 2013 the invasive calico crayfish *Faxonius immunis* (Hagen, 1870), which can drastically reduce macrophyte cover in ponds (Herrmann et al., 2022), is present in Neu-Woerr.

Study site Silene is boreal (*Fig. 3.1a*) and has a semi-continental climate with an average (1991-2020) annual air temperature of 6.6 °C (-4.1 °C in January and 18.1 °C in July) and average precipitation of 635 mm per year (Latvijas Vides ģeoloģijas un meteoroloģijas centrs, 2022). After snowmelt in spring, many pools form which dry before summer (M. Pupiņš, pers. obs.). Silene is located within Silene Nature Park, southeast Latvia on the border with Belarus (*Fig. 3.1d,e*). The park contains lakes and bogs, and is dominated by coniferous and broad-leaved forests. In the past, wetlands have been drained for agricultural purposes and several farm ponds were dug. However, since the 1990's the region experiences agricultural decline, and meadows are becoming overgrown with woody plants (Vides Konsultāciju Birojs, 2019). In Silene, in total 26 ponds were made in 2006, 2013 and 2018, mainly in drained wetlands with former agricultural use, with some of them on locations of former farm ponds (Pupins et al., 2023). Crayfish are not known to occur in the Silene ponds.

In both study sites 13 man-made ponds that are permanent or only dry up in exceptionally dry years were selected (*Table 3.1*, Appendix 3.A). The ponds were chosen for our study to represent the widest range of pond macrophyte diversity in each site. In both sites, pond bases were generally sandy or silty, mixed with gravel for some Neu-Woerr ponds. A few Silene ponds had thick layers of oozy sediment. In a minority of studied ponds fish were observed: *Lepomis gibbosus* (L., 1758*), Proterorhinus semilunaris* (Heckel, 1837) (cf. Manné et al., 2013) and small cyprinidae in Neu-Woerr and *Percottus glennii* (Dybowski, 1877) (cf. Pupina et al., 2018) and *Tinca tinca* (L., 1758) in Silene.

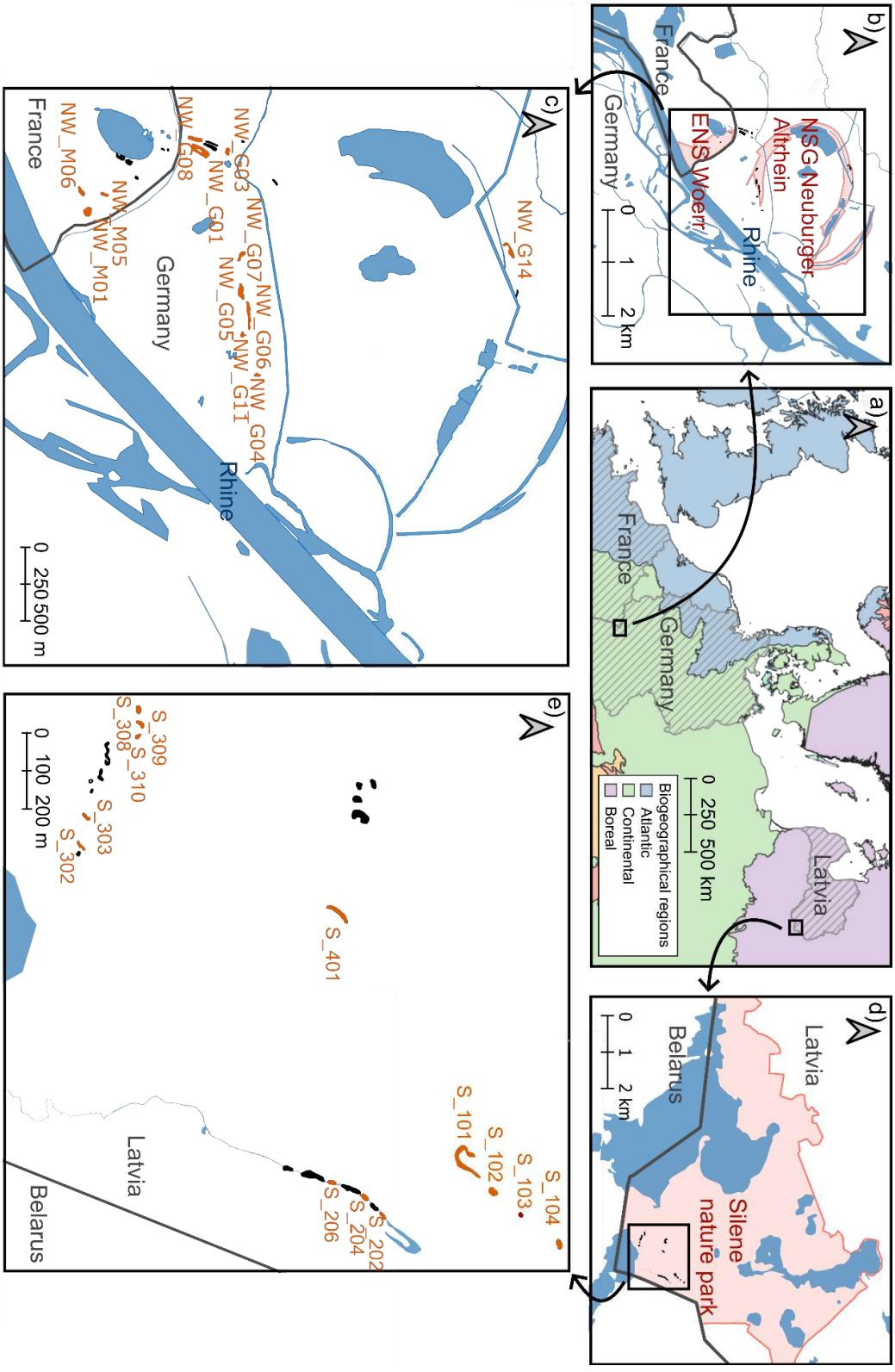


Fig. 3.1. a) Site locations within the continental (green) and boreal (violet) biogeographical regions of Europe, and within France, Germany and Latvia (hatched grey). b) Neu-Woerr and its connected protected areas Naturschutzgebiet (NSG) Neuburger Altrhein and Espace Naturel Sensible (ENS) Woerr (red). c) Studied Neu-Woerr ponds (orange). d) Silene nature park (red). e) Studied Silene ponds (orange). b-e) Borders are represented with black lines and waterbodies *with dark blue polygons. Maps were made in QGIS 3.28 with data from the European Environment Agency (2016; 2022).*

Table 3.1. Characteristics of studied Neu-Woerr (NW) and Silene (S) ponds. Codes refer to Fig. 3.1. Area is the surface area at the high water level.

Code	Latitude	Longitude	Area (m^2)	Year made	Original land occupation pond location
NW_G01	48.97708°	8.22260°	4640	2011/2012	Former gravel sieving platform
NW_G03	48.97874°	8.22285°	1010	2011/2012	Former gravel sieving platform
NW_G04	48.97964°	8.24374°	540	2011/2012	Location of former pond in reedbed and willow stand
NW_G05	48.97899°	8.23593°	2600	2011/2012	Willow and reed stands
NW_G06	48.97944°	8.23887°	2400	2011/2012	Willow and reed stands
NW_G07	48.97916°	8.23256°	1290	2011/2012	Willow and reed stands
NW_G08	48.97681°	8.22148°	2020	2013	Former gravel extraction platform
NW_G11	48.97892°	8.23992°	610	2013	Meadow on former agricultural field
NW_G14	48.99556°	8.23376°	2040	2013	Reedbed
NW_M01	48.96997°	8.22756°	2400	2011/2012	Reedbed
NW_M04	48.97511°	8.22232°	120	2011/2012	Forest
NW_M05	48.97106°	8.22607°	830	2015	Meadow on former corn field
NW_M06	48.96966°	8.22567°	1300	2015	Reedbed
S_101	55.69296°	26.78692°	1050	2013	Overgrown natural wetland
S_102	55.69361°	26.78832°	290	2018	Overgrown natural wetland
S_103	55.69425°	26.78933°	70	2018	Overgrown natural wetland
S_104	55.69513°	26.79063°	220	2018	Overgrown natural wetland with channel
S_202	55.69099°	26.78913°	110	2013	Wetland drainage channel
S_204	55.69058°	26.78833°	180	2013	Wetland drainage channel
S_206	55.68982°	26.78766°	150	2013	Wetland drainage channel
S_302	55.68420°	26.77315°	110	2013	Wetland drainage channel
S_303	55.68435°	26.77194°	130	2013	Wetland drainage channel
S_308	55.68569°	26.76755°	180	2018	Fallow meadow
S_309	55.68573°	26.76815°	170	2018	Fallow meadow
S_310	55.68565°	26.76867°	90	2018	Fallow meadow
S_401	55.69023°	26.77639°	530	2018	Natural wetland

3.2.2. Macrophyte surveys and metric calculations

Macrophyte surveys were performed in 2023, in June for Neu-Woerr and early July for Silene. In 2023, none of the studied ponds were flooded or dried up. Macrophyte surveys consisted in identifying all helophytes and hydrophytes growing in the usually wet area of the ponds. Bryophytes occurred at very low cover in some ponds and were not taken into account in the analyses. Taxonomy of the United States National Center for Biotechnology Information (NCBI) was used. For each pond, a map was drawn dividing the pond in sections with relatively homogeneous vegetation. The proportion of the surface area of the pond covered by each section was estimated, and within each section, the percentage cover of each macrophyte taxon was estimated visually. Based on this, the percentage cover of each taxon per pond was calculated.

The macrophyte percentage cover values were used to calculate multiple taxonomic and functional metrics (*Table 3.2*). Species richness is a focal metric in community ecology (Fleishman et al., 2006), but other metrics may capture different community responses to environmental variables (Beisel et al., 1998; García-Girón et al., 2019; Heino et al., 2005, 2007; Manzo et al.,

2020). Therefore, we used multiple metrics to describe the macrophyte communities. As diversity metric we chose Hill Shannon diversity, which is the Shannon entropy expressed as effective number of species (Jost, 2006). For evenness we used "Simpson evenness", the index that is obtained by dividing the reciprocal of the Simpson concentration index by the number of taxa. Simpson evenness has the advantage over Pielou's evenness that it is independent of taxonomic richness, also for low taxonomic richness (Smith & Wilson, 1996). We calculated Warwick and Clarke's (1995) taxonomic distinctness, which is the average path length through a taxonomic tree connecting two randomly chosen individuals from different taxa. The path length weights given to two taxa belonging to the same genus, family, order and class, were respectively 20, 40, 60 and 80.

As functional metrics we used the total macrophyte cover and the relative cover of anchored emergent, anchored floating leaved, anchored submerged and free-floating life forms. Life form data was retrieved from books (Muller et al., 2021; Schou et al., 2023) and the Ecological Flora Database (Fitter & Peat, 1994) using the R package TR8 (Bocci, 2015). Species could be assigned to multiple life forms (Appendix 3.B).

Table 3.2. Macrophyte metrics, with T the total number of taxa, p_i the relative cover of taxon i, w_{ij} the path length weight between taxa i and j, x_i the cover of taxon i, x_{em} the sum of the covers of emergent taxa, x_{fl} of anchored floating taxa, x_{su} of anchored submerged taxa, and x_{fr} of free-floating taxa.

Metric	Abbreviation	Formula	Reference
Taxonomic richness	Rich	Number of taxa	
Hill Shannon diversity	Shan	$e^{-\sum_{i=1}^{T} p_i ln(p_i)}$	Jost, 2006
Simpson evenness	Even	$\frac{1}{T \sum_{i=1}^{T} {p_i}^2}$	Smith & Wilson, 1996
Taxonomic distinctness	Dstar	$\frac{\sum\sum_{i<j} w_{ij} x_i x_j}{\sum\sum_{i<j} x_i x_j}$	Warwick & Clarke, 1995
Total cover	Cove	Total % of pond surface covered by macrophytes	
Relative cover emergent macrophytes	Emer	$\frac{x_{em}}{x_{em} + x_{fl} + x_{su} + x_{fr}}$	
Relative cover anchored floating macrophytes	Floa	$\frac{x_{fl}}{x_{em} + x_{fl} + x_{su} + x_{fr}}$	
Relative cover anchored submerged macrophytes	Subm	$\frac{x_{su}}{x_{em} + x_{fl} + x_{su} + x_{fr}}$	
Relative cover free-floating macrophytes	Free	$\frac{x_{fr}}{x_{em} + x_{fl} + x_{su} + x_{fr}}$	

3.2.3. Environmental variable assessment

Assessment of local abiotic and biotic variables and estimation of land cover in the 5 m buffer around ponds was performed in the same time period as the macrophyte surveys. The percentage of the pond surface area that would be shaded at midday, as well as the contribution of reeds, willows and shrubs, high trees, wetlands and meadows to the land cover of the 5 m buffer around the pond were estimated. Water depth was measured at the deepest point in the pond and transparency was determined using a turbidity tube. To measure specific conductivity and pH, a

WTW MultiLine 3630 IDS sonde was used in Neu-Woerr and an OTT Hydrolab DS5 sonde in Silene. For Silene, the portable sonde was used to measure chlorophyll-a concentrations, while for Neu-Woerr chlorophyll-a concentrations were measured in the laboratory using spectrophotometry following AFNOR standard NF T 90-117. Pond isolation, measured as the distance to the nearest waterbody, as well as spatial coordinates and the pond surface area at the high water level, were derived from digitized features based on Google Satellite maps in QGIS 3.28 using projections EPSG:2154 and EPSG:3059.

To estimate the abundance of calico crayfish in each Neu-Woerr pond, four littoral sweep net samples were taken in different mesohabitats (*sensu* Labat et al., 2022) and spread evenly along the pond margin. Samples covering 1 m^2 were taken by moving a 0.5 mm mesh size net with a rectangular 20 x 30 cm frame four times vigorously back and forth above the sediment with an amplitude of 1 m. Captured crayfish were identified and measured from rostrum to telson.

3.2.4. Coinertia analyses

We performed coinertia analysis (CoIA) to investigate correlation between a) environmental variables and macrophyte metrics, b) environmental variables and macrophyte taxon abundance and c) land cover around ponds and environmental variables. Because of the small number of ponds sampled per network, it was not possible to perform redundancy analysis or canonical correspondence analysis. Instead, we chose CoIA, which can be used for datasets of many variables measured on few sites (Dolédec & Chessel, 1994; Dray et al., 2003). CoIA is most commonly used to relate environmental variables to community composition, but it is a flexible method that can relate any two datasets measured on the same entities (Borcard et al., 2011). It has been used to relate environmental variables to community metrics (Beisel et al., 1998).

CoIA requires appropriate initial ordinations of the datasets to be analysed (Borcard et al., 2011). We chose Principal Component Analysis (PCA) on the correlation matrix for the environmental variables and the metrics datasets. For the macrophyte abundance dataset, a transformation based PCA (Legendre & Gallagher, 2001) was performed. To this end, a Hellinger transformation of the percentage cover data was followed by covariance matrix PCA. This is equal to a Principal Coordinates Analysis of the Hellinger distance matrix (Legendre & De Cáceres, 2013). For each CoIA, an RV-coefficient, measuring the strength of the link between the two datasets on a scale from 0 to 1, was calculated (Robert & Escoufier, 1976), and its significance was evaluated using a Monte-Carlo test with 999 permutations. All CoIA's were performed for Neu-Woerr and Silene separately, because the two sites differed in terms of environmental variables (Appendix 3.A), macrophyte communities (Appendix 3.B), and invasive crayfish occurrence.

3.2.5. Community dissimilarity

To examine how geographic distance (G_{dis}) influences macrophyte community dissimilarity (C_{dis}) between ponds, we used non-linear regression as a first approach, and generalized dissimilarity modelling as a second approach. Generalized dissimilarity modelling allows the inclusion of differences between environmental conditions, besides geographic distance, as predictor variables (Ferrier et al., 2007). For both approaches we modelled the Bray-Curtis dissimilarity calculated on the log(x+1) transformed percentage cover data (Borcard et al., 2011), but for the non-linear regression we expressed it as similarity ($C_{sim} = 1 - C_{dis}$). For the non-linear regression we fitted power functions of the form $C_{sim} = a \cdot G_{dis}^{-b}$, also referred to as log-log models (Nekola & McGill, 2014; Nekola & White, 1999; Soininen et al., 2007), to data from all pond pairs. We also tried exponential (log-linear) and linear models, but these fitted the data les well, as indicated by higher AIC values. From the power-law model, an initial C_{sim} at 10 m distance (C_{sim}_init) was calculated, as well as the halving distance (G_{dis}_half) at which C_{sim} had decayed to half the C_{sim}_init, using G_{dis}_half $= 10 \cdot 2^{1/b}$. In order to investigate whether there was still decay of C_{sim} with

G_{dis} for pond pairs separated by more than G_{dis}_half, a power function for the decay of C_{sim} with G_{dis} was also fitted on a reduced dataset with only pond pairs separated by more than G_{dis}_half.

We fitted generalized dissimilarity models (GDM) to identify both the contribution of G_{dis} and of environmental dissimilarities between ponds to C_{dis}. Generalized dissimilarity modelling is a matrix regression approach based on generalized linear modelling that accommodates for non-linearities in relationships between the predictor variables (G_{dis} and differences in environmental conditions) and C_{dis} (Ferrier et al., 2007). Following Mokany et al. (2022), we used three I-splines per predictor variable to model the Bray-Curtis macrophyte dissimilarity. The predictor variables assessed were G_{dis}, as well as differences between pond pairs in the percentage of the pond surface shaded by surrounding vegetation at midday, water depth, transparency, specific conductivity, pH, chlorophyll-a concentration, distance to the nearest waterbody, pond surface area, and for Neu-Woerr crayfish abundance. The significance of each predictor variable was tested with 999 permutations, and a backward selection procedure was used to keep only predictors with a p-value lower than 0.05. To check for correlation between the significant predictors, differences between pond pairs, in Euclidean geographic distance and in each environmental variable, were calculated, and Spearman correlation coefficients were evaluated. Furthermore, the importance of each of the remaining predictors for the predicted macrophyte community dissimilarity was evaluated as the sum of the three predicted I-spline coefficients (Mokany et al., 2022).

Data analysis and visualisation were performed in R version 4.4.0 with R studio 2023.06.0 using packages HillR, vegan, taxize, ade4, adegraphics, adespatial, gdm and the tidyverse.

3.3. Results

3.3.1. Macrophyte communities

In total, 81 macrophyte taxa were identified to the species level, of which 35 were detected in Neu-Woerr and 63 in Silene (Appendix 3.B). Some *Carex* had neither flowers nor fruits at the time of sampling and were only identified to genus level. In Neu-Woerr, the most common species were *Phragmites australis*, *Veronica anagallis-aquatica* and *Juncus acutiflorus*, observed in respectively 100, 85 and 69% of the ponds. In Silene, the most common species *Typha latifolia*, *Alisma plantago-aquatica*, *Carex acutiformis* and *Lemna minor*, were each recorded in 85% of the ponds. In terms of conservation status, the species *Hippuris vulgaris*, observed in Neu-Woerr, is classified as Near Threatened on the French Red List, but as Least Concern on both the European and global Red Lists. *Sparganium natans*, occurring in Silene, is Near Threatened following the European Red List, and of Least Concern according to the global Red List (Bilz et al., 2011; Lansdown, 2014; UICN France et al., 2018). All vascular plant species were native to the location they were observed, except for *Acorus calamus*, found in Silene, which was introduced into Europe in the 16th century (Dykyjová, 1980; Royal Botanic Gardens, 2017; Schou et al., 2023).

Per pond, taxonomic richness (5 to 17 in Neu-Woerr, and 11 to 30 in Silene) and Hill Shannon diversity were lower in Neu-Woerr than in Silene (Appendix 3.B). Furthermore, total macrophyte cover was on average 49% in Neu-Woerr and 81% in Silene (*Fig. 3.2*, Appendix 3.B). Whereas Neu-Woerr ponds had almost no floating anchored and few free-floating plants, and their submerged vegetation consisted primarily of charophytes, the relative cover of different life forms in the Silene ponds varied among ponds.

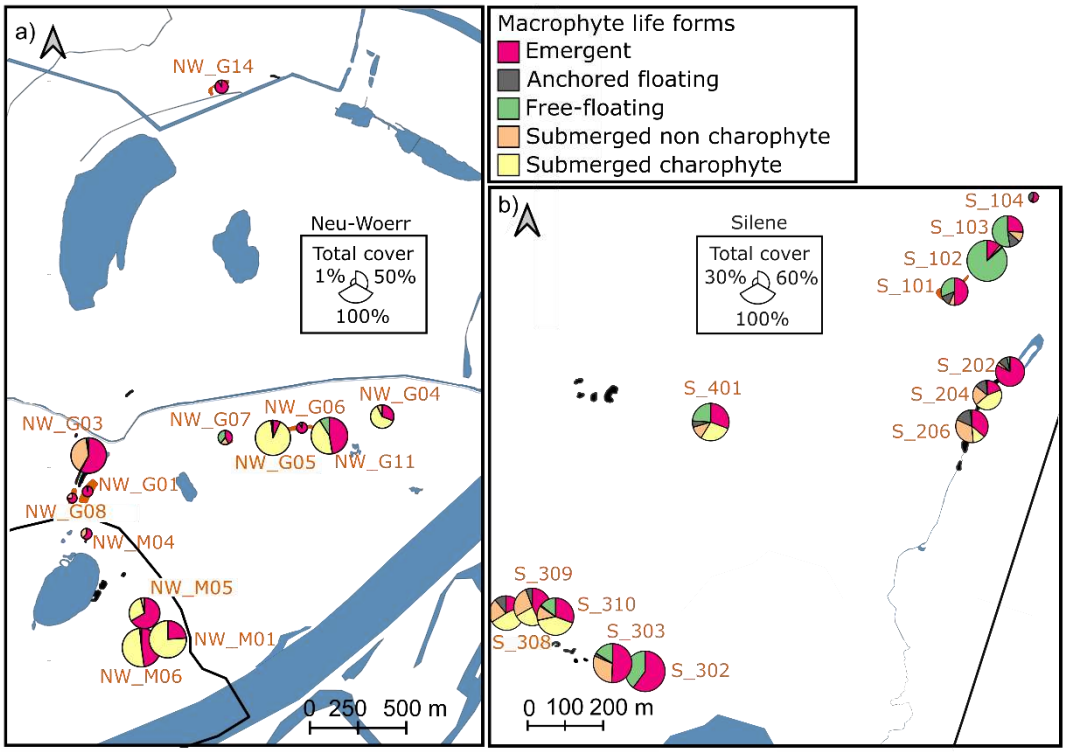


Fig 3.2. Pond locations and pie charts showing the contributions of emergent, anchored floating, free-floating, submerged charophyte and submerged non-charophyte life forms to the total macrophyte cover of the ponds in a) Neu-Woerr and b) Silene. The radius of the pie chart indicates the total cover of macrophytes with the radius scales in the insert. Note that to improve readability different scales were used for the two sites.

3.3.2. Coinertia macrophytes, environmental variables and land cover

For both sites, CoIA showed significant correlations between local environmental variables and the macrophyte metrics (*Fig. 3.3a,d*), with a major contribution of light-influencing variables to the first coinertia axis (*Fig. 3.3b,e*). For Neu-Woerr, ponds with transparent water (Trans) were on the right side of the coinertia plane, whereas those with reduced light conditions, namely those shaded by surrounding trees (Shade) and those exhibiting relatively high chlorophyll-a concentrations (Chl-a), were on the left side of plane. Ponds with high crayfish abundance (Cray) were also on the left side of the plane. For Silene, ponds with reduced light conditions were on the right side, and those with relatively high pH and great depth on the left side of the plane.

For Neu-Woerr, the ponds with reduced light conditions and high crayfish abundance, on the left side on the coinertia plane, exhibited high relative cover of emergent species (Emer) (*Fig. 3.3c*). Ponds with enhanced light conditions, on the right side of the coinertia plane, were characterised by high relative cover of submerged plants (Subm), total cover (Cove) and taxonomic distinctness (Dstar). In Silene, ponds with reduced light conditions, on the right side of the coinertia plane, had high relative cover of free-floating macrophytes (Free) (*Fig. 3.3f*), while those with improved light conditions, as well as high pH and greater depth, where characterised by a high relative cover of submerged plants.

For both sites, surface area (Area) and distance to the nearest waterbody (DistNWb) had low contributions to the coinertia between the environmental variables and macrophyte metrics. For Neu-Woerr, pond age (Age) did not contribute to the coinertia and for Silene pond age had a small contribution to the first axis and a more important contribution to the second coinertia axis, but this axis only projected 20% of the inertia. Specific conductivity (SpCond) also had a small

contribution to the first axis for Silene, and contributed to the second axis for Neu-Woerr, but this axis only projected 19% of the inertia. Taxonomic richness (Rich) contributed to the second axis in Neu-Woerr and only showed moderate contribution to the coinertia in Silene. Spearman rank correlations between taxonomic richness and the environmental variables studied were all lower than |0.6| and had p-values (Benjamini-Hochberg adjusted for multiple testing) higher than 0.05.

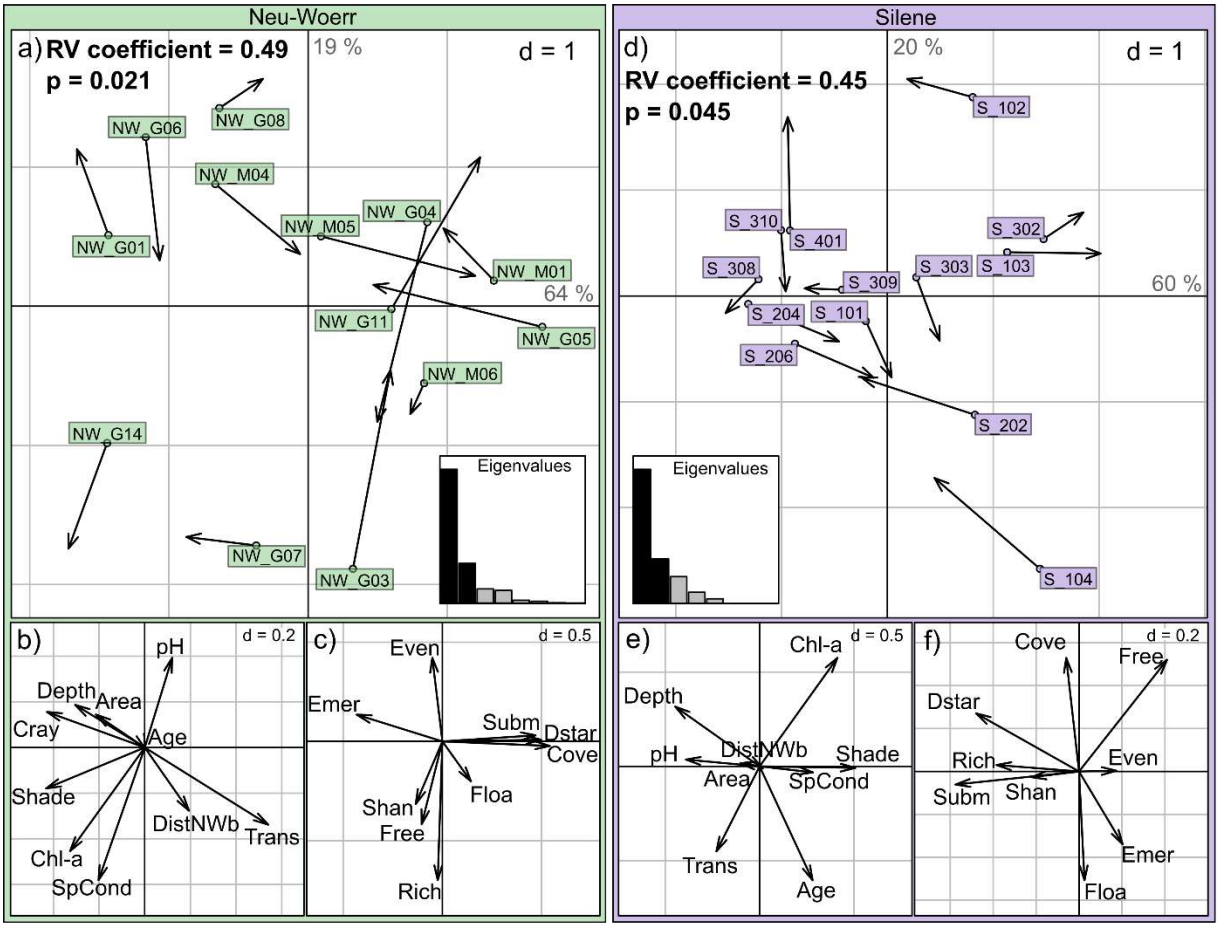


Fig. 3.3. CoIA environment – macrophyte metrics. a - c) Neu-Woerr. d - f) Silene. a, d) Positions of the ponds on the plane of the first two coinertia axes (% inertia projected by each axis in grey) according to the environmental variables (dots and labels) and according to macrophyte metrics (arrowheads). RV coefficients and p-values are in the top left corner in bold and the eigenvalue bar plots in the insets. b, e) Positions of environmental variables on the coinertia plane. c, f) Positions of the macrophyte metrics on the coinertia plane. For metric abbreviations see Table 3.2.

CoIA between macrophyte abundances and environmental variables showed high and significant correlations for both sites (*Fig. 3.4a,d*). For both sites, the first coinertia axis separated ponds with high light conditions (Trans) on the left from ponds with low light conditions (Shade, Chl-a) on the right side of the coinertia plane (*Fig. 3.4b,e*). For Neu-Woerr, crayfish abundance (Cray) and specific conductivity (SpCond) were with the low light conditions on the right side of the plane. This was also the case for depth, while for Silene depth was associated with improved light conditions, and with younger pondage.

For both sites, ponds with extensive *Chara* cover, of *Chara vulgaris* (CharVulg) in Neu-Woerr and *Chara globularis* (CharGlob) in Silene (*Fig. 3.4c,f*), had transparent water, and in Silene relatively high pH as well. In Silene, ponds with *Lemna minor* (LemnMino) were characterized by high shade from surrounding trees, high chlorophyll-a concentration, low transparency and low pH. In Neu-

Woerr, *Eleocharis uniglumis* (EleoUnig) and *Carex rostrata* (CareRost) only occurred in pond NW_G14, and *Ricciocarpos natans* (RiccNata) only in pond NW_G07, and both these ponds were characterized by high chlorophyll-a concentrations and specific conductivity.

For both sites, pond surface area only had a small contribution to the correlation between environmental variables and macrophyte taxon abundance. For Neu-Woerr, age did not contribute to the correlation, but for Silene age contributed to both axes. For both sites the distance to the nearest waterbody, and only for Silene specific conductivity, contributed mainly to the second coinertia axis. However, the second axes only projected 23% and 17% of the inertia for Neu-Woerr and Silene, respectively.

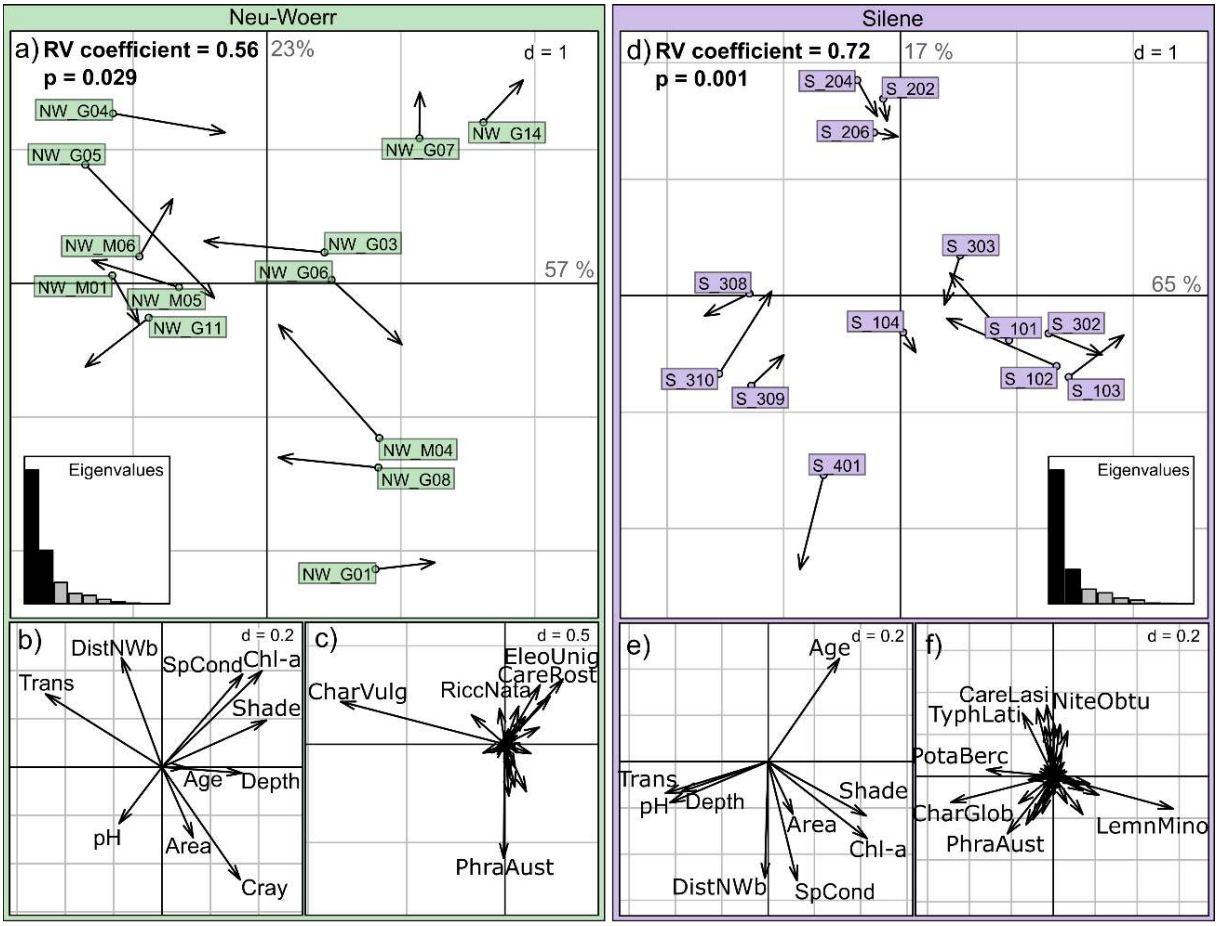


Fig. 3.4. CoIA environment – macrophyte taxa. a - c) Neu-Woerr. d - f) Silene. a, d) Positions of the ponds on the plane of the first two coinertia axes (% inertia projected by each axis in grey) according to the environmental variables (dots and labels) and according to taxon abundances (arrowheads). RV coefficients and p-values are in the top left corner in bold and the eigenvalues in the insets. b, e) Positions of environmental variables on the coinertia plane. c, f) Positions of the macrophyte taxa on the coinertia plane. For taxon name abbreviations see Table S.3.2 (Appendix 3.B).

In both sites, ponds were surrounded by shrubs and willows, reeds, meadows and high trees. In Silene, four ponds bordered bogs as well (Appendix 3.C). CoIA showed significant correlation between land cover in 5 m around ponds and selected environmental variables for Silene (RV coefficient = 0.56, p = 0.001), but not for Neu-Woerr (RV coefficient = 0.34, p = 0.094). In Silene, ponds surrounded by reeds and meadows were associated with relatively high pH and

transparent water, whereas those surrounded by trees were more shaded and had higher chlorophyll-a concentrations (Appendix 3.C).

3.3.3. Community dissimilarity

Power function models fitted on all pond pairs (Full), showed marginally non-significant decay of community similarity with geographic distance for Neu-Woerr, as indicated by the p-value of regression parameter b ($p = 0.064$), but highly significant decay for Silene ($p < 0.001$ for parameter b) (*Fig. 3.5*, *Table 3.3*). For Silene the halving distance (G_{dis}_half, the distance at which community similarity equals half its initial value at 10 m distance) was 300 m. Reduced models (Red), fitted only on pairs of ponds separated by more than G_{dis}_half, did not show a significant decay of community similarity with geographic distance. This indicates that excluding pairs of ponds separated by less than G_{dis}_half effectively removed the spatial correlation that was observed in Silene.

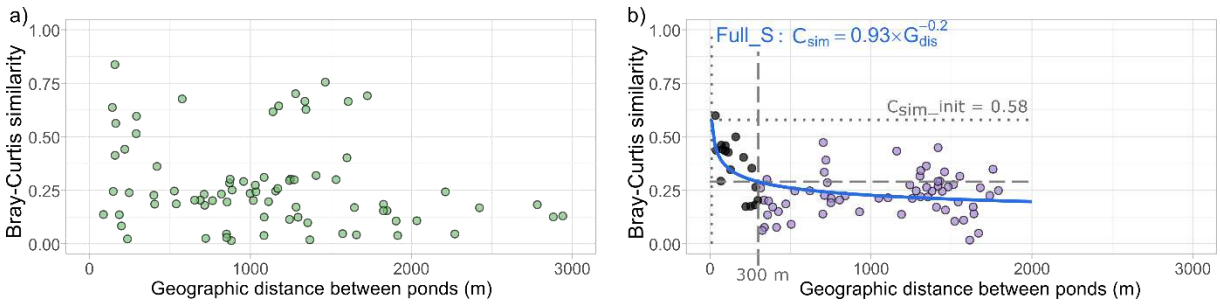


Fig. 3.5. Pairwise Bray-Curtis similarity between macrophyte communities as function of geographic distance between the ponds. a) Neu-Woerr. b) Silene with the power function fitted to all pairs of ponds and the regression equation (Full_S) in blue. Initial similarity (C_{sim}_init) is indicated with grey dotted lines and the halving distance (G_{dis}_half) with grey dashed lines. Pond pairs further apart from each other than the halving distance are presented in violet and pairs of ponds separated by less than the halving distance are in black.

*Table 3.3. Regression parameters of full (Full) and reduced (Red) power functions describing decay of community similarity with geographic distance, following $C_{sim} = a \cdot G_{dis}^{-b}$, for Neu-Woerr (_NW) and Silene (_S). Significance is indicated as *** for p < 0.001, ** for p < 0.01, * p < 0.05, and (n.s.) for p > 0.05.*

Parameter	Full_NW	Full_S	Red_NW	Red_S
a	0.86 (n.s.)	0.92 ***	-0.02 (n.s.)	0.08 (n.s.)
b	0.17 (n.s.)	0.20 ***	0.08 (n.s.)	-0.15 (n.s.)

For Neu-Woerr, no generalized dissimilarity model (GDM) of the Bray-Curtis dissimilarity could be fitted, because none of the predictor variables were significant. For Silene, however, a GDM was fitted with as predictor variables the differences between ponds in shade from surrounding trees and water transparency (model $p < 0.001$, explained deviance = 45%, *Fig. 3.6*). The Silene model did not include geographic distance between pond pairs as predictor variable. It had been excluded by the backward selection procedure, and would be marginally non-significant if added to the model (p would be 0.061). The pond pair differences in shade from surrounding trees and water transparency were not correlated (Spearman $\rho = 0.16$). Shade from surrounding trees was more important than water transparency for the community dissimilarity (C_{dis}), as indicated by the higher sum of the I-spline coefficients. The influence of shade on C_{dis} was larger between 0 and

50% of the pond surface shaded, indicated by the steep slope of the I-spline curve in this range, than between 50 and 90% shaded, where the slope was flatter. The influence of transparency on C_{dis} was most important between 70 and 120 cm transparency.

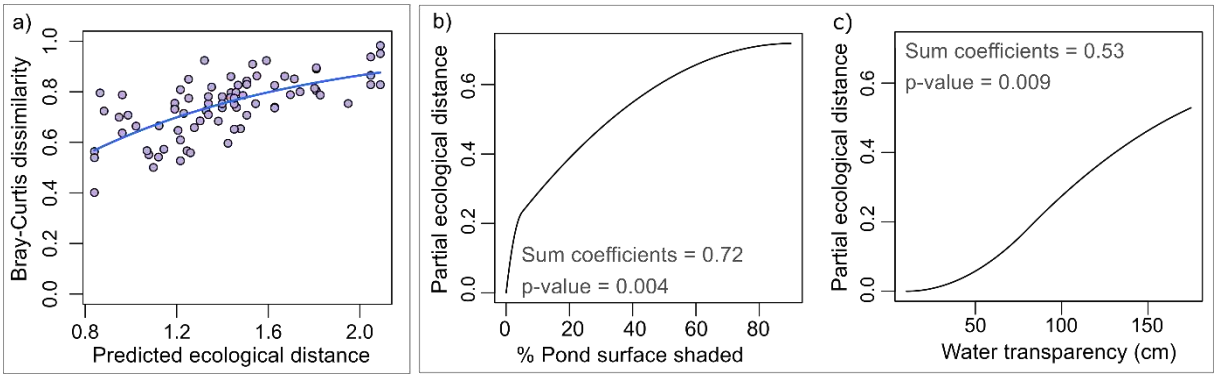


Fig. 3.6. Silene GDM. a) Observed Bray-Curtis dissimilarity as function of predicted ecological distance for each pair of ponds (points), and predicted Bray-Curtis dissimilarity as function of predicted ecological distance (line). b, c) I-spline functions for the predictor variables, with the slope indicating the rate of community composition change along the variable gradient, and in text the sum of coefficients, indicating the importance of the variable, and its significance.

3.4. Discussion

To help inform the design of pondscapes for biodiversity conservation and restoration, we studied environmental variables and spatial processes that structure macrophyte communities in two pond networks in Europe with contrasting conditions. Ponds in the western European network Neu-Woerr had low macrophyte species richness, while those in northeastern European Silene had moderately high species richness compared to ponds studied in European surveys (Hassall et al., 2011; Oertli et al., 2002; Williams et al., 2008). In Neu-Woerr, macrophyte cover was low and consisted mainly of emergent and submerged charophyte life forms. In Silene, macrophyte cover was abundant and contributions of emergent, submerged, anchored floating and free-floating life forms to the total macrophyte cover varied among ponds. In both pond networks, taxon abundances and macrophyte metrics describing community structure and function were correlated with environmental variables. Varying responses of the different macrophyte metrics highlight the importance of evaluating multiple metrics. However, the metrics taxonomic distinctness and relative cover of submerged life forms provided similar information in our study, since they were both heavily influenced by the cover of charophytes.

3.4.1. Environmental correlates

Variables affecting light conditions were important for the macrophyte community composition and relative cover of different life forms, which is in line with literature (Bornette & Puijalon, 2011; Kautsky, 1988; Lacoul & Freedman, 2006). In Silene, ponds with clear water and no shading from trees exhibited high cover of submerged macrophytes, whereas ponds with turbid water and shading from trees were dominated by free-floating plants including *Lemnaceae*. Free-floating macrophytes have a competitive advantage over submerged macrophytes at low light conditions because their leaves are on top of the water surface (Scheffer et al., 2003). The dominance of *Lemnaceae* in shaded turbid ponds in Silene is probably due to a combination of low light conditions with an absence of wind and high nutrient levels, that comes together in ponds surrounded by trees. In windy conditions *Lemnaceae* are blown to the shore and cannot dominate (Sayer et al., 2012), but in shaded ponds surrounded by trees, which act as windbreaks, they can form mats. Unlike anchored macrophytes, *Lemnaceae* cannot take nutrients from the sediment

but only from the water column, which is why they require high water nutrient levels (Scheffer et al., 2003). In Silene, ponds dominated by free-floating plants had high chlorophyll-a concentrations, indicating high nutrient levels, which was potentially related to historical farming activities, and maybe also to riparian trees. Our analysis on the correlation between the land cover in the 5 m buffer around ponds and in-pond environmental variables showed that ponds surrounded by willows, shrubs and high trees had high chlorophyll-a concentrations and low water transparency. Riparian trees and shrubs can contribute leaf litter to the ponds, which increases organic matter and nutrient concentrations (Sayer et al., 2012). The enhanced nutrient levels could allow *Lemnaceae* to take up sufficient nutrients and could stimulate algal growth, thus increasing chlorophyll-a concentrations and turbidity, which could give *Lemnaceae* a competitive advantage over submerged macrophytes.

In Neu-Woerr, ponds with low light conditions had low macrophyte cover of merely emergent life forms, whereas ponds with clear water and little tree shade had extensive beds of submerged charophytes. In Neu-Woerr, low light conditions were not only caused by algal biomass and tree shade, but also by calico crayfish. Calico crayfish resuspend sediment when digging, which causes turbidity, and they graze on macrophytes (Herrmann et al., 2022). Without macrophytes, algal growth increases, which adds to the turbidity (Scheffer & Van Nes, 2007). Turbidity, both from algal growth and sediment resuspended by crayfish, in turn impedes macrophytes from establishing. This positive feedback mechanism can maintain a pond in a crayfish induced turbid alternative stable state (Scheffer & Van Nes, 2007; Twardochleb et al., 2013). At the time of sampling, the cover of *Chara vulgaris* was quite extensive in Neu-Woerr. However, later in the season the charophyte populations collapsed and the water became turbid and devoid of macrophytes (K. van der Zon, pers. obs.). *Chara vulgaris* is a quick growing species that is resilient to disturbances (Bornette & Arens, 2002; Wade, 1990). It might be that the charophytes established in spring, when the crayfish activity was probably low. Calico crayfish hide in burrows during the winter and their eggs hatch in spring (Tack, 1941; Chucholl, 2012). At the time of sampling the crayfish activity was presumably not important enough to tip the ponds into a turbid state. Later in the season, however, increased crayfish activity could have induced the turbid state without charophytes.

In Silene, pH was positively correlated with relative cover of submerged life forms including *Chara globularis*. At high pH, inorganic carbon is primarily present in the form of bicarbonate. Charophytes can use bicarbonate more effectively for photosynthesis than angiosperm bicarbonate users. Consequently, at high pH charophytes have competitive advantage over angiosperms (Iversen et al., 2019; Maberly & Madsen, 2002; Sand-Jensen et al., 2021).

Surprisingly, whereas in Neu-Woerr pond depth seemed to be negatively correlated with charophyte cover, in Silene charophytes were relatively more abundant in deeper ponds. This contrasting outcome could be explained by the positive correlation between pond age and depth in Silene, a correlation that was absent for Neu-Woerr. It is possible that in Silene the older, ten year old, ponds already lost some depth to sedimentation, and some charophytes to succession. Succession rates depend on productivity (Hassall et al., 2012), and we expect succession to occur faster in the Silene ponds, which are characterized by extensive macrophyte cover, than in the Neu-Woerr ponds, which are disturbed by crayfish. In case of classical ecological succession, following an initial colonization phase, ponds reach a stage of maximum macrophyte diversity (Barnes, 1983; Fleury & Strehler Perrin, 2004). We expect that the older Silene ponds were at this stage. After the stage of maximum diversity ponds may become poorer in species, and richer in nutrients (Fleury & Strehler Perrin, 2004; Gee et al., 1997; Sayer et al., 2012; Williams et al., 2008). Furthermore, there may be an ongoing decrease in charophyte cover and increase in emergent vegetation cover with succession (Hassall et al., 2012; Van Geest et al., 2003). Additionally, in both studied networks, willow overgrowth will increase pond shading if

unmanaged. As discussed above, this may increase the relative cover of emergent and free-floating life forms and decrease the relative cover of submerged macrophytes.

The contribution of pond surface area to the coinertia between environmental variables and macrophyte metrics was low for both sites. Some studies found macrophyte species richness to increase with pond surface area (Friday, 1987; García-Girón et al., 2019; Gee et al., 1997; Oertli et al., 2002) while others found no effect (Biggs et al., 2005; Edvardsen & Økland, 2006b; Hassall et al., 2011; Linton & Goulder, 2000). Oertli et al. (2002) studied 80 Swiss ponds and concluded that there are more macrophyte species in several small ponds than in one large pond of the same total surface area. However, since some species only occurred in the smallest or largest ponds, the authors advise to create ponds of varying sizes when possible (Oertli et al., 2002).

Our analyses did not show effects of pond isolation, measured as distance to the nearest water body, on pond macrophyte community composition and metrics. This might be due to the fact that all studied ponds were quite close to other water bodies and because we only studied permanent ponds. The most isolated Silene pond was separated from the nearest water body by 240 m, and the most isolated Neu-Woerr pond by 170 m only. Linton and Goulder (2000) studied a pondscape with ponds separated from other water bodies by more than 500 m and did find pond macrophyte species richness to increase with the number of neighbouring water bodies. Lozada-Gobilard et al. (2019), studied kettle holes that (except for one) all had other water bodies within 500 m, and did not find an effect of isolation on macrophyte richness in permanent ponds. However, they did find a negative correlation between isolation and macrophyte species richness for ephemeral ponds, which might depend on nearby ponds for recolonization after disturbance (Lozada-Gobilard et al., 2019).

3.4.2. Community dissimilarity as function of geographic distance and environmental dissimilarity

In Silene, the power model fitted on all pond pairs showed significant decay in macrophyte community similarity with increasing geographic distance between ponds, while the power model fitted only on ponds separated by more than the halving distance (G_{dis}_half = 300 m) showed no decay of community similarity with geographic distance. From this we could derive that there was spatial correlation in the macrophyte community composition between ponds separated by less than 300 m in Silene. The generalised dissimilarity model for Silene, however, showed a marginally non-significant influence of geographic distance on community dissimilarity. This non-significance might be due to the relatively low number of ponds studied, or due to the selection strategy of ponds to study, which was aimed at capturing the largest macrophyte diversity possible. The generalised dissimilarity model did show significant effects of the difference in shade from surrounding trees and difference in water transparency on community dissimilarity, from which we derive that differences in environmental variables were more important than spatial distance in explaining beta diversity.

For Neu-Woerr, the power model showed marginally non-significant decay of community similarity with geographic distance. The non-significance may be explained by the high similarity in macrophyte community composition between two clusters of ponds that were separated by a relatively large distance of 1.1 to 1.7 km (*Fig. 3.5a*). We expect that pond pairs from these clusters have similar macrophyte communities because of similar hydrological regimes, an important factor influencing macrophyte community composition (Jeffries, 2008; Kautsky, 1988), which we unfortunately did not measure. The generalized dissimilarity model for Neu-Woerr did not show significant effects of geographic distance nor of any of the differences in environmental variables on community dissimilarity. This non-significance may be due to the low number of ponds studied, short gradients, or unmeasured confounding variables. Besides hydrology, an unmeasured variable that could have influenced the Neu-Woerr macrophyte communities is pesticide runoff

from the nearby agricultural activities (Ulrich et al., 2022). Furthermore, although crayfish abundance was measured, this was biased because we only captured juvenile crayfish. Crayfish abundance was measured by sweep netting at daytime along the pond edge, a technique that does not allow for the estimation of the number of adult crayfish, who live deeper in the pond and are only active at night (Tack, 1941). To estimate the abundance of both adult and juvenile crayfish, overnight trapping should be performed, for instance with artificial refuge traps (Green et al., 2018).

3.4.3. Pondscape design for target species

First of all, pond construction should not drain wetlands *(sensu stricto)* or destroy terrestrial habitats with valuable biodiversity. Furthermore, pondscape design should be adapted to the restoration or conservation goals of the planned network. A pond network that should host specific target species of conservation concern may have different requirements than a pond network aimed to increase freshwater biodiversity on a landscape scale in general.

One of the target species in our study sites was the European pond turtle *Emys orbicularis*. *Emys orbicularis* feeds on macrophytes and invertebrates associated with them, and uses macrophytes for hiding and basking (Lebboroni & Chelazzi, 1990; Thienpont et al., 2020). Ponds designed for *E. orbicularis* should have sufficient cover of submerged and anchored floating macrophytes, as well as a border of emergent vegetation, and should be exposed to sunlight (Bensettiti & Gaudillat, 2002). Based on our results on the scale of individual ponds, it can be advised that when a pond is made or restored for *E. orbicularis,* the abundance of crayfish species that have a large influence on macrophytes, and the percentage of the pond surface that is shaded by trees, should not be too high. This may require recurrent management of surrounding trees and placement of the pond on a site without crayfish species that have a large influence on macrophytes.

On the pondscape scale, the design for target species should consider the habitat requirements of the specific species at all life stages, and should, if relevant, not only include the aquatic but also the terrestrial habitat (Pupins et al., 2023; Rannap et al., 2020). Furthermore, the placement of ponds should depend on the locations of source populations and the dispersal abilities of the target species. If the species is not dispersing by air, dispersal corridors should be considered as well (Moor et al., 2024; Noel et al., 2006). Ponds made for the common spadefoot toad, *Pelobates fuscus* (Laurenti, 1768), another target species for the Neu-Woerr site, should for example be placed less than 500 m apart, because individuals from this species rarely disperse more than 500-1000 m from the pond they were born (Nyström et al., 2002).

3.4.4. Pondscape design to enhance regional biodiversity

To enhance regional diversity, one could aim to increase the alpha diversity of ponds, or the beta diversity between ponds, whereby an increase in beta diversity should not lead to a decrease in alpha diversity or vice versa (Socolar et al., 2016). Based on the two sites studied, it is hard to generalise on how to increase pond macrophyte alpha diversity. In our study, taxonomic richness did not contribute to the correlation between macrophyte community metrics and the environmental variables studied. Furthermore, although the ponds in Neu-Woerr and Silene were made for similar target species using similar methods, macrophyte diversity was lower in Neu-Woerr. The presence of calico crayfish may in part explain the low macrophyte richness and cover in Neu-Woerr. Other factors, such as the regional macrophyte species pool, anthropogenic pressures, pond use by mammals and macroecological aspects may also explain the difference in macrophyte diversity between the study sites. To disentangle the effects of these factors, studies on larger numbers of ponds should be performed. These should span different biogeographic regions, as there are still knowledge gaps on macrophyte macroecology, with for

example contradictory results from studies on the relationship between latitude and diversity (Alahuhta et al., 2021).

Our results from Silene indicate that high macrophyte beta diversity may be obtained by creating ponds with varying levels of shade from surrounding trees and water transparency. Based on results from other studies, it can be expected that constructing both temporary and permanent ponds (Della Bella et al., 2008) of different sizes (Oertli et al., 2002) and at different points in time to attain different succession stages (Hassall et al., 2011) could enhance beta diversity as well. Pondscapes can be designed to include ponds of various ages, sizes, and levels of shade from surrounding trees. While it may be more complex to influence pond permanency and water transparency, pond placement may be informed by hydrological conditions, source water nutrient concentrations and the presence of bioengineering species.

Since pondscape design for high beta diversity should not come at the cost of decreased alpha diversity, and macrophyte metapopulations are probably more resilient when multiple suitable habitats are available within in a pondscape, it may be desirable to not only create ponds that differ in environmental conditions, but also ponds that provide similar habitats. When environmental conditions are spatially structured, a design of clusters of ponds that are close together but geographically isolated from other clusters may be optimal (Jeffries, 2008). Overall, pondscapes designed to include both ponds that differ in environmental conditions, as well as groups of ponds that provide similar conditions, may be most effective for enhancing macrophyte biodiversity on a regional scale.

Chapter 4

Life is good when you're wearing waders

Chapter 4: Macroinvertebrate biodiversity assessment of human-made ponds in a Baltic country

This chapter will be transformed in a manuscript for submission to an academic journal after the PhD defence.

4.1. Introduction

Since the start of the discipline in the late nineteenth century, freshwater science and policy have focused on rivers and lakes (Boix et al., 2012; Davies et al., 2008). Only in the last 30 years, ponds have received research attention (Boix et al., 2012; Hill et al., 2016; Oertli et al., 2002). Studies from the UK, Western Europe and Mediterranean countries have shown that ponds are important freshwater habitats (Davies et al., 2008; Della Bella et al., 2005; Williams et al., 2004). They host specific species and portray high beta diversity, thus contributing to regional freshwater diversity (De Meester et al., 2005; Hill et al., 2016, 2021). Thereby it does not fundamentally matter whether ponds are of natural or human-made origin (Céréghino et al., 2014; Coccia et al., 2016; Hill et al., 2021). However, depending on how the human-made ponds were created, their community composition may differ from natural ponds (Coccia et al., 2016; Tomingas et al., 2025, but see Sartori et al., 2014).

With the drastic loss of wetland habitats and associated freshwater biodiversity decline, wetland conservation and restoration are urgently needed (Kingsford et al., 2016). Examples from the British Isles, Mediterranean countries and Sweden show that pond creation can be an effective measure to enhance freshwater biodiversity (Coccia et al., 2016; Thiere et al., 2009; Williams et al., 2008). However, mechanisms affecting pond creation success are not fully understood, and research on the biodiversity value of human-made ponds has focussed on a few geographic regions only (Hill et al., 2021; Williams et al., 2008). The biodiversity of human-made ponds in the Baltic countries, for example, has received little attention. Pond networks have been created in these countries, mainly as habitat for herpetofauna, and their amphibian and turtle communities have been studied (e.g. Pupina & Pupins, 2014; Pupins et al., 2023; Rannap et al., 2009). However, information on other taxa living in these ponds, such as macroinvertebrates, is lacking.

Macroinvertebrates are ubiquitous and integral components of pond ecosystems (Labat & Usseglio-Polatera, 2023). They constitute a diverse group that inhabits a wide range of habitats and fulfils many functional roles (Tachet et al., 2010). In absence of fish or other predators they function as top predators, and filter feeders contribute to maintenance of a clear water state (Labat & Usseglio-Polatera, 2023). Macroinvertebrates also break down leaf litter and are of key importance for nutrient cycling. They contribute to nutrient flow across the sediment-water interface through bioturbation and bioirrigation, and provide food for consumers inside as well as outside the water (Macadam & Stockan, 2015). By exporting nutrients and energy to terrestrial food webs, emerging insects contribute to both the functioning of terrestrial ecosystems and the limitation of eutrophication in ponds (Fehlinger et al., 2023; Labat & Usseglio-Polatera, 2023). Besides, there are rare and protected macroinvertebrate species that live in ponds (Biggs & Williams, 2024). It is therefore not surprising that macroinvertebrate community metrics are used in existing pond ecological quality assessment protocols (e.g. Angélibert et al., 2010; Howard & Road, 2002; Indermuehle et al., 2010; Labat & Usseglio-Polatera, 2023; Menetrey et al., 2011; Solimini et al., 2008; Trigal et al., 2009)

To date, Baltic pond macroinvertebrate data is very scarce. Like most of the EU member states, the Baltic countries include only lakes larger than 50 ha in their ecological quality monitoring

programmes for the Water Framework Directive (WFD) (Kristensen & Globevnik, 2019). A study by Céréghino et al., (2012), which compared biological traits from pond macroinvertebrates among different biogeographic regions in Europe, did not include any Boreal data. The authors discussed the difficulty of obtaining data across Europe. As far as I am aware there are still no English language published scientific articles on macroinvertebrate communities in Latvian or Lithuanian ponds. On 1 May 2025, I performed a literature search on Google Scholar with the search term "(Latvia OR Lithuania OR Estonia OR Baltic) AND (pond OR small shallow lake OR small waterbod*) AND *invertebrate)". I found a few studies on macroinvertebrate communities in various types of waterbodies, including ponds, in Estonia (Tomingas et al., 2025; Vaikre et al., 2015, 2018), but the search did not yield any studies of pond macroinvertebrate communities from Lithuania or Latvia.

The current knowledge on macroinvertebrates in both natural and human-made Baltic ponds is clearly insufficient to guide the effective implementation of human-made ponds as solutions to enhance freshwater diversity in northeastern Europe. A standard sampling protocol is needed to obtain this knowledge. The Sampling of Small Shallow lake invertebrates (S_3i) method, recently developed in France (Labat et al., 2022), could be appropriate. The S_3i method is applicable to standing waters with a surface area up to 50 ha and 7 m deep, and can be used to calculate various macroinvertebrate metrics such as taxonomic richness and Shannon diversity (Labat et al., 2022). The method is based on the sampling strategies used in the Predictive SYstem for Multimetrics (PSYM) and Indice de Biodiversité des Etangs et Mares (IBEM) assessment methods. The S_3i, PSYM and IBEM methods all use hand netting for invertebrates and sample all mesohabitats in a pond. Yet, whereas the PSYM method allocates thee minutes of sampling time to each pond, the IBEM method requires a specific number of samples based on the surface area and the S_3i method prescribes one sample per mesohabitat type (Angélibert et al., 2010; Howard, 2002; Indermuehle et al., 2010; Labat et al., 2022). The IBEM and S_3i methods use predefined lists of mesohabitat types, which are defined by the physical structure of the vegetation, or the non-vegetated substrate, and by whether the mesohabitat occurs at the shoreline or deeper in the pond (Indermuehle et al., 2010; Labat et al., 2022).

A mesohabitat can be defined as "a visually distinct and easily identifiable habitat within the freshwater body" (Della Bella et al., 2005), and should be delimited based on the habitat requirements of the studied organism group (Brunke et al., 2001; Pringle et al., 1988). The mesohabitat approach was first developed for lotic macroinvertebrate sampling and is based on the rationale that the different physical substrates, food resources and sheltering opportunities mesohabitats provide shape the macroinvertebrate community composition (Beisel et al., 1998; Brunke et al., 2001, 2001; Harper et al., 1997; Oertli et al., 2005b; Suren & Winterbourn, 1992). Indeed in ponds, vegetation structure is a primary determinant of macroinvertebrate community composition (McAbendroth et al., 2005; Walker et al., 2013). However, pond-wide environmental variables such as, pH, nutrient levels and fish presence influence macroinvertebrate communities as well (Friday, 1987; Hassall et al., 2011; Labat et al., 2024). Knowledge on the relative importance of mesohabitat types and pond-wide variables in structuring macroinvertebrate communities is important for efficient pond creation. For instance, if mesohabitat types are of main importance, the creation of a small number of ponds with diverse mesohabitats would be optimal. If, however, pond variables are more important, a large number of ponds with varying conditions would be more efficient. For pond monitoring it is also interesting to know if less effort-intensive macrophytes surveys can be used as proxies for macroinvertebrate assessments.

As a first step to increase the knowledge on macroinvertebrates in Baltic ponds, I studied the invertebrate communities in ten human-made Latvian ponds. Our study is the first to employ the S_3i method outside of France. I investigated whether taking one sample per mesohabitat type was sufficient for detecting a large part of the macroinvertebrates present in the Latvian human-made

ponds. Furthermore, I examined whether macroinvertebrate samples differed between mesohabitat types and between ponds, where pond level differences would suggest an important influence of pond-wide variables on community composition. I also investigated which pond-scale environmental variables correlate with the macroinvertebrate community composition in the studied ponds, and to what extent the macroinvertebrate and macrophyte community composition and richness were correlated. Lastly, I compared the macroinvertebrate taxa in the ten studied human-made ponds to macroinvertebrates data from the ten closest lakes included in the national Latvian monitoring programme for the WFD.

Overall, our research questions are: 1) How complete are the macroinvertebrate inventories obtained with one sample per mesohabitat type, for individual ponds and for ten ponds from the same pond network combined? 2) Are macroinvertebrate communities more similar in samples from the same mesohabitat type than in samples from different mesohabitat types, and are macroinvertebrate communities more similar in samples from the same pond than in samples from different ponds? 3) How do pond environmental variables relate to macroinvertebrate community composition in human-made ponds? 4) Can macrophyte richness and community composition be proxies of macroinvertebrate richness and community composition in ponds? 5) How do macroinvertebrate communities compare between human-made ponds and lakes in Latvia? Based on the outcomes of the study I aim to formulate advice on the sampling, evaluation, and management of human-made ponds.

4.2. Methods

4.2.1. Pond characteristics, environmental variables and macrophytes

Ten human-made permanent ponds (*Table 4.1*) were studied in study site Silene, Latvia, described in **Chapters 2** and **3**. The ponds are characterized by rich macrophyte communities and high macrophyte cover (**Chapter 3**). Furthermore, they are little impacted by anthropogenic stressors, as they are located in a protected nature area in a zone along the border between the Schengen Area and Belarus that can only be accessed by people with a special permit. One of the ponds is inhabited by the invasive fish *Perccottus glenii* (Dybowski 1877) (Pupiņa & Pupiņš, 2012) and another by the native fish *Tinca tinca* (L., 1758) which both feed on macroinvertebrates (Bezmaternykh & Shcherbina, 2018; Kati et al., 2015). The presence of these fish may therefore impact the macroinvertebrate communities. Furthermore, some of the ponds may be impacted by localised nutrient enrichment resulting from former pig farming on the site.

From 19 to 24 July 2022, pond environmental variables were measured and macrophyte surveys were performed. Macrophyte communities were surveyed according to the method described in **Chapter 3**. The environmental variables pH, specific conductivity, chlorophyll-a concentration, maximum depth, turbidity, and the percentage of the pond surface area shaded by surrounding trees were assessed as described in **Chapter 3**. Furthermore, the oxygen concentration below the water surface was measured with the OTT Hydrolab DS5 sonde. Total nitrogen and total phosphorus data, measured in July 2023, so one year later, were added to the dataset as well. Total nitrogen was measured according to the Latvian national standard potassium peroxydisulfate – cadmium methodology LVS 340:2001 and total phosphorus was measured using the Latvian national standard spectrophotometric ammonium molybdate method LVS EN ISO 6878:2005 L

Table 4.1. Silene ponds studied. Area is the high-water surface area and age the pond age in 2022. Fish present indicates whether fish are known to be present in the pond and if so which species.

Pond	Area (m^2)	Age (year)	Fish present
S_101	1050	9	
S_102	290	4	
S_104	220	4	
S_202	110	9	
S_204	180	9	
S_206	150	9	
S_302	110	9	*Tinca tinca* (L., 1758)
S_303	130	9	
S_308	180	4	
S_401	530	4	*Percottus glennii* (Dybowski, 1877)

4.2.2. Macroinvertebrate sampling and identification

Between 19 and 22 July 2022, macroinvertebrates were sampled following the S_3i protocol (Labat et al., 2022). In short, mesohabitats from *Table 4.2* were identified and the proportion of the pond surface area covered by each mesohabitat was estimated. This was generally done from the shore before sampling, but sometimes during sampling mesohabitats that were not visible from the shore were added as well. One sample was taken per mesohabitat, either by vigorous sweep netting in mesohabitats characterized by vegetation other than duckweeds, or by scooping up sediment or duckweeds for other types of mesohabitats. A 0.5 mm mesh size hand net with a rectangular 20 x 30 cm frame was used. Sweep net samples covered an area of about 1 m^2 while sediment and duckweed samples only covered 0.05 m^2. Although the S_3i method recommends sampling three 0.05 m^2 plots for the mesohabitats H02 and H01, we only sampled one plot for these mesohabitats.

Amphibian larvae were removed from the samples and placed back in the pond. Samples were preserved in ethanol until identification in the lab. Identification was performed to the lowest taxonomic level possible with a stereo microscope of maximum 50x magnification. The keys listed in Table 4.3 were used. If more than 100 individuals of the same taxon were present in a sample their abundance was not counted, but estimated instead.

*Table 4.2. Mesohabitats from the S_3i method. Table reproduced from Labat et al., 2022. * We only sampled 1 x 0.05 m^2.*

Code	Definition	Technique	Sample area (m^2)
Shoreline habitats (<20 cm depth)			
H12a	Creeping helophytes with thread-like leaves (*Isolepis fluitans, Juncus bulbosus, Agrostis stolonife*ra, etc.)	Sweep net	1
H12b	Helophytes with no thread-like leaves (*Hypericum, Lycopus, Ludwigia, Menyanthes,* etc.)	Sweep net	1
H12c	Hydrophytes with low dissected or no thread-like leaves (*Potamogeton polygonifolius, Najas, Elodea*)	Sweep net	1
H12d	Small erect herbaceous helophytes with thread-like leaves (*Carex, Juncus,* etc.)	Sweep net	1
H12e	Hydrophytes with thread-like or dissected leaves (*Myriophyllum, Utricularia,Batrachium, Zannichellia*), Characeae	Sweep net	1
H12f	Hydrophytes with floating leaves or large straight helophytes (*Nymphaea, Typha, Phragmites*)	Sweep net	1
H12g	Bryophyta	Sweep net	1
Deep habitats (>20 cm depth)			
H11	Roots	Sweep net	1
H10	Small creeping helophytes or with no thread-like leaves	Sweep net	1
H09	Hydrophytes with low dissected or no thread-like leaves	Sweep net	1
H08	Small erect herbaceous helophytes with thread-like leaves	Sweep net	1
H07	Hydrophytes with thread-like or dissected leaves, Characeae	Sweep net	1
H06	Stones, blocks	Scoop	3 x 0.05
H05a	Large floating leaves hydrophytes or large straight helophytes	Sweep net	1
H05b	Small floating leaves (duckweeds, *Azolla*)	Scoop	0.05
H04	Filamentous algae, (only if distinct mesohabitat or >5% coverage)	Scoop	3 x 0.05
H03	Bryophyta	Sweep net	1
H02	Litter and organic sediment	Scoop	3 x 0.05*
H01	Loose mineral substrate	Scoop	3 x 0.05*
H0a	Flagstone	Scoop	3 x 0.05

Table 4.3. Keys used for macroinvertebrate identification.

Organism group	Geographic scope	Reference
Freshwater macroinvertebrates	France	Tachet et al., 2010
Freshwater macroinvertebrates	Estonia	Timm, 2015
Freshwater molluscs	Latvia	Rudzīte et al., 2010
Freshwater molluscs	Germany	Gloër & Meier-Brook, 2003
Odonata	Estonia	Martin, 2022
Amphipods	Germany	Eggers & Martens, 2001

4.2.3. Resolution of taxonomic ambiguity

Before dissimilarities between samples could be calculated and rarefaction curves could be constructed, taxonomic ambiguity had to be resolved. This is because the initial taxonomic assignment of the macroinvertebrates was performed at overlapping taxonomic levels, which is common for macroinvertebrate identification (Meredith et al., 2019). In our dataset, for example, some individuals had been identified as the species *Coenagrion hastulatum*, others as the genus *Coenagrion* and others as the family Coenagrionidae. Identification had been performed this way because the smallest individuals of Coenagrionidae in the samples could only be assigned to family level, the larger individuals had characteristics that allowed for genus level identification and only a few very large individuals could be assigned to species level. However, it would not have been right to conclude that a sample with Coenagrionidae, *Coenagrion* and *Coenagrion hastulatum* has two taxa more than a sample with only Coenagrionidae.

There are various strategies for the resolution of taxonomic ambiguity and no method is perfect (Meredith et al., 2019). I used the strategy "Assign Parents to Children, first on sample then on dataset level" (APTC-SG) described by Meredith et al. (2019). This strategy resolves taxonomic ambiguity dataset-wide, is suitable for the construction of rarefaction curves and preserves abundance and taxonomic information (Meredith et al., 2019). Here follows an example of how I resolved the overlap between the higher level 'parent' assignment *Coenagrion* and its lower level 'children' *Coenagrion hastulatum* and *Coenagrion lunulatum* following APTC-SG.

First, I identified the samples that contained both the 'parent' assignment and at least one of the 'children'. For those samples, identifications of the 'parent' *Coenagrion* were assigned to the 'child' that was most abundant in the sample. After this first step was performed for all samples with both the 'parent' and at least one 'child', in a second step, the remaining *Coenagrion* counts were assigned to the most abundant child in the entire dataset, which was *C. hastulatum.* Using this strategy almost all overlapping taxa were resolved.

It was decided to not resolve the overlap between Orthocladiinae and *Corynoneura* because the Orthocladiinae that were not identified as *Corynoneura* did not belong to the genus *Corynoneura*. I therefore treated Orthocladiinae and *Corynoneura* as different taxa. Species and genus level *Aeshna* assignments were not resolved either. This is because the *Aeshna* not identified to species level were *A. subarctica* or *A. juncea,* and did not overlap with the *A. caerulea* and *A. cyanea* in the dataset. Lastly, the overlap between Limnephilini and *Limnephilus* was not resolved because the Limnephilini not identified to genus level did not belong to the genus *Limnephilus*. The APTC-SG resolved pond dataset was used in all analyses, except in the comparison with the lake data, for which the pond dataset was further harmonized with the lake dataset, as described below.

4.2.4. Assessment of sample completeness

To answer the question of whether enough samples were taken per pond, sample-based rarefaction curves were constructed per pond. These rarefaction curves represent the number of taxa detected as function of the number samples taken in a pond. They are calculated by repeated random re-sampling samples from the same pond without replacement, and averaging the outcomes (Gotelli & Colwell, 2001). In R, rarefaction curves can be either calculated on individual-based abundance or sample-based incidence data (Chiu, 2023; Gotelli & Colwell, 2001). To be able to construct the curves for our sample-based abundance data, I transformed the data into sample-based incidence data, as is recommended (Chiu, 2023). The specaccum() function in the vegan R package with the "random" method and 999 permutations was used for all rarefaction curves.

Rarefaction curves per pond were constructed for taxonomic richness, family richness and Coleoptera genus richness. Macroinvertebrate family richness was shown to be a good indicator of pond ecological quality in the UK and Switzerland (Howard, 2002; Menetrey et al., 2011). Coleoptera richness is also a good indicator of pond ecological quality, at least in lowland Switzerland (Menetrey et al., 2011), and can indicate the success of pond creation as a mitigation measure for natural wetland loss, at least in the U.S.A. (Fairchild et al., 2000). This is probably because the order Coleoptera contains species sensitive to environmental conditions, species with varying habitat requirements, and has adult as well as larval aquatic stages (Bloechl et al., 2010; Coccia et al., 2016; Fairchild et al., 2000). Furthermore, ponds are important habitats for Coleoptera (Della Bella et al., 2005).

For an estimation of the proportion of taxa detected in the ten ponds together, a sample-based rarefaction curve was constructed based on all samples from all ponds. Furthermore, the Chao2 estimator and its confidence interval were calculated to estimate the total number of species in the ten ponds together (Chao, 1984). To this end, I used the specpool() function on the sample-based incidence data for all samples from all ponds. The Chao2 estimator is based on the number of species was found in one and two samples only (Chao, 1984). I did not calculate this nonparametric estimator, nor another estimator, for the individual ponds because this would be inappropriate with only one sample per mesohabitat.

4.2.5. Correlations with mesohabitat type and pond-scale environmental variables

To visually assess whether samples from the same pond and whether samples from the same mesohabitat had similar macroinvertebrate communities, non-metric multidimensional scaling (nMDS) was performed on the Hellinger dissimilarities calculated on the APTC-SG resolved macroinvertebrate densities. To test if samples from the same pond were significantly more similar than samples from other ponds and if samples from the same type of mesohabitat were significantly more similar than samples from different mesohabitat types, analysis of similarities (ANOSIM, Clarke & Green, 1988) was performed. Like nMDS, ANOSIM is a non-parametric rank-based method. It produces a test statistic R that indicates the strength of the separation between the samples from different groups on a scale of -1 to 1 (Clarke, 1993; Muthukrishnan et al., 2019). ANOSIM was performed on the Hellinger dissimilarities and significances were tested with 999 permutations.

The correlation between the environmental variables and the macroinvertebrate community composition was investigated with coinertia analysis (Dolédec & Chessel, 1994). I chose coinertia analysis because it is a suitable method when working with large numbers of variables measured on few sites, and because the influence of correlated environmental variables on the result is small compared to other constrained ordination techniques (Dray et al., 2003). Like in **Chapter 3**, I performed an initial correlation matrix Principal Component Analysis (PCA) on the environmental variables dataset and a covariance matrix PCA on the Hellinger transformed macroinvertebrate density dataset (Legendre & De Cáceres, 2013). The significance of the RV coefficient, which indicates the strength of the correlation between the environmental variables and the macroinvertebrate community, was calculated with 999 permutations.

4.2.6. Correlations with macrophytes

Congruence between taxonomic groups is often assessed by evaluating the correlation between the taxonomic richness of the groups, and by testing the correlation between community dissimilarities (De Morais et al., 2018; Heino, 2010). To evaluate the correlation between the macrophyte and macroinvertebrate richness, I performed Spearman rank correlation tests. For the macrophyte richness I used the number of taxa observed per pond, assuming that almost all macrophyte taxa present in a pond were detected and that the macrophyte data had no taxonomic

overlap. For the macroinvertebrate richness I used both the observed richness, as well as the richness rarefied to two samples, taken from the rarefaction curves. I did this in order to account for the differences in numbers of samples taken per pond. I also tested the correlation between the macroinvertebrate and macrophyte Shannon diversity indices, because the Shannon diversity index is less sensitive to rare taxa than species richness.

To assess the correlation between the macroinvertebrate and macrophyte communities, Mantel tests were performed for Hellinger dissimilarities calculated on 1) the macrophyte cover and the macroinvertebrate densities averaged per pond, and on 2) the presence-absence of macrophyte and macroinvertebrate taxa. The Spearman rank correlation and 999 permutations were used. Additionally, I performed a coinertia analysis on the macrophyte cover and the invertebrate the densities averaged per pond, with Hellinger transformation-based PCA as initial analysis.

4.2.7. Comparison with Latvian lake data

Lake macroinvertebrate data were obtained from the national Latvian monitoring programme for the Water Framework Directive (Latvijas Vides ģeoloģijas un meteoroloģijas centrs, 2022). For the ten monitored lakes closest to the sampled ponds, data were retrieved for the last year each lake was sampled (*Table 4.4*). The lake dataset was first APTC-SG standardized. After this, for comparison with the pond dataset, the level of taxonomic identification was harmonized between the two datasets. To this end, when for a given taxonomic group the level of taxonomic identification differed between the datasets, the highest taxonomic level was kept in both datasets. For example, *Chironomidae*, identified to family level in the lake dataset, but only to tribe level in the pond dataset, were all harmonized to family level, and leeches, identified to genus level in the pond dataset but to species level in the lake dataset, were all harmonized to genus level. To visualise the differences and similarities in taxa observed in the ponds and lakes, Krona plots (Ondov et al., 2011) were made for the harmonized pond and lake datasets. The plots were constructed based on the number of lakes and pond in which each taxon was found, and taxa were classified according to the National Center for Biotechnology Information (NCBI) taxonomy.

Table 4.4. Lakes for which monitoring data was obtained. Distance to ponds is the distance between the centre of each lake to the centre of the studied ponds. Distances and surface area obtained from digitized Google satellite features in QGIS 3.28 using projection EPSG:3059

Lake	Surface area	Distance to ponds	Sampling dates
Sitas ezers	190 ha	2 km	16/05/2016 and 12/10/2016
Riču ezers	1300 ha	4 km	20/05/2021 and 13/10/2021
Sila ezers	250 ha	4 km	20/05/2021 and 13/10/2021
Smiļģīnas ezers	50 ha	4 km	29/04/2019
Baltais ezers	90 ha	5 km	29/04/2019
Šēnheidas ezers	70 ha	8 km	25/10/2022
Abiteļu ezers	100 ha	9 km	25/10/2022
Černavu ezers	70 ha	9 km	12/06/2023
Skirnas ezers	80 ha	14 km	9/05/2018
Dārza ezers	50 ha	14 km	9/05/2018

A significance level of 0.05 was used for all tests and all analyses and visualisations were performed in Microsoft Excel 365, KRONA, Inkscape 1.3, QGIS 3.28 and R 4.4.0 with R studio 2023.06.0 using packages vegan, taxize, iNEXT, ade4, RColorBrewer and the tidyverse.

4.3. Results

In the ten studied ponds, a total of 36 samples were taken in 12 S_3i mesohabitat types (*Fig. 4.1*). In total 116 taxa were observed, across 89 genera and 53 families (*Table S.4.2*). The taxon richness per sample ranged from 0 to 47 (mean = 23, SD = 13) and was lower in deeper mesohabitat types than in the shoreline mesohabitat types (unequal variances t-test, T = - 4.7, p-value < 0.001). The density of invertebrates ranged also widely among samples, from 0 to 4419 individuals per m^2 (median = 529.5, SD = 1164), but did not differ significantly between deeper and shoreline mesohabitat types (Wilcoxon rank sum test, W = 116, p-value =0.19). Per pond, two to six samples were taken, and macroinvertebrate richness ranged from 32 to 64 taxa (median = 44, SD = 9, *Fig. 4.2*).

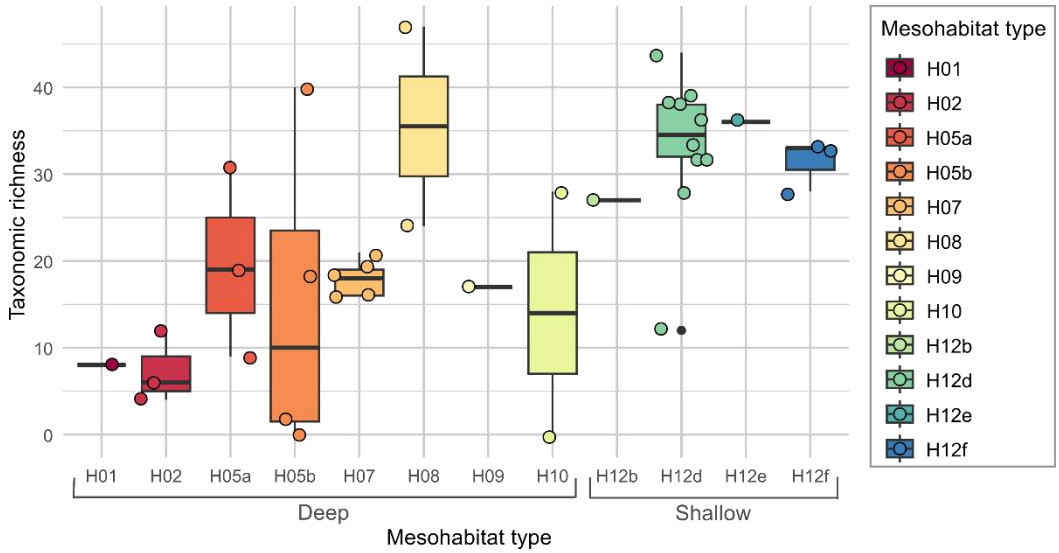


Fig. 4.1. Boxplots of taxonomic richness per mesohabitat type. Scatter points indicate individual samples and colours the different mesohabitat types.

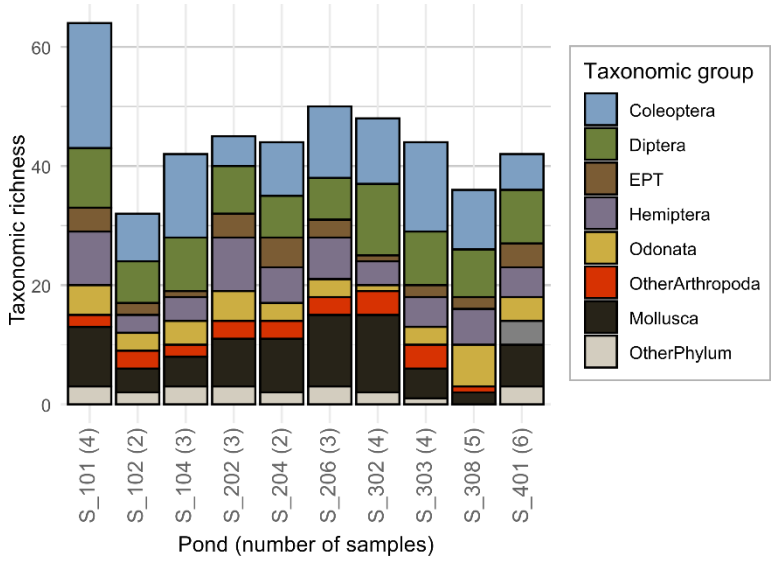


Fig. 4.2. Taxonomic richness per pond. Pond codes on the x-axis with between brackets the number of samples taken in each pond. Colours indicate the higher order taxonomic groups in the legend with EPT standing for Ephemeroptera, Plecoptera and Trichoptera.

4.3.1. Sample completeness

For some of the individual ponds, it is likely that a substantial proportion of the taxa present in the pond was not detected, as indicated by the rarefaction curves per pond (*Fig. 4.3a*), which show a substantial increase in taxonomic richness with the number of samples taken. Especially ponds S_102, S_104, S_202, S_204 and S_206, where only two or three samples were taken, have steep rarefaction curves. The rarefaction curves for ponds S_308 and S_401, where five and six samples were taken, on the other hand, seem to level off, which indicates that a large majority of taxa likely to be present in these ponds was captured. The rarefaction curves for families (*Fig. 4.3b*) flatten out more than those for taxa, whereas the Coleoptera genera curves (*Fig. 4.3c*) indicate a lower sample completeness than those for taxa.

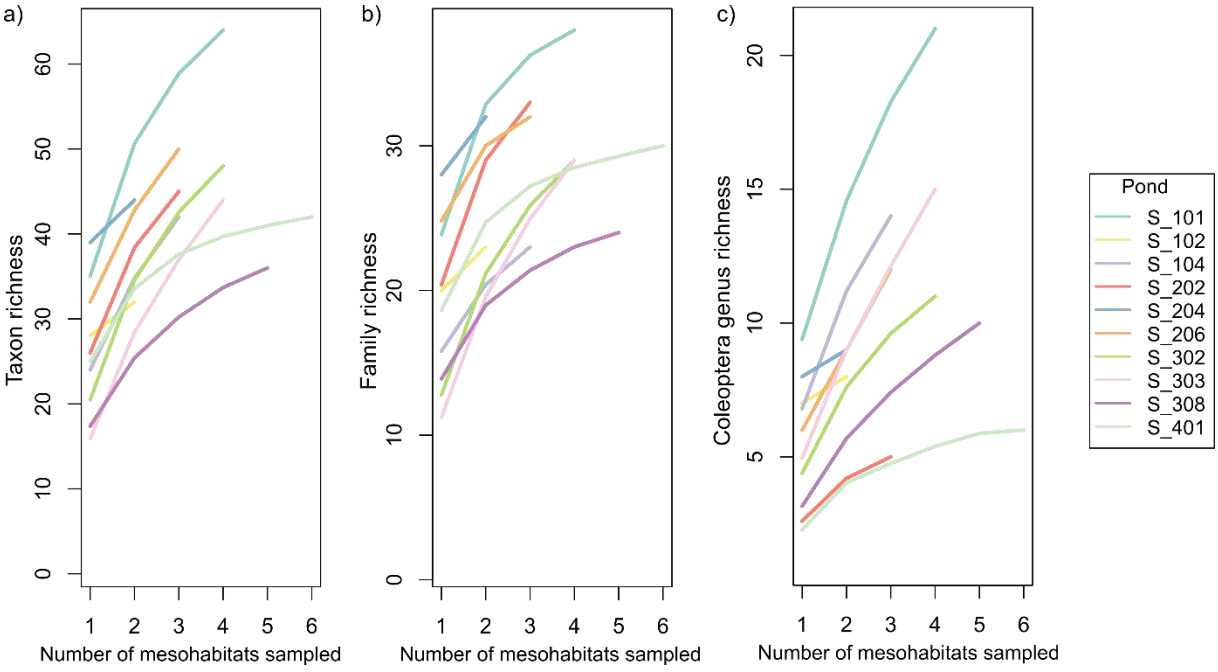


Fig 4.3. Rarefaction curves per pond for a) macroinvertebrate taxa, b) macroinvertebrate families, and c) Coleoptera genera. For clarity, standard deviations are not displayed.

The 116 taxa observed in the ten ponds together, however, likely represent the vast majority of macroinvertebrate taxa present in the ponds. This is inferred from the rarefaction curve for all samples, which approaches an asymptote (*Fig. 4.4*), and the Chao2 estimator (132, SE = 8), which is close to the observed number of taxa.

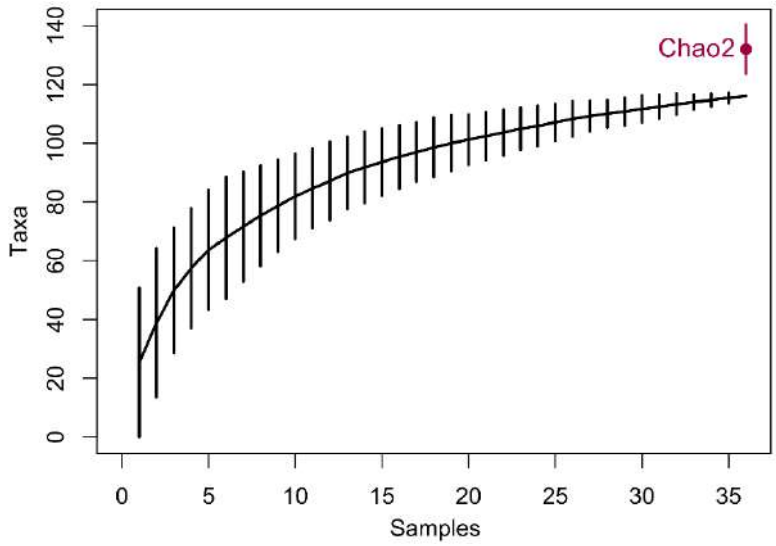


Fig. 4.4. Sample-based rarefaction curve of all samples from all ponds with vertical lines indicating the standard deviation. Above the curve in red the Chao2 estimator and its standard error.

4.3.2. Mesohabitat types and pond-wide environmental variables

Macroinvertebrate community composition was similar among samples within ponds and differed between the ponds. This can be seen from the nearness of samples from the same pond in the nMDS *(Fig. 4.5 a,b*), and the significant result of the ANOSIM of the samples grouped by pond. Some mesohabitat types, especially H12d (small erect herbaceous helophytes with thread-like leaves, for example *Carex*), hosted very different communities in the different ponds (*Fig. 4.5 c,d*). Samples from other mesohabitat types, for example H05b (small floating leaves, for example *Lemna)* were more similar. The ANOSIM showed that samples from the same mesohabitat type were not significantly more similar than samples from different mesohabitat types.

The macroinvertebrate community composition averaged per pond was significantly correlated with the environmental variables in the ponds. The RV coefficient of the coinertia analysis was high and significant (Fig. 4.6). The first coinertia axis represents the most important environmental gradient along which the macroinvertebrate communities were structured. Along this axis ponds are arranged from high levels of nutrients (total nitrogen: TN, total phosphorus: TP) and high algal biomass (chlorophyll-a concentration; Chl-a) on the left to low nutrient levels and high water transparency (Trans) on the right. Along this first axis ponds are also organised according to levels of shade from surrounding trees (Shade), with ponds with heavy shading on the left, and according to the measured concentration of dissolved oxygen (DO) with ponds that had relatively high oxygen concentrations on the right.

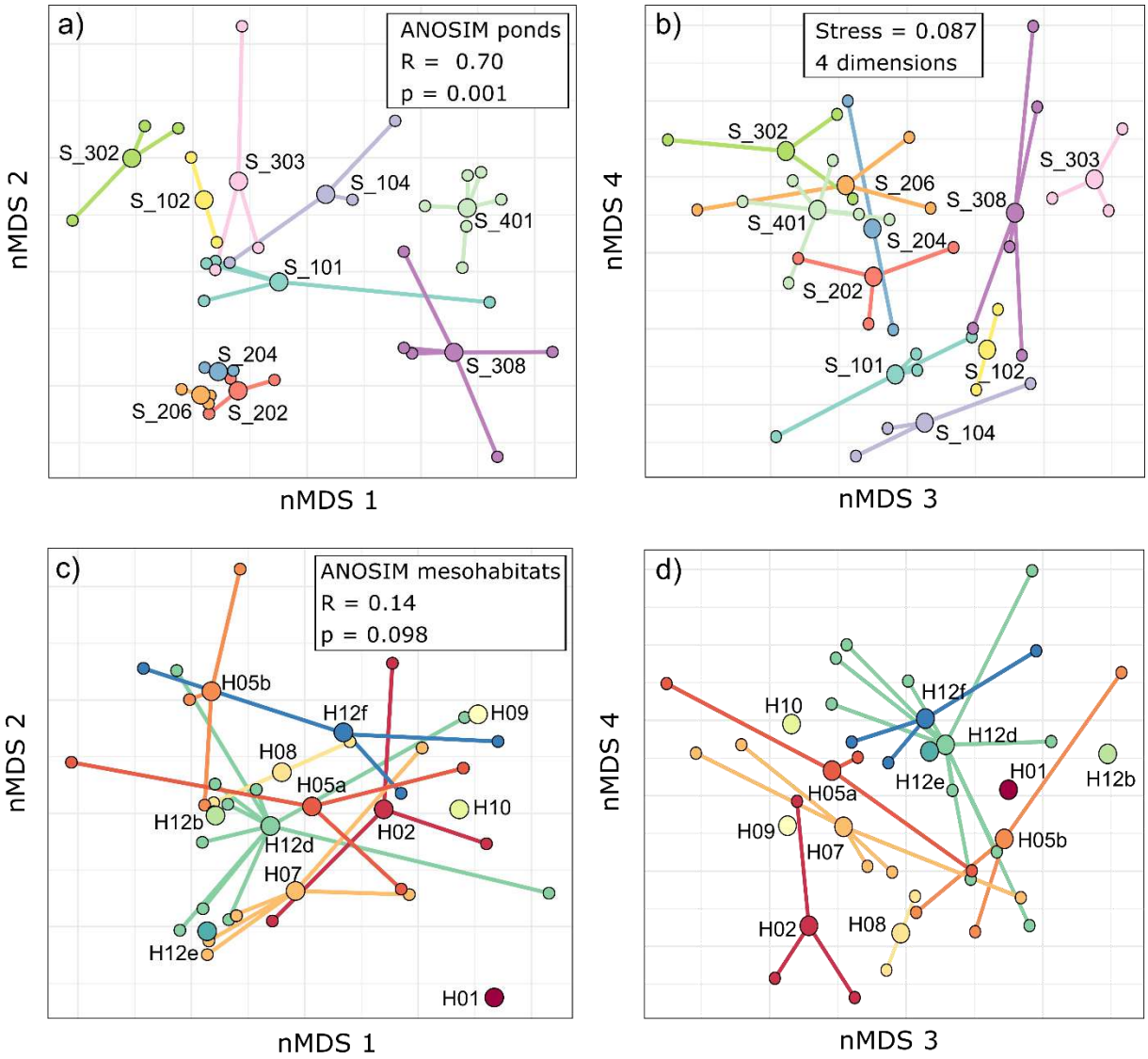


Fig. 4.5. Hellinger dissimilarity nMDS with ANOSIM results in insets. a,b) Spiders connect samples from the same pond and large dots indicate the centre of samples from the same pond. c,d) Spiders connect samples from the same mesohabitat type and large dots indicate the centre of samples from the same mesohabitat type. a,c) nMDS dimensions one and two. b,d) nMDS dimensions three and four.

The pond furthest to the left of the coinertia plane, S_302, had the highest concentrations of total nitrogen (7.1 mg L^{-1}) and total phosphorus (0.54 mg L^{-1}), the second highest chlorophyll-a concentration (the highest was 4.7 µg L^{-1}), was one of the ponds with the lowest transparency measured (10 cm), had the largest proportion of its surface shaded at midday (65%), and had the lowest oxygen concentration (0.69 mg L^{-1}) measured in all ponds. Although pond S_308, on the opposite side of the coinertia plane, had higher nutrient levels than ponds S_104 (in which TN was 1.7 mg L^{-1}), and S_202 (in which TP was 0.05 mg L^{-1}), it was one of the ponds with the lowest chlorophyll-a concentration (1.1 µg L^{-1}), and transparency (175 cm) measured, received no shade at midday, and had the second highest oxygen concentration (8.01 mg L^{-1}) (For environmental variables per pond, see Appendix 4.A).

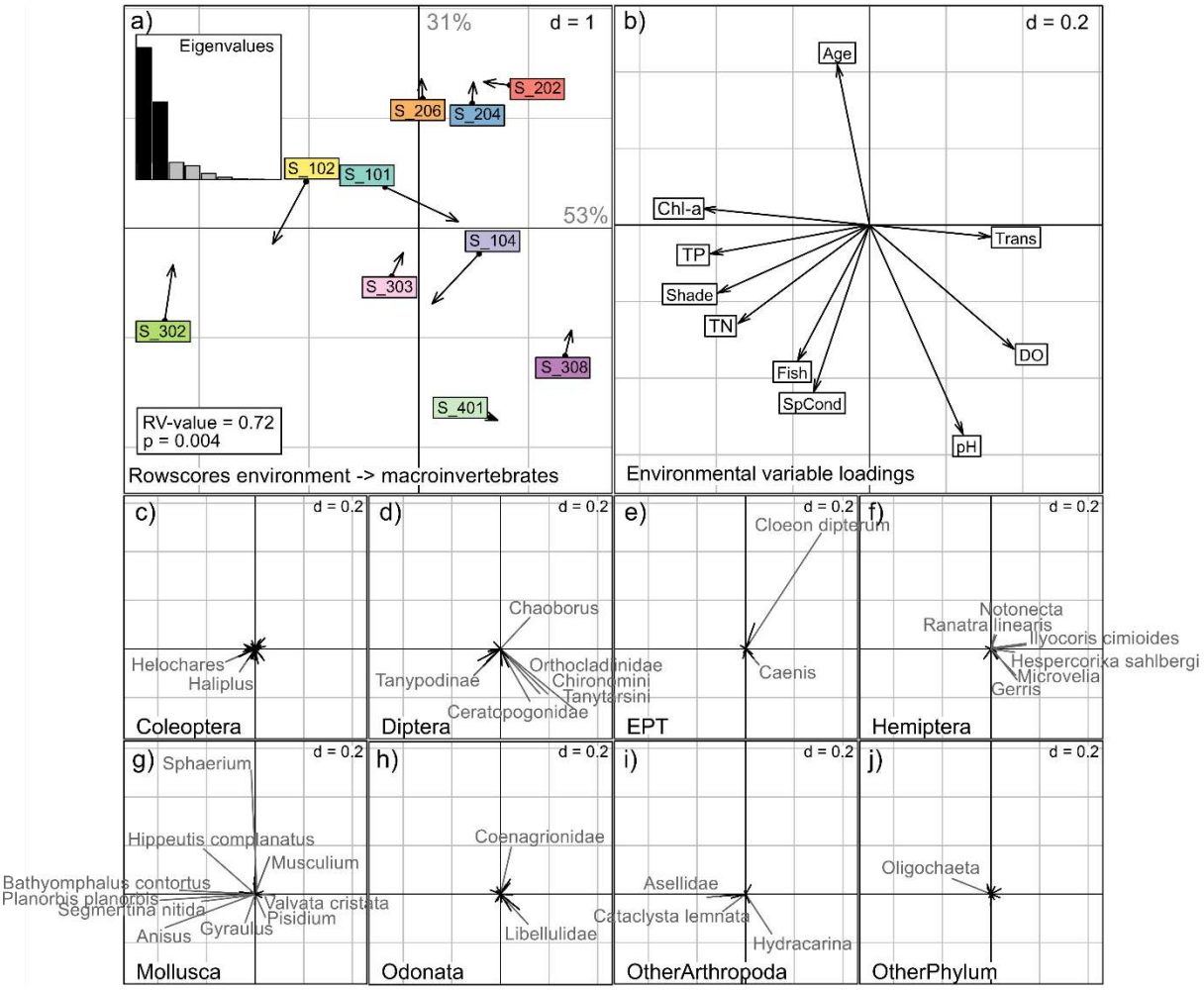


Fig. 4.6. Coinertia environmental variables – macroinvertebrates. a) Positions of the ponds on the plane of the first two coinertia axes (% inertia projected by each axis in grey) according to the environmental variables (dots and labels) and according to macroinvertebrates (arrowheads). The eigenvalue bar plot is in the upper left corner inset and the RV coefficient and p-value are in the bottom left inset. b) Positions of environmental variables on the coinertia plane. c-j) Positions of the macroinvertebrates on the coinertia plane, organised per higher order taxonomic groups. Names of taxa with large contributions are in grey.

The density of Diptera, Ephemeroptera, Trichoptera, Hemiptera and Odonata taxa was highest in the ponds with low nutrient levels and high oxygen concentrations, on the right side of the coinertia plane. However, some of the molluscs, especially the *Planorbidae,* were abundant in the ponds with low oxygen concentrations and high nutrient levels. The abundance of *Anisus* was highest in pond S_302. The *Planorbidae Segmentina nitida,* which is protected in Latvia, *Planorbis planorbis* and *Bathyomphalus contortus* reached their highest densities in ponds S_102 and S_101 and were present in pond S_302 as well. The Lepidoptera *Cataclysta lemnata* was also most abundant in ponds S_102 and S_101, and observed in pond S_302. Lastly, the *Assellidae* were most abundant in pond S_302.

The second coinertia axis, which is less important than the first axis but still projects 31% of the variance, separated ponds S_202, S_204 and S_206, which had very low specific conductivity (SpCond: 78 to 82 µS cm^{-1}) and slightly acidic water (pH 6.4), from ponds S_401 and S_308, which had the highest pH values measured (7.6 and 7.9). The four year old ponds S_102, S_104, S_308 and S_401 were mainly located on the bottom half of the coinertia plane, but the separation with the older (9 year old) ponds was not very clear and there were large differences in community

composition between ponds in the same age class. The ponds with low specific conductivity and slightly acidic water had high densities of *Sphaerium* and *Coenagrionidae*, as did pond S_101, and the highest densities of *Musculium* and *Triaenodes bicolor. Cloeon dipterum* was highly abundant in all ponds except in ponds S_302 and S_401.

4.3.3. Congruence with macrophyte surveys

The Spearman rank correlation between the macrophyte taxonomic richness and the observed macroinvertebrate richness per pond was not significant, nor was the correlation between the macrophyte richness and the richness of macroinvertebrates rarefied to two samples. The correlation between the macrophyte and macroinvertebrate Shannon diversity indices was not significant either (*Fig. 4.7*).

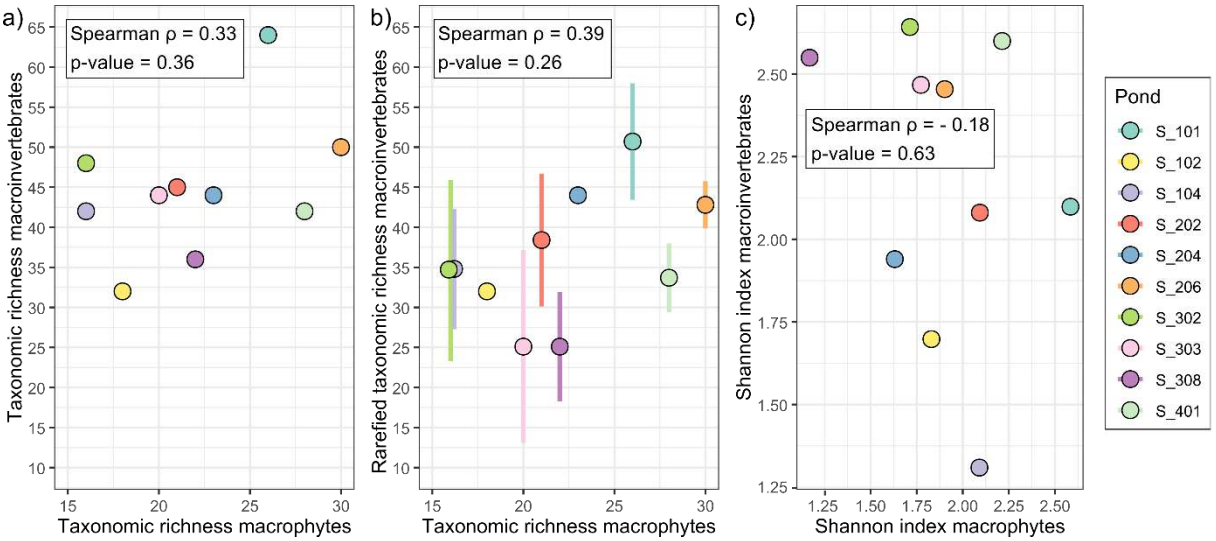


Fig. 4.7. Correlation between macrophyte and macroinvertebrate diversity per pond with results of Spearman rank correlation tests in insets. a) Observed taxonomic richness macrophytes and macroinvertebrates b) Observed macrophyte taxonomic richness and macroinvertebrate taxonomic richness rarefied to two samples with the standard deviation indicated by segments. c) Shannon diversity index macrophytes and macroinvertebrates.

Nonetheless, correlations between the macrophyte and macroinvertebrate community dissimilarities were significant, as shown by the Mantel tests (*Table 4.5*) and coinertia analysis (*Fig. 4.8*). The Mantel correlation was stronger when dissimilarities were calculated from abundance data than when calculated from presence-absence data. Coinertia analysis, only performed on the abundance data, showed strong and significant correlation between the macrophyte and macroinvertebrate communities. The pattern of the positions of the ponds according to their macrophyte and macroinvertebrate community composition on the coinertia plane is very similar to the pattern of their positions according to their environmental variables and macroinvertebrate communities (*Fig. 4.6*), but with the second axis inverted. Only ponds S_302 and S_303 are placed differently with respect to the other ponds on the macrophyte-macroinvertebrate coinertia plane than on the environment-macroinvertebrate coinertia plane.

Table 4.5. Mantel statistics and their significance for the density and presence-absence based Hellinger dissimilarities.

Dissimilarity	Mantel statistic	p-value
Density-based Hellinger	0.30	0.024
Presence-absence Hellinger	0.29	0.042

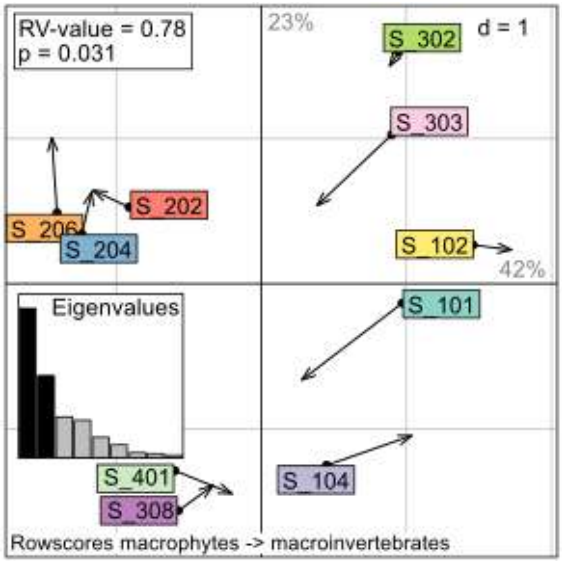
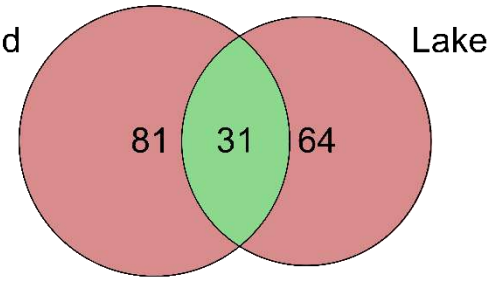


Fig. 4.8. Coinertia macrophytes – macroinvertebrates. Positions of the ponds on the plane of the first two coinertia axes (% inertia projected by each axis in grey) according to the macrophytes (dots and labels) and according to macroinvertebrates (arrowheads). The eigenvalue bar plot is in bottom left corner inset and the RV coefficient and p-value are in the top left inset.

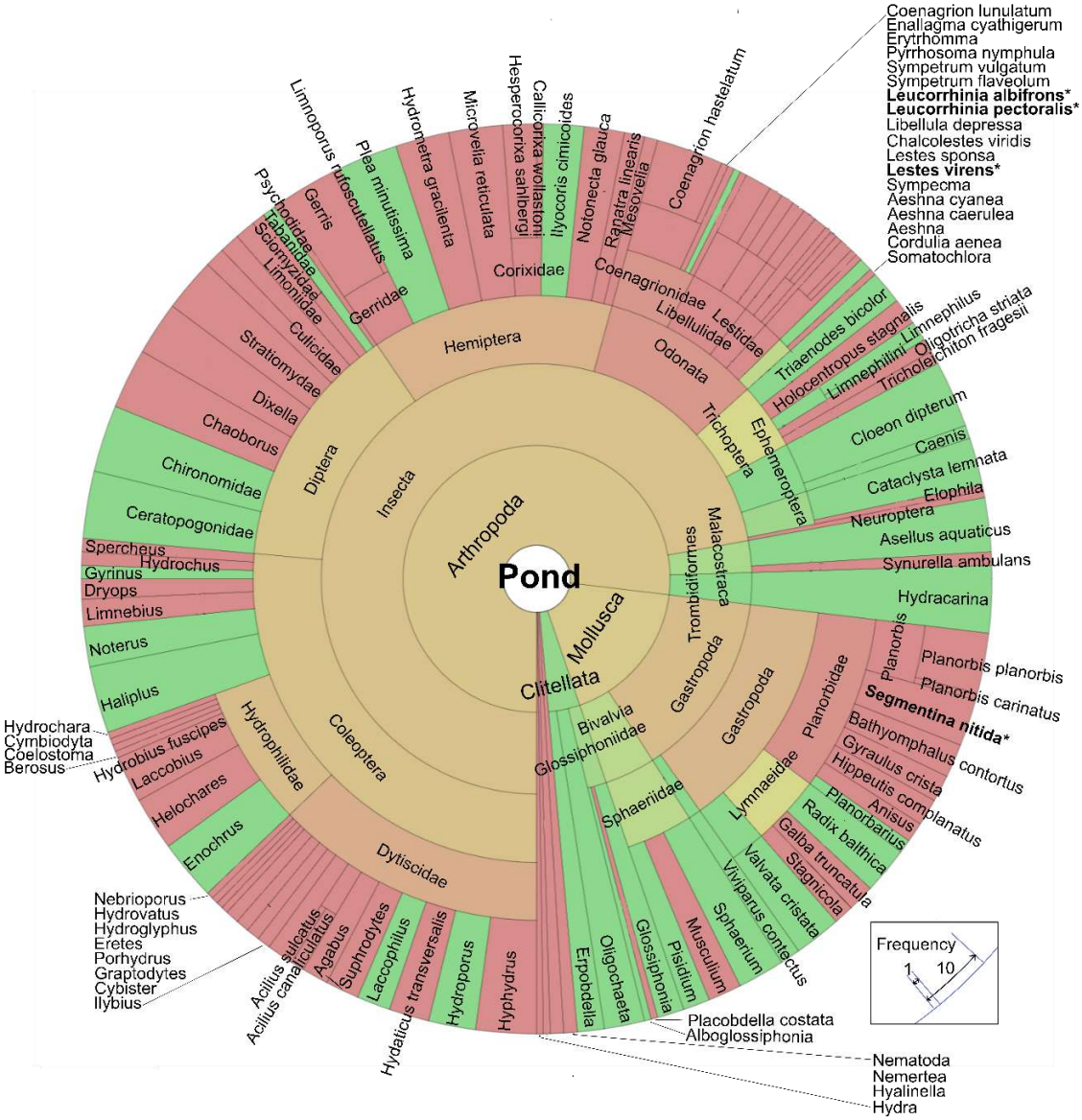
4.3.4. Comparison with lakes

In the ponds and lakes combined, 176 taxa were observed (*Fig. 4.9*). Most taxa were uniquely recorded in the ponds (46% of taxa) or in the lakes (36% of taxa). Only a modest proportion of the taxa (18%) was present in both the pond and lake datasets. For both datasets, the large majority of taxa belonged to the class of insects (75% of taxa in ponds and 69% of taxa in lakes). However, for the pond dataset the order of Coleoptera was most taxon rich (32 taxa), while for the lake dataset the Trichoptera were most diverse (22 taxa). In the ponds more taxa of Coleoptera, Odonata and Hemiptera were detected than the lakes. The lake dataset, on the other hand, included more Trichoptera and Ephemeroptera. The in Latvia protected species *Lestes virens, Leucorrhinia albifrons, Leucorrhinia pectoralis* and *Segmentina nitida* were detected uniquely in the ponds. Protected species in the lake dataset were *Anax imperator* and *Libellula fulva* (Ministru kabineta, 2000).

a)


Pond
Lake
81
31
64

b)


Pond
Arthropoda
Mollusca
Clitellata
Insecta
Malacostraca
Trombidiformes
Gastropoda
Bivalvia
Hemiptera
Odonata
Ephemeroptera
Trichoptera
Diptera
Coleoptera
Corixidae
Coenagrionidae
Libellulidae
Lestidae
Gerridae
Hydrometridae
Notonectidae
Mesoveliidae
Psychodidae
Tabanidae
Sciomyzidae
Limoniidae
Culicidae
Stratiomyidae
Chironomidae
Ceratopogonidae
Dytiscidae
Hydrophilidae
Haliplidae
Noteridae
Limnebiidae
Dryopidae
Gyrinidae
Hydrochidae
Spercheidae
Glossiphoniidae
Sphaeriidae
Lymnaeidae
Planorbidae
Planorbis
Hydracarina
Asellidae
Neuroptera
Elophilidae
Caenidae
Cloeon dipterum
Leptophlebiidae
Limnephilini
Limnephilus
Oligotricha striata
Triaenodes bicolor
Holocentropus stagnalis
Tricholeiochiton fagesii
Coenagrion hastelatum
Coenagrion lunulatum
Enallagma cyathigerum
Erythromma
Pyrrhosoma nymphula
Sympetrum vulgatum
Sympetrum flaveolum
Leucorrhinia albifrons*
Leucorrhinia pectoralis*
Libellula depressa
Chalcolestes viridis
Lestes sponsa
Lestes virens*
Sympecma
Aeshna cyanea
Aeshna caerulea
Aeshna
Cordulia aenea
Somatochlora
Hesperocorixa sahlbergi
Callicorixa wollastoni
Ilyocoris cimicoides
Notonecta glauca
Ranatra linearis
Mesovelia
Microvelia reticulata
Hydrometra gracilenta
Plea minutissima
Limnoporus rufoscutellatus
Gerris
Nebrioporus
Hydrovatus
Hydroglyphus
Eretes
Porhydrus
Graptodytes
Cybister
Ilybius
Acilius sulcatus
Acilius canaliculatus
Agabus
Suphrodytes
Laccophilus
Hydaticus transversalis
Hydroporus
Hyphydrus
Hydrochara
Cymbiodyta
Coelostoma
Berosus
Hydrobius fuscipes
Laccobius
Helochares
Enochrus
Spercheus
Hydrochus
Gyrinus
Dryops
Limnebius
Noterus
Haliplus
Chaoborus
Dixella
Stratiomyidae
Limoniidae
Culicidae
Sciomyzidae
Tabanidae
Psychodidae
Erpobdella
Oligochaeta
Glossiphonia
Pisidium
Musculium
Sphaerium
Viviparus contectus
Valvata cristata
Stagnicola
Galba truncatula
Radix balthica
Anisus
Planorbarius
Hippeutis complanatus
Gyraulus crista
Bathyomphalus contortus
Segmentina nitida*
Planorbis carinatus
Planorbis planorbis
Synurella ambulans
Placobdella costata
Alboglossiphonia
Nematoda
Nemertea
Hyalinella
Hydra
Frequency
1
10

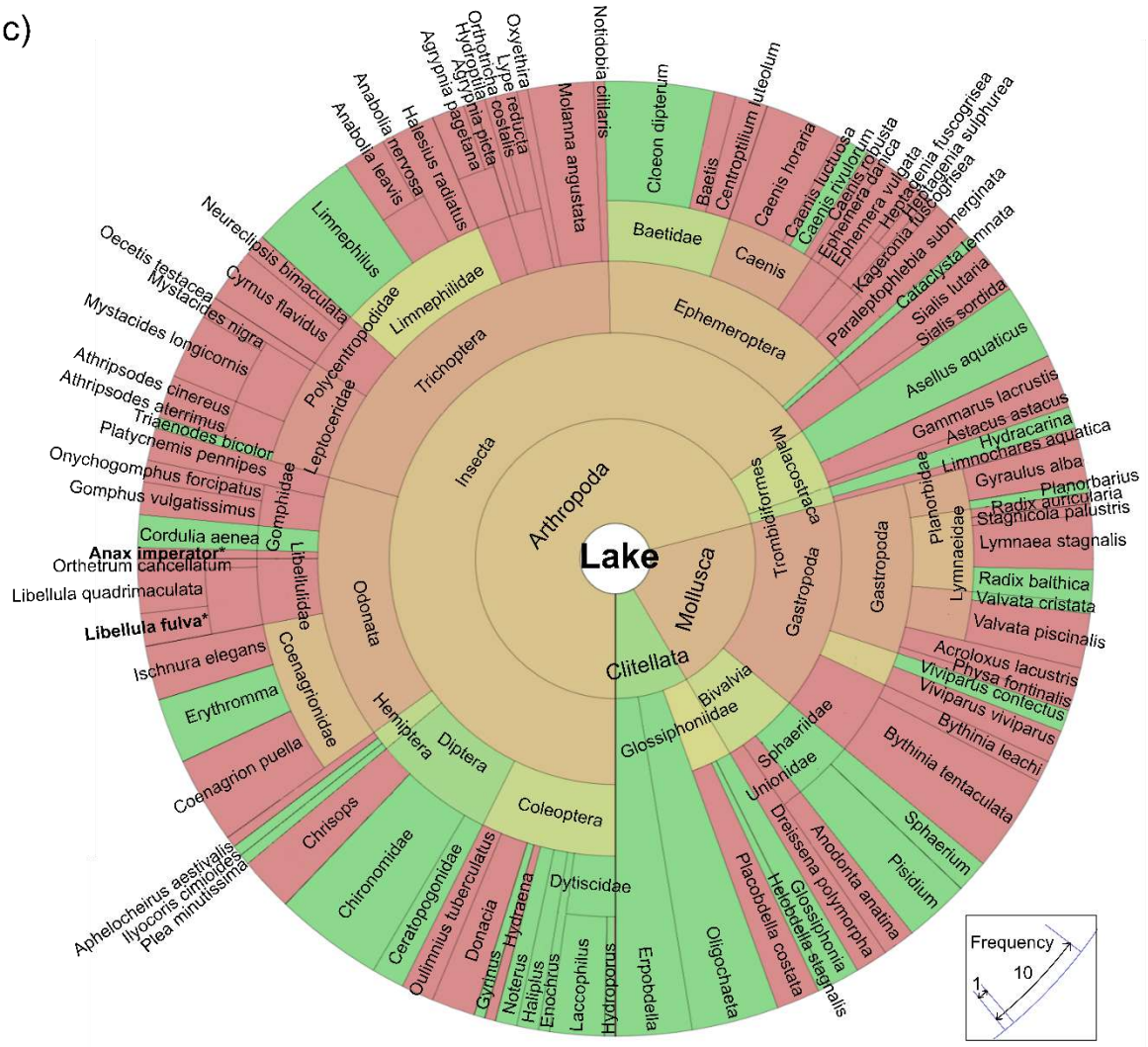


Fig. 4.9. Plots comparing pond and lake macroinvertebrates with taxa occurring in both the lake and pond dataset in green and taxa only occurring only in one of the datasets in red. a) Venn diagram overlap of taxa in studied ponds and WFD lakes. b) Krona plot of taxa in all studied ponds. c) Krona plot of taxa in the ten lakes monitored for the WFD. The outside width of the Krona plot for each of the lowest level taxa indicates the frequency of occurrence, according to the scale in the insets. Species protected in Latvia are indicated in bold and have an asterisk.

4.4. Discussion

Although macroinvertebrates are of key importance for pond ecosystems, and are widely used for pond ecological quality assessment, there is a lack of published pond macroinvertebrate data for many parts of Europe, including the Baltic countries. As an early attempt to collect macroinvertebrate community data for Latvian ponds, ten human-made ponds were sampled following the recently developed S_3i protocol (Labat et al., 2022). In the ten ponds, we took overall 36 samples in 12 distinct S_3i mesohabitat types and detected 116 taxa, which is the vast majority of the 132 ± 8 taxa present according to the Chao2 estimator.

For the individual ponds, the median taxonomic richness was 44. This is higher than the median taxonomic richness of 268 French ponds (median richness = 35, SD = 15, min = 5, max = 79) and not lower than the median taxonomic richness of 112 French least impacted lowland ponds (median = 41, SD = 11, min = 18, max = 68) which were also sampled using the S_3i protocol

(Labat et al., 2022, 2024). Moreover, the median number of samples taken in both French studies was five (Labat et al., 2022, 2024), whereas the median number of samples in our study was 3.5.

I could not estimate the actual number of taxa present in the individual ponds, because richness estimators, such as Chao2, and extrapolation methods would be biased for the low number of samples per pond and the mesohabitat-based sampling. The sample-based rarefaction curves for the individual ponds seemed to indicate that the ponds where five or six samples were taken, were sampled better than the ponds where only two or three samples were taken. Furthermore, the rarefaction curves suggested that for sampling Coleoptera genera, which are relatively rare, more samples would be needed than for the entire macroinvertebrate community, to reach the same degree of completeness. We took one sample per mesohabitat type. To properly estimate how many samples should be taken in individual ponds to reach a certain sample completeness, larger numbers of samples, for example 12 or 15, should be taken in one pond.

4.4.1. Macroinvertebrate structuring at the mesohabitat and pond scale

ANOSIM showed that macroinvertebrate samples differed significantly per pond, but not significantly per mesohabitat type (see *Fig. 4.5*). Multiple explanations are possible for these findings. Firstly, the sampled ponds were densely vegetated and multiple macrophyte species grew next to or on top of each other. We assigned the sampled mesohabitat type according to the dominant growth form, but macrophytes with other growth forms often occurred in the sampled location as well. This mixing of mesohabitat types could explain the lack of distinction between macroinvertebrate communities from the different assigned mesohabitat types. Secondly, the same mesohabitat type may have consisted of different species or densities of macrophytes in different ponds, providing different resources to the invertebrates (Cheruvelil et al., 2002; Rennie & Jackson, 2005).

A third explanation could be that pond-wide variables were more important than mesohabitat type in structuring the macroinvertebrate communities. This explanation is supported by the strong and significant correlation between environmental variables and macroinvertebrate communities per pond, as shown by coinertia analysis. Other studies also found that pond or ditch wide environmental variables were more important than mesohabitat type or macrophyte habitat structure in shaping macroinvertebrate communities (Della Bella et al., 2005; Verdonschot et al., 2012). Furthermore, there are studies indicating that macroinvertebrate communities in a sample could be influenced by macrophytes elsewhere in the waterbody (Cottenie et al., 2001; Cyr & Downing, 1988).

4.4.2. Correlations with environmental variables and macrophytes

Dissolved oxygen concentration, nutrient levels, chlorophyll-a concentration, water transparency and shade from surrounding trees all contributed strongly to the first axis of the coinertia between pond-wide environmental variables and macroinvertebrate community composition (see *Fig. 4.6*). Dissolved oxygen concentration was probably a major factor structuring the macroinvertebrate communities in the studied ponds. We measured the oxygen concentrations only once at the water surface during daytime and it would be interesting to measure it near the sediment and at nighttime, and to measure the biological oxygen demand. However, oxygen concentrations below 3.5 mg L^{-1} were recorded in half of the ponds (*Table S.4.2*), and such low concentrations are limiting for several macroinvertebrates, for example species belonging to the orders of Ephemeroptera and Trichoptera (Gaufin et al., 1974; Resh & Cadré, 2009).

The ponds with low oxygen concentrations were abundant in snails of the family *Planorbidae*, including the protected *Segmentina Nitida*. *Planorbidae* are well-adapted to low-oxygen environments as they take their oxygen from the surface, have lungs and carry haemoglobins with high affinity for oxygen (Jones 1961, Lieb 2006). In pond S_302, which had the lowest

oxygen concentration measured, 0.69 mg L^{-1}, the fish *T. tinca* occurred, which is also adapted to low oxygen conditions. The muscles of *T. tinca* require relatively little oxygen, the fish can obtain oxygen by gulping air above the water surface, and can even enter into a dormant condition where it needs very little oxygen. *Tinca tinca* can survive oxygen concentrations as low as 0.4 mg L^{-1} (Beelen, 2008).

To further explore relationships between macroinvertebrate communities, mesohabitat types and pond-wide environmental variables, I performed a principal component analysis of the community weighted means of a range of macroinvertebrate traits that hypothetically could relate to pond-wide environmental variables and mesohabitat type (Appendix 4.B). Sample community weighted means were also more clearly separated according to pond than according to mesohabitat type (Appendix 4.B, *Fig. S.4.2m,n*). Communities of different ponds had distinct dominant respiration types, but this was not clearly organised according to measured dissolved oxygen concentration (*Fig. S.4.2k*). It is likely that the fuzzy coded trait respiration type and the restricted number of trait modalities in the trait database used (Tachet et al., 2010) did not allow for clear separation according to oxygen levels. For example, atmospheric breathing (Chapman et al., 2004) is not included in the database, and *Planorbidae* are assigned tegument breathing.

Effects of oxygen depletion and eutrophication on pond macroinvertebrate communities are hard to disentangle (Biggs & Williams, 2024). The two ponds with the lowest oxygen concentrations S_302 and S_102 had extensive cover of free floating macrophyte cover (**Chapter 3**, *Fig. 3.2*). Floating macrophytes can deplete oxygen levels in the water beneath them (Caraco et al., 2006). As described in **Chapter 3**, free floating macrophyte dominance in Silene may have been related to a combination of high nutrient levels and surrounding trees which generate shade and shelter the pond from wind. Dissolved oxygen concentration was only moderately correlated with nutrient levels (Spearman's ρ with TN -0.35; with TP -0.51). This is perhaps because high sediment oxygen demand may have been another main reason for low oxygen levels. Sediment oxygen demand can cause hypoxia (Cross & Summerfelt, 1987). Except for pond S_102, ponds with low oxygen concentrations had thick layers of oozy organic sediment (K. van der Zon, pers. obs.).

Relations between nutrients and macroinvertebrates are complex and may be mediated by macrophytes (Declerck et al., 2011). In the studied ponds nutrients concentrations had a major contribution to the strong coinertia between the environmental variables and macroinvertebrate communities, and coinertia analysis and mantel tests showed that the macroinvertebrate and macrophyte communities were strongly correlated. It could be that 1) nutrient levels influenced macrophyte communities, which influenced the macroinvertebrate communities, or that 2) nutrients may have both influenced the macroinvertebrate and macrophytes communities simultaneously. The relationship between nutrient levels and macrophyte communities was probably mediated by increased algal levels, hence turbidity, and floating macrophyte dominance (**Chapter 1**, *Fig 1.6*, **Chapter 3**, Scheffer & Van Nes, 2007). In the studied ponds, nutrient and chlorophyll-a concentrations were positively correlated with each other and negatively correlated with water transparency (Spearman's ρ>0.7, Appendix, 4.A, *Fig S.4.1*). However, it is not sure if the nutrient levels themselves impacted the macroinvertebrates or that this relationship was mediated by the macrophytes. In a factorial experiment on the influence of nutrient levels and dominant macrophyte growth form on macroinvertebrate community composition, Declerck et al. (2011) found that only macrophyte growth form had a direct effect on epiphytic macroinvertebrate community composition and nutrient levels had not. The study by Declerck et al. (2011) was relatively short but points at explanation 1) a macrophyte mediated relation between nutrient levels and macroinvertebrate community composition.

4.4.3. Comparison between human-made ponds and lakes

The studied ponds hosted in total 81 taxa that were not present in the most nearby lakes monitored by the Latvian government. Four species among these, *Lestes virens*, *Leucorrhinia albifrons*, *Leucorrhinia pectoralis*, and *Segmentina nitida,* were protected in Lativa. Differences in taxa present in the pond and lake datasets may be related to differences between the sampling protocols used, or to different sampling seasons and years. Whereas the human-made ponds were sampled in July 2022, the lakes were sampled in months varying from April to October and in the years 2016 to 2023 (*Table 4.4*). Furthermore, both datasets were probably not exhaustive, as indicated by the substantial proportion of observed taxa only found in a single waterbody.

However, it is possible that the ponds hosted different macroinvertebrate communities than the lakes. All lakes contained multiple species of fish (between five and 18 species observed since 1972, Latvijas ezeri, n.d.). Fish are known to have a large influence in macroinvertebrate community structure (Wellborn et al., 1996, Marklund, 2002, Scheffer 2006), and many Coleoptera, and some Hemiptera species do not coexist with fish (Biggs and Williams 2024, Cook 1984, de Mendoza 2012, Wellborn et al., 1996). It may be that the ponds had richer Coleoptera and Hemiptera communities than the lakes because the ponds were mostly fishless. Studied ponds S_302 and S_401, did contain *Tinca tinca* and *Perccottus glenii*, respectively, and did have Coleoptera and Hemiptera. However, pond S_401 had the lowest Coleoptera richness of all ponds studied, and despite the rich vegetation and large number of mesohabitats sampled, this pond was not particularly rich in macroinvertebrates.

The observation that the lakes were richer in Ephemeroptera and Trichoptera than the ponds is harder to explain. Ephemeroptera and Trichoptera are sensitive to dissolved oxygen concentrations (Gaufin et al., 1974; Resh & Cadré, 2009), but some ponds did have high oxygen concentrations and oxygen concentrations varied highly between the lakes and over time (Latvijas Vides ģeoloģijas un meteoroloģijas centrs, 2020). Other studies also found low Ephemeroptera richness in ponds (Friday, 1987; Menetrey et al., 2008). Gee et al., (1997) found that the species richness of Ephemeroptera and Trichoptera in ponds decreases as the percentage of the pond margin that is shaded by surrounding trees increases. They give several potential explanations for this finding, including the possibility that it is related to the behaviour of the flying adults. Future research on the diversity of Colopetera, Heteroptera, Ephemeroptera and Trichoptera in different types of aquatic habitats could be performed to verify if these groups use different types of habitats. If this is the case, mechanisms behind this pattern, for example relating to the behaviour of flying adults, could be investigated.

4.4.4. Implications for pond macroinvertebrate surveys

Sampling and identifying macroinvertebrates are time consuming, and I investigated whether macrophyte surveys could be a good proxy for macroinvertebrate assessments. Although the community composition of the macroinvertebrates and macrophytes in the studied ponds was correlated, there was no significant correlation between their taxonomic richness or their Shannon diversity index. It could be that I found no significant relation because of the small number of ponds studied. Studies on larger number of ponds in the United Kingdom found either no significant correlation between the richness of macrophytes and the richness of molluscs, dragonflies and beetles, when studying 83 ponds (Law et al., 2019), or a significant but weak correlation between macrophyte and macroinvertebrate richness, when studying 425 ponds (Hassall et al., 2011). Law et al. (2019) showed that the richness of molluscs, dragonflies and beetles could be predicted from the macrophyte richness when accounting for water quality, pond physical morphology and land use around ponds. However, accounting for these variables may be impractical. In general there is little support for the use of surrogates in freshwater systems (Heino 2010, de Morais 2018). Even though macrophytes are principal determinants of

macroinvertebrate communities, macrophyte and macroinvertebrate diversity may respond to different drivers and stressors (Friday, 1987; Hassall et al., 2011). Furthermore, the communities have different successional trajectories (Hassall et al., 2012). Therefore, I would not recommend the use of macrophyte surveys surrogates for macroinvertebrate assessments.

A potential solution to time constraints in macroinvertebrate community assessment could be use of environmental DNA (eDNA) metabarcoding (Harper et al., 2019). Although eDNA metabarcoding methods for pond amphibian detection can be as reliable as traditional amphibian surveying methods (Moss et al., 2022; Schwesig et al., 2025), eDNA methods for the detection of pond macroinvertebrates communities still have to be improved (Harper et al., 2019; Hill et al., 2021; Schwesig et al., 2025). The use of eDNA metabarcoding to study pond communities will be further discussed in **Chapter 5**.

For the moment I would recommend traditional sampling for the study of pond macroinvertebrate communities. Methods using hand netting are the most reliable for this (García-Criado & Trigal, 2005). Based on our observations the S_3i sampling method is suitable for the study of Baltic ponds, as long as a minimum number of samples is taken. The S_3i protocol is based on the sampling methods in the PSYM and IBEM protocols, which are also tested and standardized protocols that employ hand netting of invertebrates (Angélibert et al., 2010; Howard, 2002; Indermuehle et al., 2010; Labat et al., 2022). Depending on the goal of the survey, the sampling methods used in the three protocols may appropriate for Baltic ponds. Macroinvertebrate sampling methods that allocate equal sampling effort to each pond, such as in the PSYM protocol (Howard, 2002) have the advantage that they simplify the comparison of diversity among ponds. Advantages of the S_3i sampling protocol over the PSYM sampling protocol are, that it describes a specific way of back and forth sweeping that creates a vortex which seems to dislocate the invertebrates effectively from their substrate, and that it explicitly samples shallow mesohabitats, which have a higher taxonomic richness than deeper mesohabitats. An advantage of the S_3i over the IBEM sampling method is that it often requires less effort (Labat et al., 2022).

4.4.5. Implications for the evaluation of pond creation

After determining the macroinvertebrate richness and community composition of human-made ponds, the value of this diversity needs to be evaluated to assess the benefit of the pond creation intervention. Several strategies could be envisaged to evaluate the biodiversity value of human-made ponds. Most commonly, the success of pond creation is evaluated by comparing human-made ponds to natural ponds in the same area (Coccia et al., 2016; Kentula, 2000; Reyne et al., 2021; Ruhí et al., 2013; Thiere et al., 2009). A second strategy is to measure the quality of the human-made ponds using a suitable index that is calibrated on good quality and degraded ponds in the study region. The S_3i sampling method was developed with the BECOME multimetric index which is calibrated for France (Labat & Usseglio-Polatera, 2023). Multimetric pond quality indices exist also for the UK, Swiss lowlands, central Italian mountains and North Iberian Spain (Howard, 2002; Menetrey et al., 2011; Solimini et al., 2008; Trigal et al., 2009), but not for the Baltic countries. To develop an ecological quality index for Baltic ponds, metrics should be selected that respond to stressors that impact ponds in this region, and calibrated on sets of ponds in good and in degraded ecological states (Labat & Usseglio-Polatera, 2023; Menetrey et al., 2011; Solimini et al., 2008; Trigal et al., 2009).

These two evaluation strategies are, however, not entirely satisfactory, because they only focus on the human-made ponds themselves, and not on their contribution to the maintenance or recovery of freshwater diversity in the region. A third and more relevant strategy is therefore to evaluate the freshwater diversity in human-made ponds in the context of other freshwater habitats in the landscape, such as lakes, temporary pools, swamps, ditches, rivers, backwaters and streams (Kentula, 2000; Remm et al., 2015; Sayer, 2014). By comparing the taxa found in human-

made ponds to nearby lakes I started employing such a landscape approach. To proceed in this direction other types of water bodies should be included, and a standardized sampling protocol should be employed (such as in Davies et al., 2008; Vaikre et al., 2018; Williams et al., 2004). Furthermore, it would be instructive to study the trajectories of human-made ponds over time (Kentula, 2000).

4.4.6. Implications for pond creation and management

The human-made ponds studied had median species richness similar to that of well-preserved natural ponds in France. This finding adds to the literature showing that human-made ponds can provide habitat for diverse macroinvertebrate communities (Coccia et al., 2016; Thiere et al., 2009; Williams et al., 2008). Furthermore, the human-made ponds hosted species that were not found in the most nearby lakes monitored by the Latvian government, including the protected species *Lestes virens*, *Leucorrhinia albifrons*, *Leucorrhinia pectoralis*, and *Segmentina nitida*. Pond creation may therefore be a tool to enhance macroinvertebrate diversity. The macroinvertebrate communities were more structured per pond than per mesohabitat-type. This indicates that pond creation should focus on the creation of a large number of ponds. Furthermore, there was a strong correlation between pond-wide environmental variables and macroinvertebrate communities. This correlation suggests that creating ponds varying in their environmental conditions can, perhaps partly mediated by macrophyte communities, contribute to increasing macroinvertebrate gamma diversity. Even ponds with low oxygen levels supported protected species, which underlines the value of ponds with various environmental conditions.

Chapter 5

Bleaching, filtering, bleaching, filtering. Iris and Melina kept smiling while sampling eDNA

Chapter 5: Benchmarking environmental DNA metabarcoding in European freshwater ponds

5.1. Introduction

Macrophytes, macroinvertebrates and amphibians are suitable indicator groups for the evaluation of created and restored ponds (**Chapter 1**). However, macrophyte and macroinvertebrate monitoring using the methods described in **Chapters 3** and **4** require taxonomic expertise. The same is true for amphibian monitoring using visual and audio encounter surveys and larval sampling (Bálint et al., 2018). Furthermore, macroinvertebrate identification is time consuming and requires the sacrificing of sampled specimens.

Evaluation of pond creation and restoration could entail the monitoring of ponds over time, or comparison with natural ponds or other freshwater habitats (**Chapter 4**). Ponds may be difficult to access and even nearby ponds can differ substantially in their community composition, which necessitates the inclusion of a substantial part of them in monitoring strategies (Harper et al., 2019; Hill et al., 2021; Robertson, 2024). When involving multiple surveys and samplings over time, or numerous ponds are to survey and sample, the effort required to evaluate pond creation and restoration may be considerable. As conventional methods are time consuming, faster methods that enable the monitoring of multiple taxonomic groups in large numbers of ponds by few operators would be highly beneficial.

Environmental DNA (eDNA) metabarcoding was announced to revolutionize aquatic biodiversity monitoring by providing a non-invasive, sensitive, cost and time efficient complement to conventional monitoring. It would also allow the detection of multiple taxonomic groups from a single sample (**Chapter 1**, Baird & Hajibabaei, 2012; Blackman et al., 2024a; Schenekar, 2023). The number of eDNA metabarcoding studies for biomonitoring has been growing considerable since 2012, and there have been many comparisons between eDNA metabarcoding and conventional methods (Blackman et al., 2024a; Iacaruso et al., 2025; Keck et al., 2022). However, despite its potential as a valuable tool for monitoring large numbers of ponds at multiple points in time, there is a lack of studies comparing eDNA metabarcoding and conventional methods in ponds (Harper et al., 2019; Hill et al., 2021; Robertson, 2024).

In ponds, there have been single species studies (i.e. not employing metabarcoding) on the detection of specific invasive and threatened amphibians, fish, reptiles, invertebrates and plants (e.g. Ficetola et al., 2008; Fujiwara et al., 2016; Mauvisseau et al., 2018; Mayne et al., 2024; Raemy & Ursenbacher, 2018). In the UK, single species eDNA detection from pond water has even been implemented in great crested newt (*Triturus cristatus*) surveys (Rees et al., 2014). However, metabarcoding of pond eDNA has been primarily restricted to communities of fish, amphibians and microorganisms (e.g. Bálint et al., 2018; Duleba et al., 2021; Evans et al., 2017; Gumińska et al., 2021; Li et al., 2019; Peixoto et al., 2023; Skelton et al., 2023). To my knowledge, only four studies (Harper et al., 2021; Krol et al., 2019; Robertson, 2024; Schwesig et al., 2025) compared invertebrate communities and only one PhD thesis compared macrophyte communities (Robertson, 2024) identified with conventional methods and eDNA metabarcoding in ponds.

The dynamics of environmental DNA may differ markedly among study systems (Harper et al., 2019; Schenekar, 2023). In ponds, especially in summer, water mixing may be limited, and eDNA can be heterogeneously distributed. Because of this heterogeneous distribution, the detection of caged salamanders, for example, depends on the depth, distance to the cage, wind direction and presence of vegetation at the location where eDNA samples are taken (Mayne et al., 2024). Furthermore, the turbidity of pond water could restrict the volume of water that can be passed

through a filter to collect the eDNA (Harper et al., 2019). As a consequence of characteristics such as heterogeneity and turbidity, results from comparisons between eDNA metabarcoding and conventional methods in rivers and lakes cannot directly be translated to ponds (Harper et al., 2019). Pond specific studies on eDNA metabarcoding for biomonitoring are therefore still needed.

This study aims to help fill in the knowledge gap regarding eDNA heterogeneity by comparing the composition of unassigned DNA sequences between samples from different mesohabitats in nine ponds. Also with regards to heterogeneity, it is evaluated if aquatic and amphibian species are detected in multiple samples from the same pond. Furthermore, conventional macrophyte, macroinvertebrate, and amphibian inventories are compared with eDNA metabarcoding outcomes. Lastly, this study aims to detect fish from the eDNA samples, as fish can have large impacts on pond invertebrate and amphibian communities (Hecnar & M'Closkey, 1997; Labat et al., 2024).

My research questions are: 1) do unassigned DNA sequences differ between samples from different ponds and between mesohabitats within ponds? 2) Are the same aquatic and amphibious species detected in samples from the same mesohabitat, and in samples from the same pond? 3) How do conventional and eDNA metabarcoding inventories compare in the composition of detected macrophyte, macroinvertebrate and amphibian species and genera? 4) Which fish species are detected by eDNA metabarcoding in the studied ponds?

5.2. Material and methods

5.2.1. Sampling

In July 2022, macroinvertebrate, macrophyte and eDNA surveys and samplings were performed in nine ponds in study site Silene, described in **Chapter 2** and **Chapter 3** (*Table 5.1*). In five of these ponds, larval caudata were sampled with ten hand net sweeps per pond, as described in (Pupins et al., 2023). To minimise cross-contamination and disturbance of sediment, eDNA samples were taken before invertebrate sampling and macrophyte surveys were performed. In case caudata larva and eDNA sampling were performed on the same day, the eDNA sampling was performed first. Vocalizing amphibian monitoring data, collected by Čeirāns et al., (2024) earlier in the year, was available for four of the studied ponds.

Before sampling, mesohabitats were identified according to the Sampling of Small Shallow lake invertebrates (S_3i) protocol (Labat et al., 2022). A mesohabitat is a visually distinct habitat within the pond that can be identified based on the plant and sediment structure (Della Bella et al., 2005). The S_3i protocol provides a list of potential mesohabitats (**Chapter 4,** Table 4.2, Labat et al., 2022). In each mesohabitat, first duplicate surface water samples for eDNA metabarcoding where taken, followed by one macroinvertebrate sample. Macroinvertebrate sampling procedure is described in **Chapter 4**. After the sediment disturbed by the macroinvertebrate sampling had settled, macrophyte surveys were performed according to the methods described in **Chapter 3**.

Table 5.1. Environmental DNA (eDNA) and conventional surveys and samplings of macroinvertebrates (MI), macrophytes (MP), caudata larvae (CL) and vocalising anurans (VA) in Silene ponds, with the number of mesohabitats (MH) sampled per pond and the dates as dd-mm. All sampling and surveying was performed in 2022.

Pond	Latitude	Longitude	Pond surface area (m^2)	Number MH	Date eDNA and MI	Date MP	Date CL	Date VA
S_101	55.69296°	26.78692°	1050	4	22-07	22-07	22-07	17-04; 26-04; 28-05; 05-06
S_102	55.69361°	26.78832°	290	2	21-07	22-07		
S_104	55.69513°	26.79063°	220	3	22-07	22-07		
S_202	55.69099°	26.78913°	110	3	21-07	21-07	22-07	17-04; 26-04; 28-05; 05-06
S_204	55.69058°	26.78833°	180	2	21-07	24-07		
S_206	55.68982°	26.78766°	150	3	21-07	24-07	22-07	17-04; 26-04; 28-05; 05-06
S_302	55.68420°	26.77315°	110	3	19-07	19-07	22-07	
S_303	55.68435°	26.77194°	130	3	19-07	19-07	22-07	17-04; 26-04
S_308	55.68569°	26.76755°	180	4	20-07	21-07		

Duplicate surface water eDNA samples were taken from the shore using two bleached 250 mL Nalgene bottles per mesohabitat (see Appendix 5.A for the sampling protocol). When mesohabitats could not be reached from the shore, samples were taken by wading into the pond and quickly taking the two samples as far away as possible from the places where the operator had walked. Samples were kept cool until filtering on the same day. Gloves worn during eDNA sampling were changed between each mesohabitat. Gloves were worn in all subsequent eDNA handling steps and changed between each step. All material in contact with eDNA samples was new or had been immersed in 10% bleach solution for at least 15 minutes. Only the waders could, for logistical reasons, not be bleached between each mesohabitat and had only been bleached once before the sampling campaign.

Enclosed filter capsules, such as Sterivex capsules, have been recommended for aquatic eDNA sampling as they pose less contamination risk than open or housed filters that require handling of the filter membrane (Bruce et al., 2017). Samples were filtered through 0.45 µm PVDS Millipore Sterivex filter capsules using a Bürkle vampire sampler peristaltic pump. In case a filter capsule clogged before the entire sample was filtered (i.e. when only single droplets would come out), filtration was stopped, and the filtered volume was noted. After filtration, 2 mL Longmire's buffer was added to each filter capsule (Longmire et al., 1997). It has been shown that eDNA on filters preserved in Longmire's buffer at room temperature does not degrade within two weeks (Spens et al., 2017). Each filter capsule was sealed with a Luer inlet stopper and parafilm, as for logistical reasons no fitting Luer outlet stoppers were available to close the filter capsules in the field. Sealed filters were placed in a plastic bag and frozen at -80 °C within two weeks

Every sampling day a negative rinsing water control and a negative air control were taken. The rinsing water control consisted of a 250 mL sample of the tap water that was used to rinse the material after bleaching. This sample was taken using one of the bleached Nalgene bottles, carried into the field, opened and closed and then treated like the pond water samples. The negative air control was taken by filtering air for five minutes through a clean filter capsule.

5.2.2. Environmental DNA metabarcoding

DNA extraction was performed in a laboratory in Germany dedicated to the handling of DNA and RNA samples before polymerase chain reaction (PCR). All pipetting steps were performed in a VWR PCR Workstation, which was decontaminated with UV light and PanReac AppliChem DNA/RNA-ExitusPlus before and after every extraction batch. Extraction was performed following a protocol adapted from the modified version of Aljanabi and Martinez's (1997) “high salt” protocol described in Kusanke et al., (2020), with adjustments for Sterivex filter capsules stored in Longmire’s buffer (see Appendix 5.B for the extraction protocol). The extraction involved lysis with Longmire’s buffer and proteinase K inside the Sterivex capsule, followed by NaCl addition, centrifugation and isopropanol precipitation of DNA from the supernatant. Precipitated DNA was washed twice with ethanol and eluted in water. In every extraction batch, one negative extraction control, consisting of a Sterivex capsule filled with Longmire’s buffer, was included.

The concentration of DNA from samples, negative field and negative extraction controls was measured using a Quantus Fluorometer and QuantiFluor double stranded DNA kit. All field negative controls were included in the following steps, but extraction negative controls were only included if the DNA concentration was higher than the Quantus Fluorometer detection limit. For a selection of samples DNA purity was assessed with a Implen NanoPhotometer Pearl spectrophotometer. Eluted DNA was stored at -20 °C until PCR amplification.

A multiplex metabarcoding strategy was employed, which combines multiple primer pairs per PCR (De Barba et al., 2014). Seven primer pairs were used to amplify plant, invertebrate and vertebrate DNA in four PCR reactions (*Table 5.2*, Werner et al., Submitted). For plants, multiple markers were used, as recommended by Espinosa Prieto et al., (2024). The internal transcribed spacer 2 (ITS2) nuclear ribosomal DNA marker was chosen because of its good taxonomic resolution for seed plants. This marker was complemented by two common plant markers that are more universal, but have less discriminatory power, namely the chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene and the chloroplast trnL (UAA) intron (Espinosa Prieto et al., 2024). For each marker we chose a primer pair targeting a short region (<400 base pairs), to enable amplification of degraded DNA fragments (Ficetola et al., 2021; Taberlet et al., 2018), and sequencing on 150 and 250 base pair (bp) paired-end systems.

For invertebrates, a part of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified. The COI barcode has good taxonomic resolution, and is the marker with the most complete refence databases for invertebrates. However, COI is a protein-coding gene with codon degeneracy, which complicates universal invertebrate primer design (Leese et al., 2021; Taberlet et al., 2018). Aquatic insect metabarcoding is nevertheless most commonly performed by amplifying regions of the COI gene using highly degenerate primer pairs (Takahashi et al., 2023). For molluscs, however, the 16S mitochondrial ribosomal RNA (16S) gene is often preferred. This is because there are many mollusc reference sequences available for the 16S marker, as it is commonly used for freshwater bivalve phylogenies (e.g. Bespalaya et al., 2024), and because the 16S gene has conserved regions for which metabarcoding primers have been designed (Klymus et al., 2017; Mulero et al., 2021; Prié et al., 2021). For fish, it has become an established practice to amplify the mitochondrial 12S ribosomal RNA gene (Blackman 2024a). The 12S-V5 primer pair (Riaz et al., 2011) was chosen for our study because it amplifies both fish and amphibian DNA (Brys et al., 2021).

Table 5.2. Metabarcoding primer pairs used in the four multiplex (MP) reactions with reference expected length and target taxonomic group.

MP	Primer	Sequence (5'->3')	Reference	Expect length	Taxon. group
1	ITS-3p62p1F1	ACBTRGTGTGAATTGCAGRATC	Kolter & Gemeinholzer, 2021	183-271 bp	Vascular plants
	ITS-4unR1	TCCTCCGCTTATTKATATGC	Kolter & Gemeinholzer, 2021		
	Unio01_F	GCTGTTATCCCCGGGGTAR	Prié et al., 2021	137 bp	Unionid mussels
	Unio01_R	AAGACGAAAAGACCCCGC	Prié et al., 2021		
2	rbcL mini	CTTACCAGYCTTGATCGTTACAAAGG	Erickson et al., 2017	379 bp	Vascular plants
	rbcL a-R	GTAAAATCAAGTCCACCRCG	Kress & Erickson, 2007		
	mlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC	Leray et al., 2013	350 bp	Meta-zoans
	jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA	Folmer et al., 1994		
3	12S-V5 (fwd)	TAGAACAGGCTCCTCTAG	Riaz et al., 2011	98 bp	Verte-brates
	12S-V5 (rev)	TTAGATACCCCACTATGC	Riaz et al., 2011		
4	MOL16S_F	RRWRGACRAGAAGACCCT	Klymus et al., 2017	183-310 bp	Molluscs
	MOL16S_R	ARTCCAACATCGAGGT	Klymus et al., 2017		
	MOL16S_FISBLOCK	AGGTCG TAACCCCCTRG/3SpC3/	Klymus et al., 2017		
	trnL c	CGAAATCGGTAGACGCTACG	Taberlet et al., 2007	150-200 bp	Plants
	trnL h	CCATTGAGTCTCTGCACCTATC	Taberlet et al., 2007		

Single-step PCR with tagged primer pairs was performed in four PCR replicates. The combination of forward and reverse tags indicated from which sample or control PCR replicate amplicons originated (*Fig. 5.1*, Taberlet et al., 2018). Tags were eight bases long and had at least five differences between them (Coissac et al., 2012). The PCRs were carried out as described in (Werner et al., Submitted), except that DNA concentrations were not normalized. Instead, DNA extracts were diluted ten times to avoid PCR inhibition (McKee et al., 2015). This dilution ratio was chosen after qPCR testing of a dilution series (1, 10, 50 and 100 times diluted) using iTaq Universal SYBR Green Supermix on a Bio-Rad CFX Opus 96 Real-Time PCR System, with the PCR conditions described in Werner et al. (Submitted). Final PCRs were performed using the Qiagen multiplex PCR Kit in 15 µL reaction volumes on 96 well plates. PCR mixes were pipetted using the Beckmann Coulter Biomek i7 pipetting robot and PCRs were run on Eppendorf Mastercycler X50 and Bio-Rad CFX Opus 96 Real-Time machines.

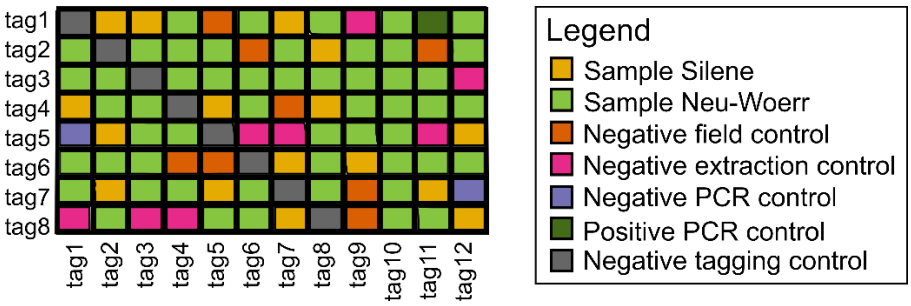


Figure 5.1. Example of PCR plate layout. Positions of samples, negative field and extraction controls, as well as negative tagging controls were varied between plates.

PCRs of the samples and negative field and extraction controls from the Silene sampling campaign were performed on the same plates as those of samples and controls taken on the Neu-Woerr site (*Fig 5.1*, Appendix 5.C, *Table S.5.1*). The Neu-Woerr samples were taken to detect potential food items for the European pond turtle (*Emys orbicularis,* L. 1758*)*, which is reintroduced on the study sites. These samples are used in work package 2 of the EMYS-R project (**Chapter 1**). They were not taken to evaluate eDNA metabarcoding, and the data from these samples is not analysed in this chapter. The order of all samples and controls on the plates was randomized. Also 15 samples from another project were added to the plates, but these only occupied the last wells. In total, for each of the four primer multiplexes, 300 samples, 16 negative field controls and 24 negative extraction controls were cycled. Of these, 69 samples, eight negative field controls and 11 negative extraction controls were taken in or associated with the Silene site. Per primer multiplex, 16 plates were cycled. On each plate, two negative PCR controls, that had nuclease free water instead of sample DNA extract, and one positive PCR control, that had a mixture of DNA from known species (Appendix 5.D, *Table S.5.3*) instead of sample DNA extract, was added. Furthermore, eight wells per plate were kept empty as "negative tagging controls", leaving some tag combinations unused to allow detection of tag jumps (Schnell et al., 2015).

PCR success was evaluated for about ten percent of reactions on agarose gel. Amplicons of the same primer multiplex were pooled without normalisation and purified using the Qiagen MinElute PCR Purification Kit. The DNA concentration and amplicon size of purified pools were measured on an Agilent TapeStation. PCR free library preparation and Illumina Novaseq sequencing according to conditions in *Table 5.3* were performed by Novogene in the UK. Based on earlier experience (Werner et al., Submitted), more reads were asked per sample for multiplex 4 (MP4) than for the other multiplexes.

Table 5.3. Sequencing conditions four the four multiplexes (MPs) with the number of reads per sample per marker, number of reads in total and data size asked.

	Read length	No. reads per sample per marker	No. reads total	Data size (Gb)
MP1	250 paired end	150000	408000000	204
MP2	250 paired end	150000	408000000	204
MP3	150 paired end	150000	204000000	51
MP4	150 paired end	225000	612000000	306

5.2.3. Bioinformatics all datasets

Sequencing data was initially processed using the OBITools4 (Boyer et al., 2016) amplicon sequence variant (ASV) pipeline described in Werner et al. (Submitted) et Romahn et al. (Submitted) (*Fig. 5.2*). Forward and reverse reads, with a minimum overlap of 10 bp and minimum alignment of 80% for the overlapping region, were merged, and other reads were discarded. The merged reads were demultiplexed, i.e., assigned to PCR replicates based on the unique tag combinations. Afterwards, the reads were dereplicated and denoised with the obiclean procedure (with r = 0.05) to formASVs.

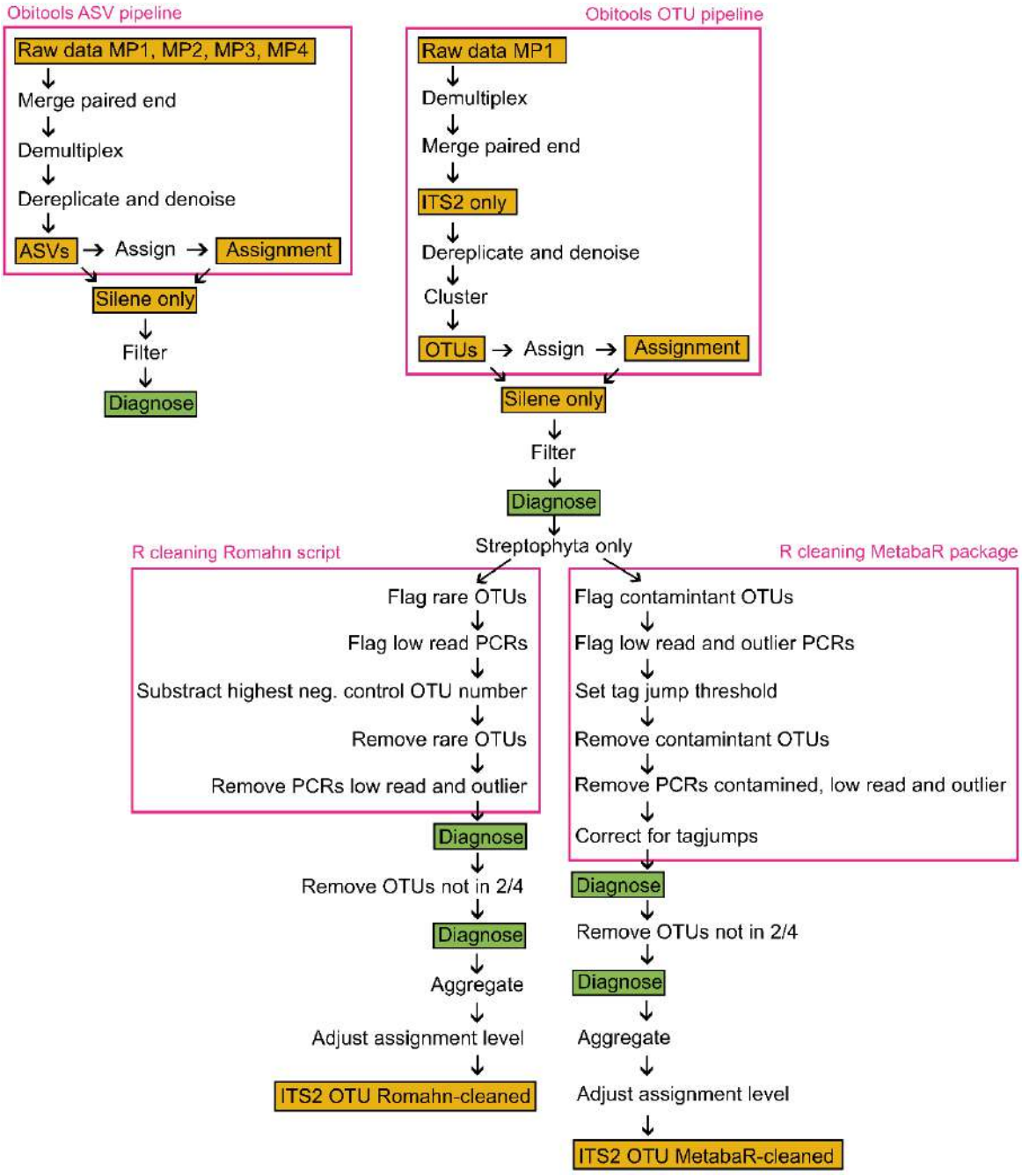


Fig. 5.2. Bioinformatics and data cleaning pipelines with datasets in yellow boxes and diagnostics presented in this chapter in green boxes. The OBITools amplicon sequence variant (ASV) pipeline was performed for all multiplexes (MPs, see Table 5.3), whereas the OBITools operational taxonomic unit (OTU) pipeline was uniquely performed for MP1. In the OBITools OTU pipeline only the ITS-3p62p1F1 & ITS-4unR1 (ITS2) dataset was selected. Two cleaning procedures, "R cleaning Romahn script" and "R cleaning MetabaR package" were performed on the ITS2 OTU dataset. Subsequently, in both datasets, OTUs were removed from the samples in which they were not preset in at least two of the four PCR replicates. Data was aggregated per sample, and levels of taxonomic assignment were adjusted, resulting in the "ITS2 OTU Romahn-cleaned" and "ITS2 OTU MetabaR-cleaned" datasets, which were both analysed to answer the research questions.

Taxonomic assignment of ASVs was preformed using the Least Common Ancestor-based obitag command and the custom reference databases described in Werner et al. (Submitted). The custom reference databases were generated in CRABS (Jeunen et al., 2023) and included sequences from the National Center for Biotechnology Information (NCBI) GenBank (Benson et al., 2002), the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database (Stoesser et al., 2002), the German Barcode of Life reference library (German Barcode of Life Consortium, 2011), the Barcode Of Life Data system (Ratnasingham & Herbert, 2007), the Mitochondrial Genome Database of Fish (Zhu et al., 2023) and the curated plant database described in Espinosa Prieto et al. (2024). With the aim to improve taxonomic resolution, for the plant and vertebrate databases, only species from genera known to occur in Europe, including those of invasive species present in the DAISIE database (Roy et al., 2020) were included. Because we expected the that sequences would not be available for a considerable proportion of macroinvertebrate genera occurring in Europe (Weigand et al., 2019), no such selection was applied to the invertebrate database (Werner et al., Submitted). After assignment, the datasets were split and only the data from the samples, negative field and extraction controls associated with the Silene sampling campaign, as well as all negative PCR, negative tagging and positive PCR controls were processed further.

All subsequent steps were performed in R version 4.4.0 with R studio 2023.06.0 and the packages vegan, ade4, adegraphics, cowplot, metabar, reshape 2, tr8, RColorbrewer, eulerr and the tidyverse. In the "filter" step of *Fig. 5.2*, only ASVs with the expected length (*Table 5.4*), assignment at least to phylum level, and a minimum similarity of 80% to the most similar sequence in the reference database were kept. The resulting datasets were diagnosed by comparing the number of reads and ASVs across the samples and different types of controls. Statistical differences were assessed using a Kruskal–Wallis test. When results were significant, post hoc pairwise comparisons were performed across samples and the different control types with Dunn's test using the Benjamini–Hochberg correction for multiple comparisons. For all statistical tests α was 0.01. Furthermore, the Bray-Curtis dissimilarity among ASV communities from samples and the different control types was visualised with Principal Coordinate Analysis (PCoA). Lastly, the presence of sequences assigned to the species and genera of positive control taxa was verified.

Table 5.4. Filtering thresholds for the expected sequence length used in the "filter" step (Fig. 5.2).

Primer pair	Expected length (bp)	Min. length accepted (bp)	Max. length accepted (bp)
ITS-3p62p1F1 & ITS-4unR1	183-271	170	-
Unio01_F & Unio01_R	137	100	200
rbcL mini & rbcL a-R	379	320	-
mlCOIintF & jgHCO2198	350	300	-
12S-V5 (fwd) & 12S-V5 (rev)	98	80	120
MOL16S_F & MOL16S_R	183-310	150	-
trnL c & trnL h	150-200	100	-

5.2.4. Bioinformatics and data cleaning ITS2 dataset

The raw MP1 sequences were additionally processed using another OBITools pipeline that generated operational taxonomic units (OTUs) (*Fig 5.2*). In contrast to the OBITools ASV pipeline, this OTU pipeline performed assignment to samples before the merging of forward and reverse reads. Furthermore, after retaining uniquely ITS2 sequences, OTUs were generated using VSEARCH (Rognes et al., 2016) with a clustering threshold of 97%, and these OTUs were assigned taxonomically, in the same way as in the ASV pipeline. The data from the Silene samples, negative field and extraction controls, as well as all negative and positive PCR controls

were retained and cleaned further in R. As before, only OTUs with the expected length and assignment at least to phylum level and a minimum similarity of 80% to the most similar sequence in the reference database were kept. The same diagnostics were performed as for the ASV datasets.

Afterwards, the OTUs assigned to the clade Streptophyta were retained, and the resulting dataset was cleaned according to two different methods (*Fig. 5.2*). The first "Romahn" method is based on recommendations in Taberlet et al. (2018) and was similar the one applied in Werner et al. (Submitted) and the second was based on the MetabaR package (Zinger et al., 2021). The two different methods were used because they are based on different principles and it was not clear which one was most appropriate for our data. In the Romahn method, based on data visualisations, OTUs with less than 29 reads (0.0001% of total reads), and PCR replicates with less than 20.000 reads were flagged. Subsequently, for each OTU in each sample PCR replicate, the highest number of reads in any of the negative controls was subtracted. The flagged OTUs and PCR replicates were removed and PCR replicates with less than 1000 reads left were discarded. Based on Bray-Curtis nMDS ordinations, 13 PCR replicates that were dissimilar from the other replicates of the same sample, and seven PCR replicates that were dissimilar from the entire set of sample PCR replicates, were removed.

In the MetabaR cleaning method, "contaminant" OTUs were flagged. These "contaminants" were the OTUs that had the highest number of reads in a negative control PCR replicate. Then, based on data visualisations, PCR replicate with less than 2500 reads and PCR replicates with a Bray-Curtis dissimilarity to the other PCR replicates from the same sample higher than 0.5 were flagged. Subsequently, based on data visualisations, a "tagjump" threshold of 3% for the relative abundance of OTUs was set. Contaminant OTUs, PCR replicates with more than 10% contaminant OTUs, and the flagged low read number and outlier PCR replicates were discarded. Then, OTUs were removed from PCR replicates where the read number of an OTU was lower than 3% of the total read number of this OTU in the entire dataset.

Subsequently, for both the Romahn- and MetabaR-cleaned datasets, OTUs were removed from samples if they were present in less than two PCR replicates from the same sample. Both before and after this step Bray-Curtis PCoA was performed and the number of reads and OTUs was visualised. Afterwards, OTU read counts were aggregated per sample by summing over PCR replicates. Subsequently, levels of OTU taxonomic assignment were adjusted using thresholds for the similarity between OTUs and their best match reference sequence. Species, genus and family level detection were only allowed with similarities higher than 96%, 94% and 90%, respectively. Samples from the nine study ponds in *Table 5.1* were selected and analysed.

5.2.5. Statistical analyses of cleaned ITS2 datasets

Both the Romahn- and MetabaR-cleaned datasets were analysed to answer the research questions. Analyses were presence-absence based, because the relationship between the abundance of macroorganisms in study systems and the read abundance from eDNA metabarcoding is complex and variable (Blackman et al., 2024a; Elbrecht & Leese, 2015). The dissimilarity between samples in terms of detected OTUs was visualised by Nonmetric Multidimensional Scaling (nMDS) of the Dice-Sørensen dissimilarity using the metaMDS() function. To test whether OTU communities differed between samples taken in different ponds, and between different mesohabitats within ponds, permutational analysis of variance (PERMANOVA) was performed (Anderson, 2017). A two-way nested model of the Dice-Sørensen dissimilarity between samples as function of the pond and the mesohabitat within the pond was tested with a sequential (type 1) sum of squares. PERMANOVAs were run with 9999 permutations using the function adonis2().

For the assigned species, Ellenberg's soil moisture indicator (F) values were downloaded from the Ecological Flora of the British Isles database (Fitter & Peat, 1994) using the tr8() function. Furthermore, the distribution of the assigned species was looked up on the Global Biodiversity Information Facility (*Global Biodiversity Information Facility*, z.d.) and Plants Of the World Online (Royal Botanic Gardens, 2017). Using this information, the species were classified as aquatic if Ellenberg's F was 11 or higher, amphibious if Ellenberg's F was nine or ten, terrestrial if it was eight or lower, and exotic when the species had no known observation in Latvia.

Comparison between conventional and eDNA inventories for the nine ponds combined was performed for 1) all species, 2) uniquely aquatic and amphibious species, and 3) all genera. For each of these categories, the number of taxa detected uniquely in the conventional, uniquely in the Romahn-cleaned eDNA metabarcoding and uniquely in the MetabaR-cleaned eDNA metabarcoding inventories was calculated. The number of taxa detected with any combination of these methods was calculated as well. These numbers were visualised with Venn diagrams. For the aquatic and amphibian species, the presence or absence in the Romahn- and MetabaR-cleaned datasets was analysed per sample.

5.3. Results

With the conventional methods, in total 45 species, 78 genera and 47 families of macroinvertebrates, and 58 species, 43 genera and 27 families of macrophytes were detected in the nine study ponds (see Appendix 5.E, *Table S.5.4*, for the macrophytes). In the five ponds where amphibians were surveyed and sampled, eight amphibian species were detected (M. Pupiņš, pers. data).

During the Silene sampling campaign, 69 water samples were taken, and on average 235 mL was filtered per sample (coloured points in *Fig. 5.3*, 250 mL samples were taken). DNA extracted from these samples had an average concentration of 12 ng/µL, and DNA extracted from the eight Silene negative field controls had an average concentration of 0.06 ng/µL. Of the 27 negative extraction controls taken when the Silene samples were extracted, 11 had a DNA concentration higher than the limit of detection (on average 0.03 ng/µL).

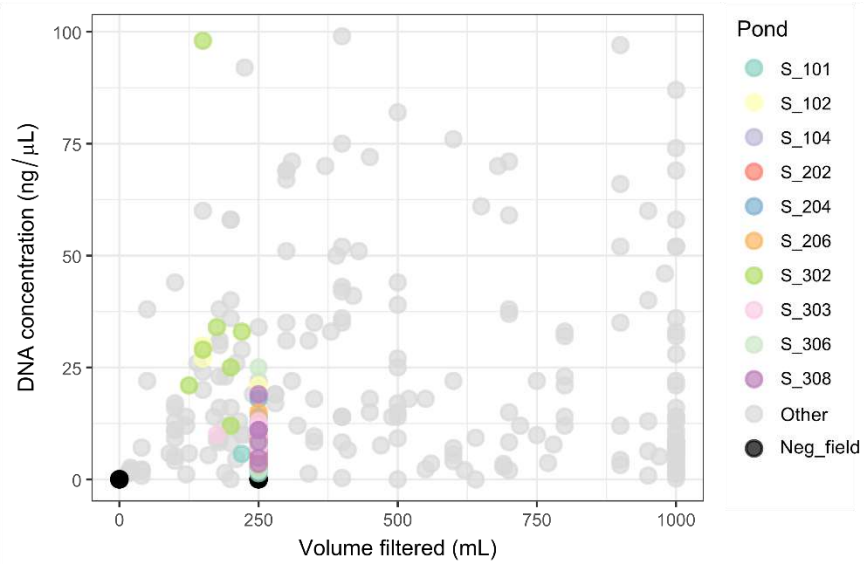


Fig. 5.3. Concentration of extracted DNA as function of filtered volume. Silene samples coloured according to pond (see legend). Neu-Woerr samples on the PCR plates in grey and negative field controls in black.

In total, for the four multiplexes and 6144 PCR reactions from the Silene samples as well as from the Neu-Woerr samples cycled on the same PCR plates, we received 6,208,661,158 reads (Appendix 5.C, *Table S.5.2*). The quality of the sequencing data was good, since 93.5% of bases had a Phred score higher than 30.

5.3.1. Diagnostics all datasets after ASV pipeline

After processing with the OBITools ASV pipeline, the datasets for the four multiplexes from the Silene samples and controls (528 PCR reactions) contained in total 575,154,212 reads and 2,164,274 ASVs (*Fig. 5.4*). The number of reads and ASVs obtained differed substantially between the markers that were combined in the same multiplex. The plant markers received more reads and more ASVs than the invertebrate markers. After filtering based on expected size and assignment to phylum level and a minimum similarity to a reference database sequence of 80%, a total of 427,832,128 reads and 1,026,402 ASVs were left. For the Unio01_F & Unio01_R primer pair, this filtering step removed all reads.

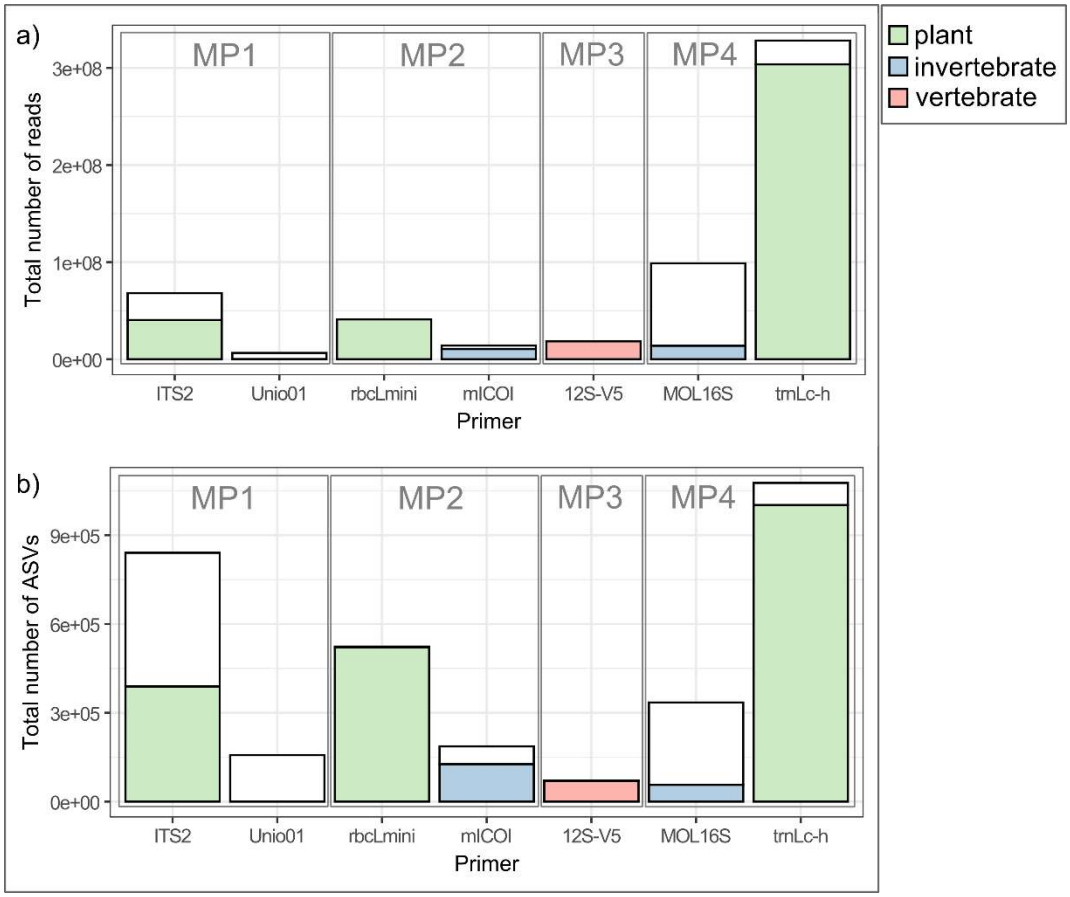


Fig. 5.4. Total number of a) reads and b) ASVs per primer pair for Silene samples and controls. Total bars (coloured and white) show counts before filtering. The filtering is based on expected amplicon length, assignment to phylum level and a minimum similarity to a reference database sequence of 80%. Coloured parts of bars show counts after this filtering step. Colours indicate the target group for each primer pair (see legend).

In the ITS-3p62p1F1 & ITS-4unR1 (ITS2) amplicons, significantly more reads and ASVs were obtained from the PCR replicates of the samples than of the negative controls (*Fig. 5.5* and *5.6*, Kruskal-Wallis test, and if significant Dunn tests with Benjamin-Hochberg correction for multiple testing, $\alpha = 0.01$). For the other primer pairs, there was either no significant difference between the PCR replicates of samples and controls in terms of read counts and ASVs, or the positive control PCR replicates resulted in more reads or ASVs than the sample and negative control PCR

replicates. For the rbcL mini & rbcL a-R (rbcLmini) and trnL c & trnL h (trnLc-h) primer pairs, positive control PCR replicates generated significantly more ASVs than sample PCR replicates (*Fig. 5.6*, Dunn test with Benjamin-Hochberg correction, α = 0.01). Only for the ITS2 dataset, PCoA ordinations of the Bray-Curtis dissimilarity between the PCR replicates (*Fig. 5.7*) show a separation between sample PCR replicates and negative control PCR replicates. For the other datasets, PCoA shows overlap in the ASV composition of PCR replicates from samples and negative controls. Positive control PCR replicates do stand out in the rbcLmini, mlCOlintF & jgHCO2198 (COI), MOL16S_F & MOL16S_R (MOL12S) and trnLc-h datasets.

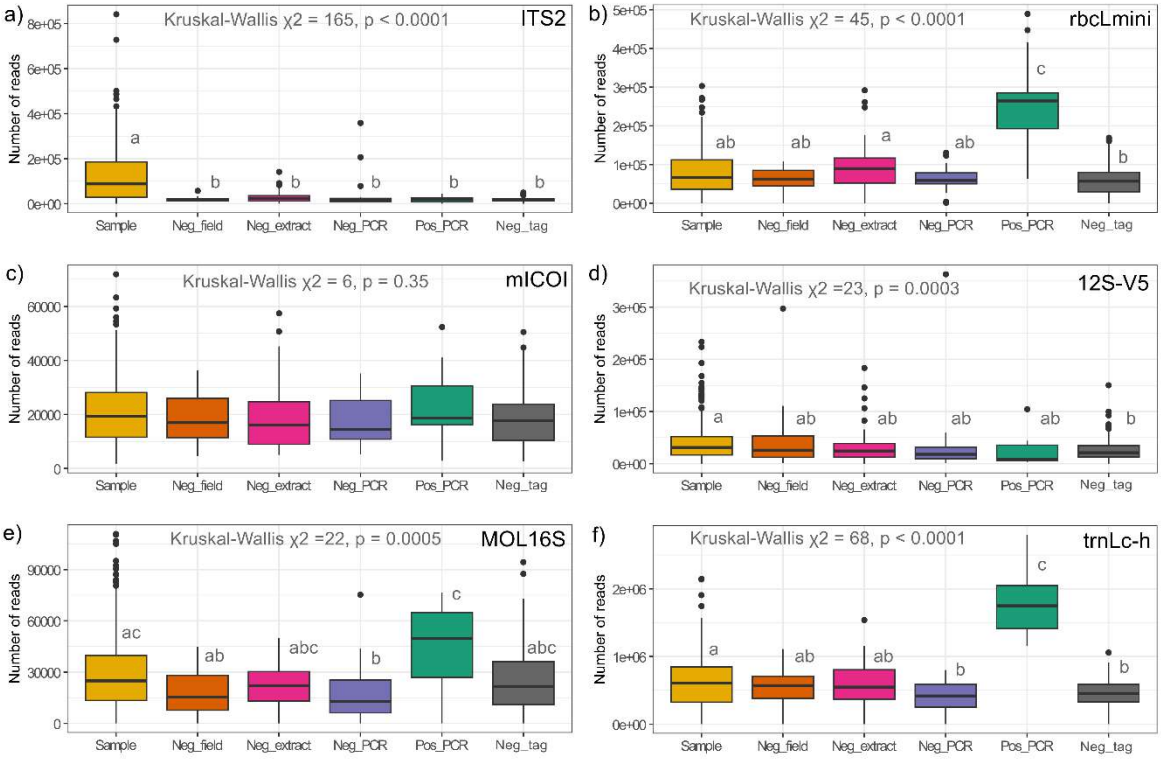


Fig. 5.5. Boxplots (fences for the 25th percentile, median and 75th percentile) of the number of reads in PCRs of different types of samples and controls (negative field, negative extraction, negative PCR, positive PCR and negative tagging). Outcomes of Kruskal-Wallis tests of differences in read counts across PCR of different types of samples and controls in text above the boxes. Outcomes of pairwise Dunn tests with Benjamin-Hochberg correction for multiple testing and α = 0.01 in compact letter display. a) ITS-3p62p1F1 & ITS-4unR1 b) rbcL mini & rbcL a-R c) mlCOlintF & jgHCO2198 d) 12S-V5 (fwd) & 12S-V5 (rev) e) MOL16S_F & MOL16S_R f) trnL c & trnL h

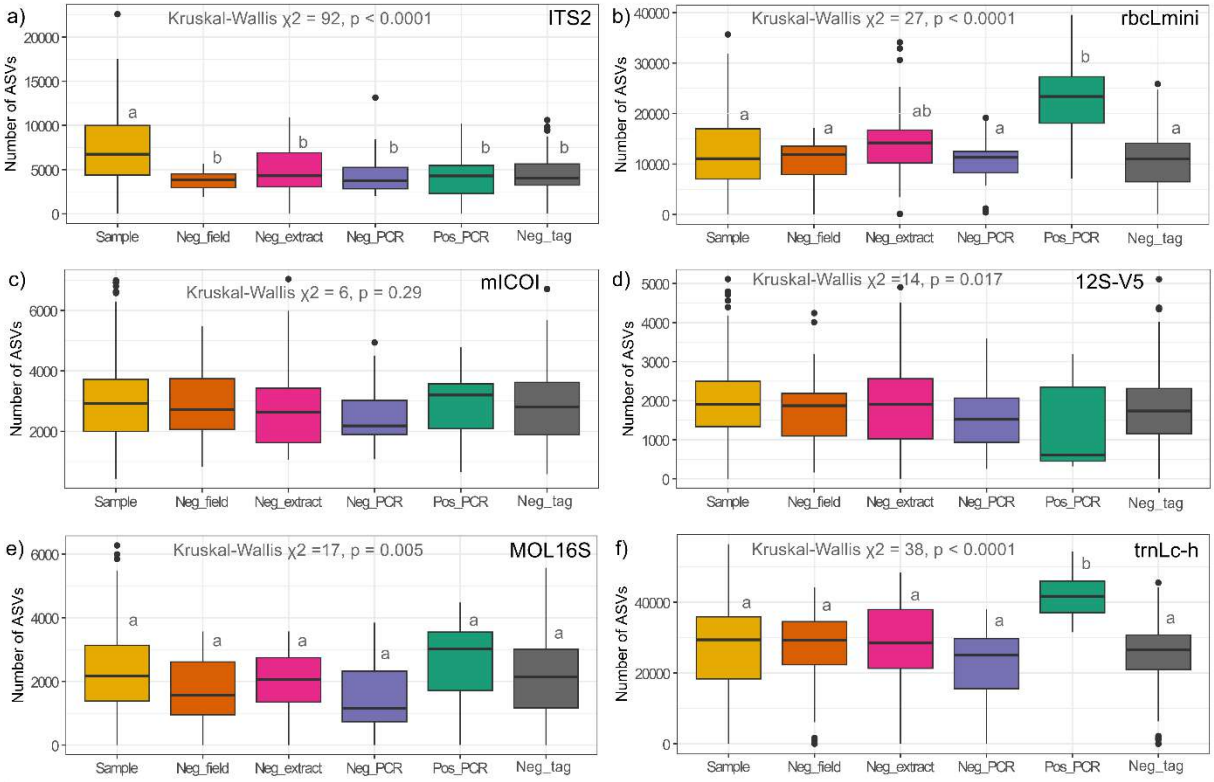


Fig. 5.6. Boxplots (fences for the 25th percentile, median and 75th percentile) of the number of ASVs in PCRs from different types of samples and controls (negative field, negative extraction, negative PCR, positive PCR and negative tagging). Outcomes of Kruskal-Wallis tests of differences in ASV number between PCRs of different types of samples and controls in text above the boxes. Outcomes of pairwise Dunn tests with Benjamin-Hochberg correction for multiple testing and α = 0.01 in compact letter display. a) ITS-3p62p1F1 & ITS-4unR1 b) rbcL mini & rbcL a-R c) mlCOIintF & jgHCO2198 d) 12S-V5 (fwd) & 12S-V5 (rev) e) MOL16S_F & MOL16S_R f) trnL c & trnL h.

The COI dataset contained ASVs assigned to the positive control species *Gammarus fossarum*, *Folsomia candida*, *Baetis rhodani*, *Rhyacophila nubile*, and the trnLc-h and rbcLmini datasets included ASVs assigned to the positive control genus *Coffea*. These datasets did not contain ASVs of the other positive control taxa (Appendix 5.D, Table S.5.3). None of the positive control taxa were detected in the 12S-V5, ITS2 and MOL16S datasets. ASVs assigned to positive control taxa were primarily present in the data from the positive control PCR reactions (Appendix 5.D, Fig. S.5.3). However, relatively low numbers of reads of ASVs assigned to positive control taxa were present in the PCR replicates of samples and negative controls (up to 10% of the maximum number of reads of ASVs assigned to positive control taxa in the positive control PCR replicates).

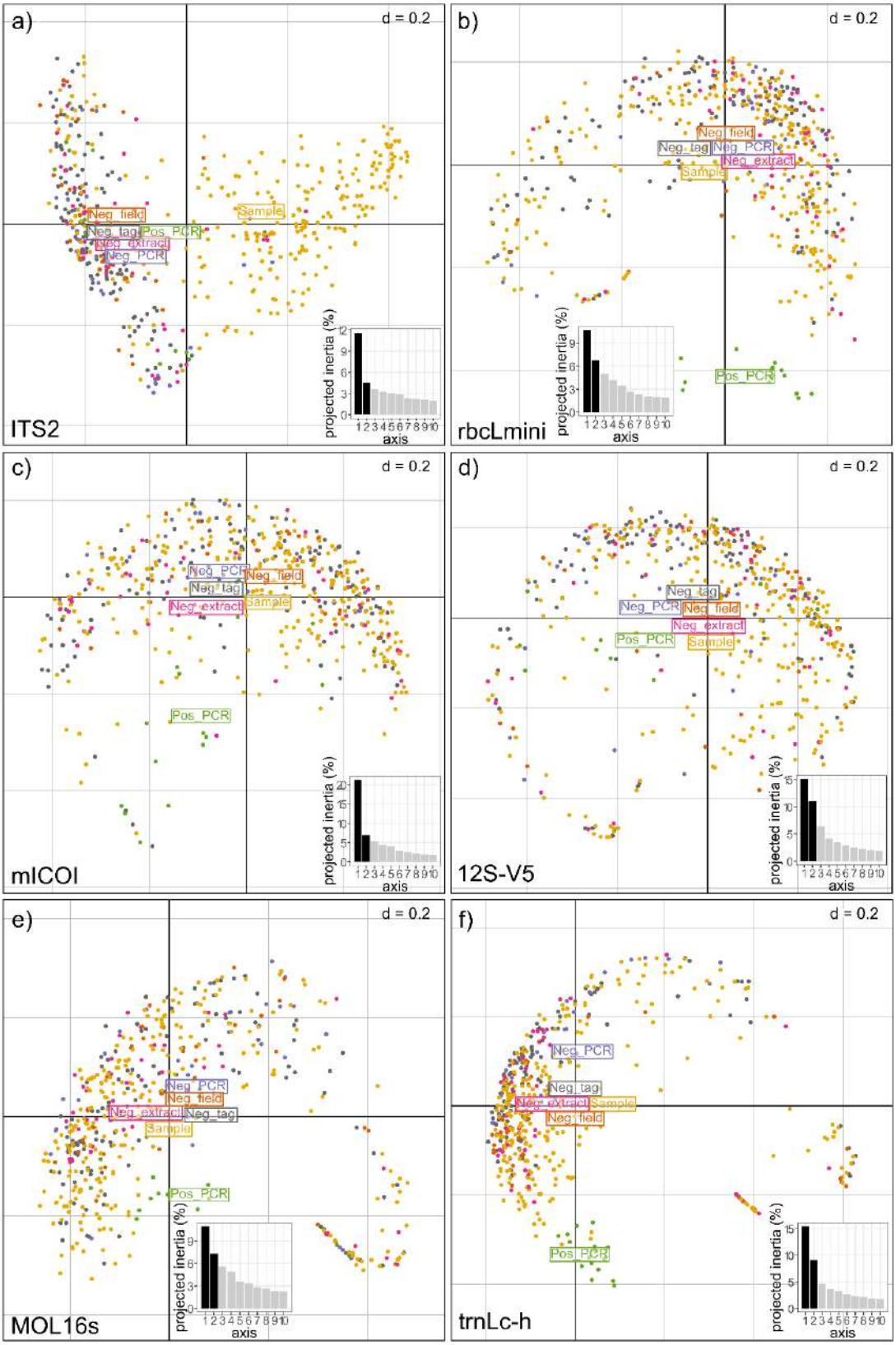


Fig. 5.7. First two Bray-Curtis PCoA axes of ASV communities per PCR reaction. Colours indicate the type of sample or control of the PCR reaction (negative field, negative extraction, negative PCR, positive PCR and negative tagging PCR), with the approximate centre of the reactions from the same type in text with a box of matching colour. Bar plots of percentage projected inertia for the first ten axes in insets. a) ITS-3p62p1F1 & ITS-4unR1 b) rbcL mini & rbcL a-R c) mlCOIintF & jgHCO2198 d) 12S-V5 (fwd) & 12S-V5 (rev) e) MOL16S_F & MOL16S_R f) trnL c & trnL h.

5.3.2. Diagnostics ITS2 dataset after OTU pipeline and subsequent cleaning

After processing with the OBITools OTU pipeline and selection of Silene data only, the ITS2 dataset contained 45,292,231 reads clustered into 11,210 OTUs (Appendix 5.F, *Table S.5.4*). Subsequent filtering based on expected sequence length, assignment to at least phylum level and similarity to a reference sequence of at least 80%, resulted in 29,479,693 reads forming 3669 OTUs. The number of reads in the sample and positive control PCR replicates was significantly higher than the number of reads in the negative control replicates (pairwise comparison with Dunn's test, p-values adjusted with Bengamini-Hochberg method, α = 0.01, *Fig. 5.8a*). The number of OTUs in the samples and positive control replicates was significantly higher than in the field negative control replicates, but not significantly higher than in the other types of negative control replicates (*Fig. 5.8b*).

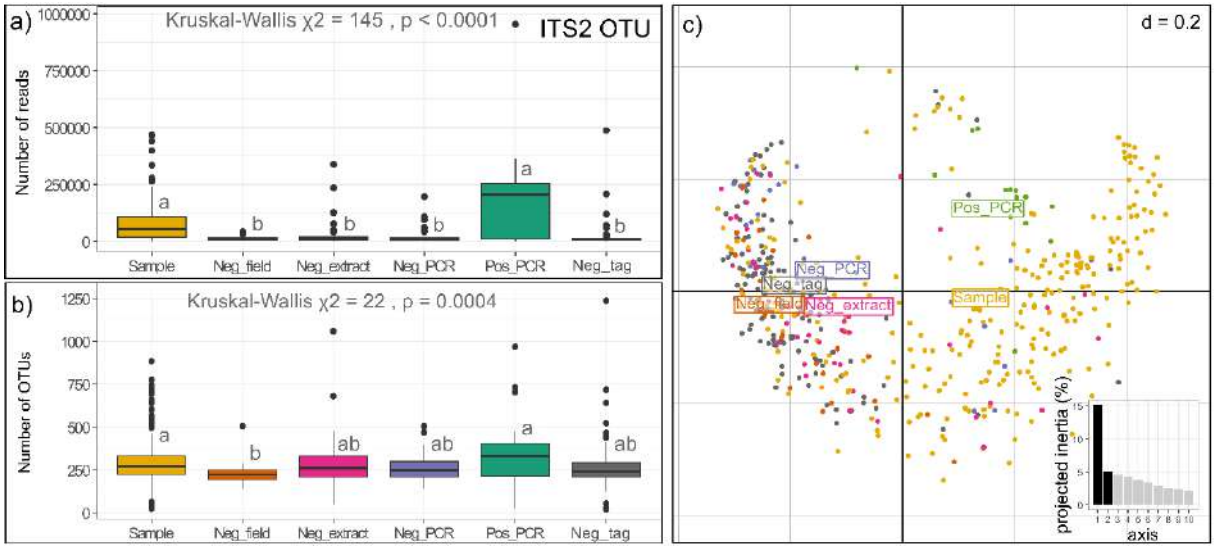


Fig. 5.8. Diagnostics for the ITS-3p62p1F1 & ITS-4unR1 (ITS2) OTU Silene dataset after OBITools processing and filtering based on expected amplicon length, assignment to phylum level and similarity to a reference sequence of at least 80%. a,b) Boxplots (fences for the 25th percentile, median and 75th percentile) of the number of a) reads and b) OTUs in PCRs of different types of samples and controls (negative field, negative extraction, negative PCR, positive PCR and negative tagging). Outcomes of Kruskal-Wallis tests of differences in numbers of reads in PCRs from different types of samples and controls in text above the boxes. Outcomes of pairwise Dunn tests with Benjamin-Hochberg correction for multiple testing and α = 0.01 in compact letter display. c) First two Bray-Curtis PCoA axes of OTU communities per PCR reaction. Colours indicate sample or control type, with the approximate centre of the reactions from the same type in text with a box of matching colour. Bar plot of percentage projected inertia for the first ten axes in inset.

PCoA shows dissimilarity between the OTU communities int the negative control PCR replicates on the left side of the plane formed by the first two PCoA axes and the positive control and sample PCR replicates on the right side of the plane (*Fig. 5.8c*). In the ITS2 OTU dataset the positive control taxon *Vanilla planifolia* was detected at species level and *Piper* at genus level. ASVs assigned to these taxa were most abundant in the positive control PCR replicates, but also present at very low abundance in some sample and negative control replicates (Appendix 5.D, *Fig. S.5.4*).

After selection of Streptophyta OTUs only, there were 29,357,171 reads forming 3555 OTUs in the ITS2 dataset (Appendix 5.F, *Table S.5.4*). Of these reads, 72% resulted from sample PCRs, 17% from the different types of negative control PCRs combined, and from 11% from positive control PCRs (Appendix 5.F, *Table S.5.5*). Cleaning of this dataset with the Romahn method

resulted in reductions of 42% in the number PCR replicates for which there was data, of 56% in the number of reads, and of 74% in the number of OTUs in the data from the sample PCR replicates (Appendix 5.F, *Table S.5.4*). These reductions were primarily the result of the correction with the negative controls, which subtracted from each OTU in each sample PCR replicate the highest number of reads in any of the negative control PCR replicates. The MetabaR cleaning method was less severe and resulted in reductions of 20% in the number PCR replicates with data, of 13% in the number of reads, and of 39% in the number of OTUs in the data from all sample PCR replicates together.

For both the Romahn- and MetabaR-cleaned datasets, removal of OTUs from samples where they were not present in at least two PCR replicates resulted in a small reduction of the total number of reads and median number of reads per PCR (Appendix 5.F, *Table S.5.4*, *Fig. S.5.6*). This step also did not have a large influence on the similarity between PCR replicates of the same sample (Appendix 5.F, *Fig. S.5.7*). However, it did reduce the total number of OTUs and median number of OTUs per PCR by more than 50% (Appendix 5.F, *Table S.5.4*, *Fig. S.5.6*)

5.3.3. Analyses cleaned ITS2 OTU dataset

After all cleaning steps, including data aggregation per sample, of the 54 samples in *Table 5.1*, there was still data for 36 samples in the Romahn-cleaned dataset, and for 52 samples in the MetabaR-cleaned dataset. The Romahn-cleaned dataset contained 5,662,468 reads clustered into 257 OTUs and the MetabaR-cleaned dataset 12,067,929 reads clustered into 853 OTUs. The rarefaction curves of both datasets show that after cleaning, the number of reads per sample sufficed to reach a representative OTU diversity (Appendix 5.F, *Fig. S.5.7*). The number of OTUs detected per pond differed markedly between the Romahn- and MetabaR-cleaned datasets (Appendix 5.F, *Fig. S.5.8*).

When comparing the nMDS plots of the Dice-Sørensen dissimilarity between samples for the Romahn- and MetabaR-cleaned datasets (*Fig. 5.9*), it is clear that the Romahn dataset contained less samples and exhibited less overlap in OTU composition between samples from different ponds than the MetabaR-cleaned dataset. Both for the Romahn- and MetabaR-cleaned datasets, PERMANOVA showed that samples from the same pond had more similar OTU compositions than samples from different ponds (*Table 5.5*, $p = 0.0001$ for both datasets). However, only in the Romahn- and not in the MetabaR-cleaned dataset, samples from the same mesohabitat within a pond were more similar than samples from different mesohabitats within a pond ($p = 0.0001$ for the Romahn- and $p = 0.13$ for the MetabaR-cleaned dataset). For the Romahn-cleaned dataset, the proportion of the variation in dissimilarity explained was highest for the mesohabitats within ponds (40%), while for the MetabaR-cleaned dataset the largest proportion was unexplained (residuals: 46%) .

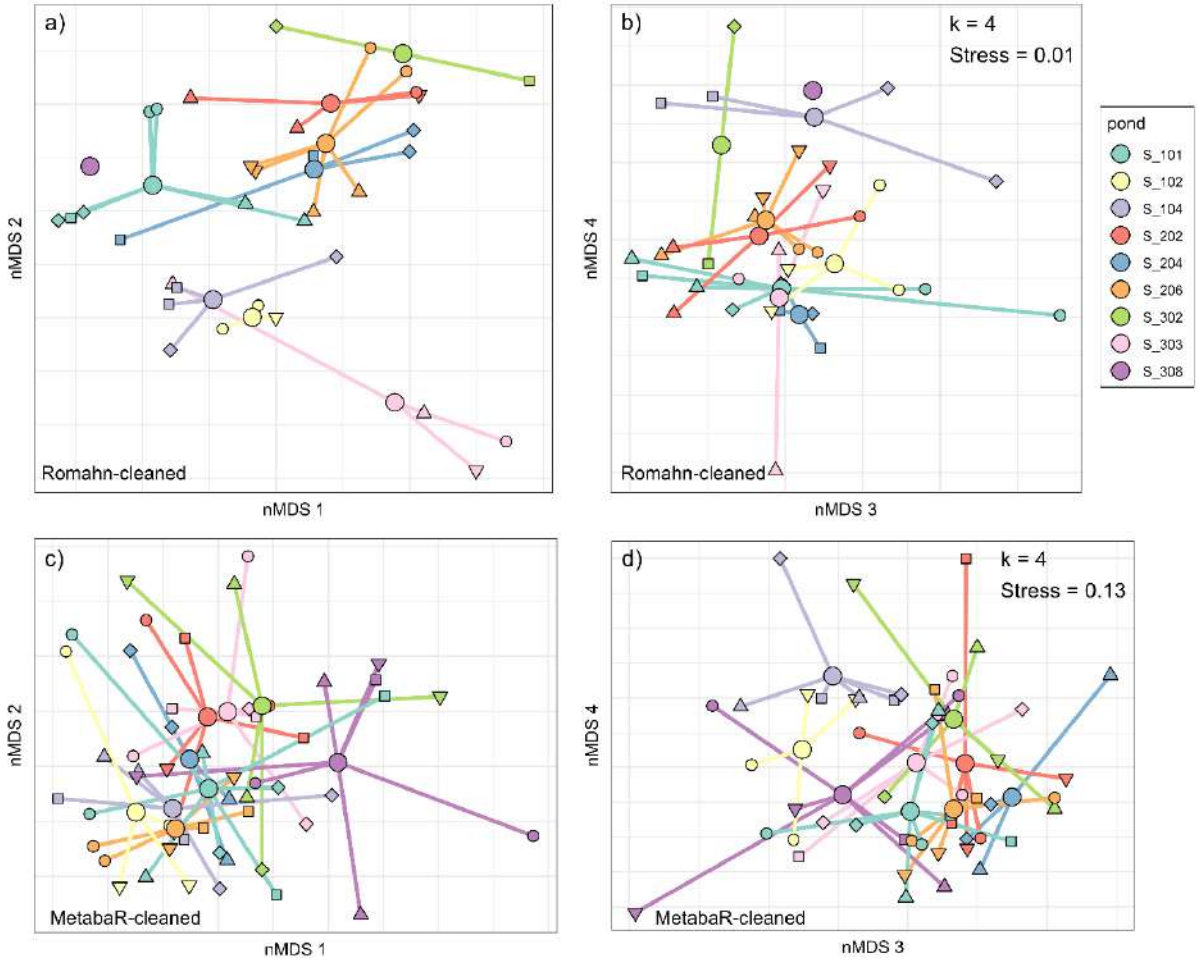


Fig. 5.9. Four-dimensional nonmetric multidimensional scaling (mMDS) ordination of Dice-Sørensen dissimilarity between OTUs detected in samples, with small symbols indicating samples and large filled circles the centroids of samples from the same pond. Colours indicate ponds and within ponds small symbols (filled circles, squares, rhombi, normal, tilted and upside-down triangles) indicate duplicate samples from the same mesohabitat. a, b) Romahn-cleaned, and c, d) MetabaR-cleaned datasets, with the stress for the ordination of each dataset indicated in text. a, c) nMDS axes 1 and 2, b, d) nMDS axes 3 and 4.

Table 5.5. PERMANOVA results for the Romahn- and MetabaR-cleaned datasets based on the Dice-Sørensen dissimilarity between samples. Percentage of variation in dissimilarity explained by each term is the partial R^2 expressed as percentage. Statistically significant differences ($p < 0.01$) are shown in bold.

Romahn	**D.f.**	**Sum of squares**	**Percentage of variation**	**F statistic**	**p-value**
Pond	**8**	**5.5**	**33%**	**2.21**	**0.0001**
Pond:Mesohabitat	**13**	**6.8**	**41%**	**1.68**	**0.0001**
Residual	14	4.4	26%		
Total	35	16.7	100%		
MetabaR	**D.f.**	**Sum of squares**	**Percentage of variation**	**F statistic**	**p-value**
Pond	**8**	**3.5**	**19%**	**1.33**	**0.0001**
Pond:Mesohabitat	18	6.3	35%	1.05	0.13
Residual	25	8.3	46%		
Total	51	18.1	100%		

The OTUs in the Romahn dataset were assigned to 77 taxa, and the OTUs in the MetabaR dataset were assigned to 183 taxa (*Fig. 5.10*). The proportions of OTUs assigned to species, genus and family level were similar for the two datasets, with for example 17% of the OTUs in the Romahn dataset and 16% of the OTUs in MetabaR dataset assigned to species level. However, the proportion of taxa assigned to species level was lower in the Romahn dataset (30%) than in the MetabaR dataset (40%).

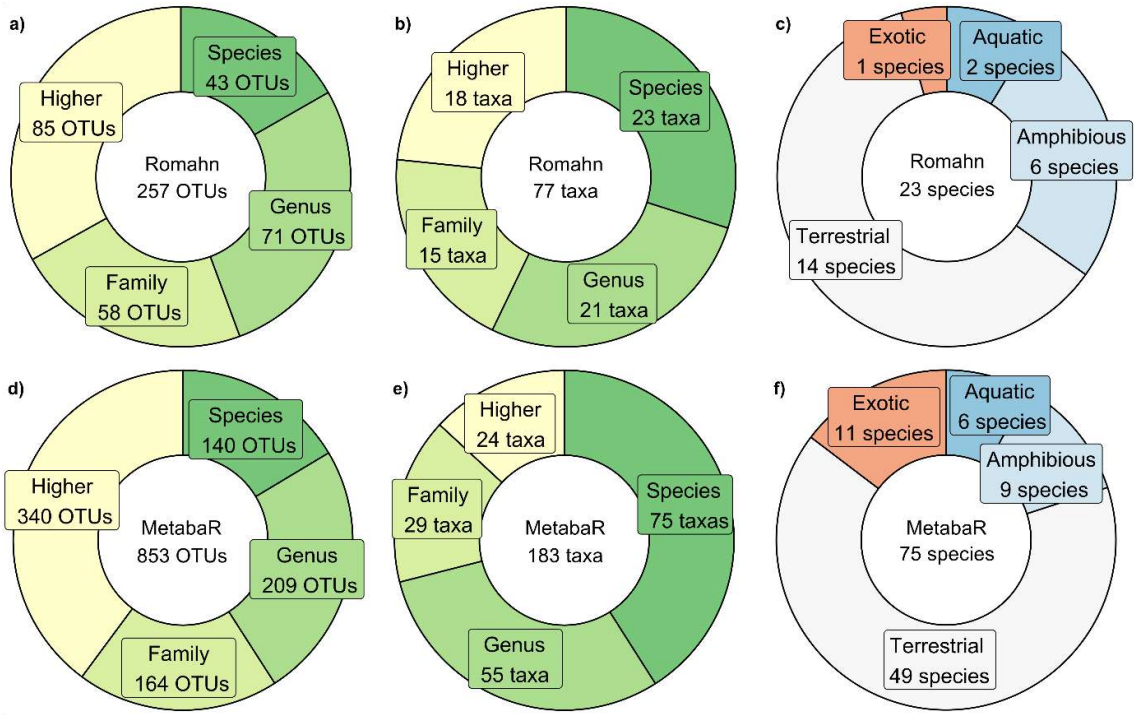


Fig. 5.10. Assignments of a-c) the Romahn-cleaned and d-f) the MetabaR-cleaned ITS2 OTU datasets from the nine studied ponds. Proportions of a,d) Operational Taxonomic Units (OTUs) and b,e) taxa assigned to species, genus, family or higher taxonomic levels. c,f) proportions of assigned species that are aquatic, amphibious, terrestrial or without known occurrence in Latvia (exotic). Inside circles in text the total numbers of OTUs, taxa or species. See Appendix 5.E for taxon identities.

Of the 23 species in the Romahn-cleaned dataset, 9% were aquatic and 26% amphibious. One of the terrestrial species in the dataset was the crop species hemp (*Cannabis sativa)* and one of the assigned species, *Carex stipata*, was labelled as "exotic" because it has no known occurrence Latvia. In the MetabaR-cleaned dataset, 8% of the 75 detected species were aquatic and 12% amphibious. Several crop species were present in this dataset. Furthermore, besides *Carex stipata*, ten other species not known to occur in Latvia were detected (Appendix 5.E, *Table S.5.7*, *Table S.5.10*)

Only a small number of species and genera was detected by both the conventional and eDNA methods the nine ponds combined (*Fig. 5.11*, five species and ten genera for the Romahn-cleaned dataset, and seven species and 14 genera for the MetabaR-cleaned dataset). All species and genera present in the Romahn-cleaned dataset were also present in the MetabaR-cleaned dataset. When considering all species, the number of species uniquely detected with conventional methods (n=51) was higher than with Romahn-cleaned eDNA metabarcoding (n=18) but lower than with MetabaR-cleaned metabarcoding (n=68). When considering only aquatic and amphibian species, the number of species uniquely detected with conventional methods (n=51) was much higher than with eDNA metabarcoding (three for the Romahn- and eight for the MetabaR-cleaned dataset).

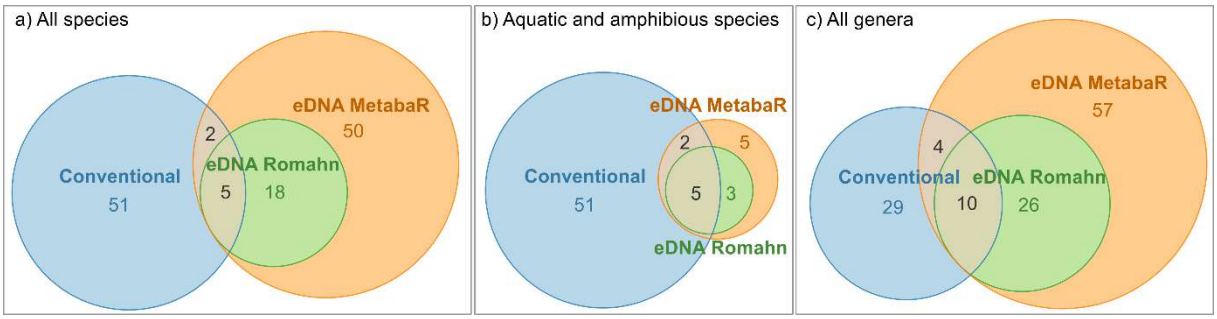


Fig. 5.11. Venn diagrams of a) all species, b) uniquely aquatic and amphibious species and c) genera present in the nine studied Silene ponds according to the conventional macrophyte surveys (blue) and ITS2 eDNA metabarcoding datasets cleaned with the Romahn method (green) and MetabaR package (orange). Numbers indicate numbers of species or genera.

Not only less aquatic and amphibian plant species were present in the Romahn-cleaned dataset than in the MetabaR-cleaned dataset, but they were also detected in less samples (*Fig. 5.12*). Some macrophytes were only detected in one of the duplicate samples taken next to each other at the same time in the same mesohabitat. The aquatic and amphibian species detected with eDNA metabarcoding, but not with conventional methods, were *Ceratophyllum demersum, Cicuta virosa* and *Juncus articulatus* for both the Romahn- and MetabaR-cleaned data, and additionally *Hippuris vulgaris, Myriophyllum spicatum, Potamogeton nodosus, Rorippa islandica* and *Utricularia australis* for the MetabaR cleaned data. *Hippuris vulgaris, Myriophyllum spicatum, Potamogeton nodosus* and *Utricularia australis,* although not detected by conventional surveys in the Silene site, had been detected by conventional surveys in the Neu-Woerr site in 2023 (**Chapter 3**). Samples from 2023 from the Neu-Woerr site had been cycled on the same PCR plates as the Silene samples (Appendix 5.C, *Table S.5.1*).

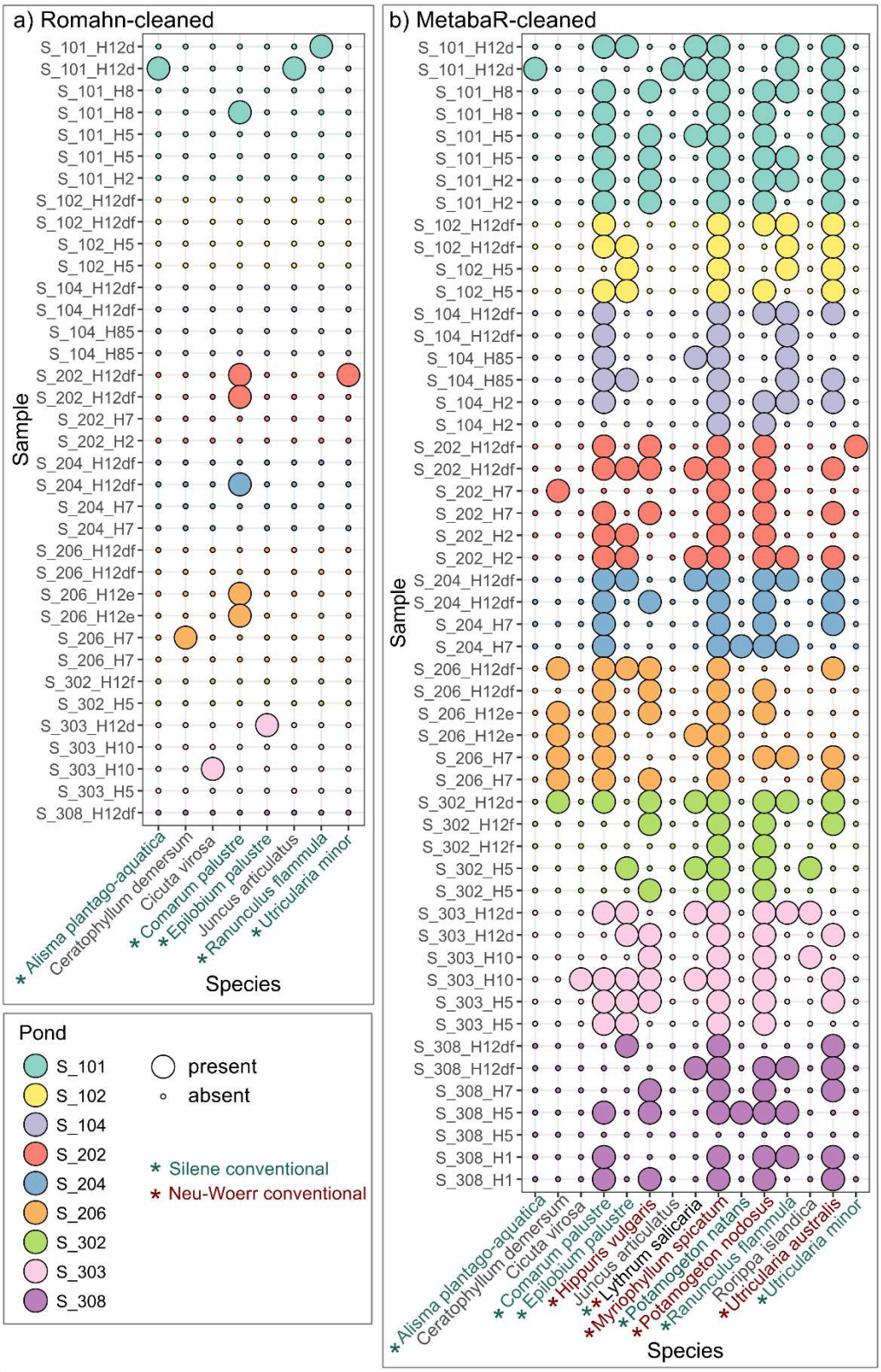


Fig. 5.12. Presence and absence, indicated by large and small filled circles, respectively, of detected aquatic and amphibious plants from samples in a) the Romahn-cleaned and b) the MetabaR-cleaned datasets. Samples taken from the same mesohabitat have the same name and colours indicate the pond samples came from. Species detected by conventional methods in the Silene site in green and with a green asterisk, and species detected by conventional methods on the Neu-Woerr site in dark red. Lythrum salicaria *was detected by conventional surveys on both sites.*

5.4. Discussion

The goal of this study was to benchmark eDNA metabarcoding as a biomonitoring tool for pond macroorganisms. I therefore aimed to study the heterogeneity of macroorganismal eDNA in ponds and to compare eDNA metabarcoding with conventional methods for the identification of pond macroorganisms. Seven primer pairs, targeting macrophytes, macroinvertebrates, amphibians and fish, were combined in four metabarcoding multiplexes. The Unio01_F & Unio01_R primer pair did not amplify target eDNA. This is likely because no *Unionidae* were present in the studied ponds **(Chapter 4**). Five primer pairs did not generate significantly more reads and ASVs in sample PCR replicates than in negative control PCR replicates, and did not result in ASV communities that differed between sample and negative control replicates (*Fig. 5.5*, *Fig. 5.6*). Because of this lack of difference between samples and negative controls, I decided to not analyse the data from these five primer pairs.

Only one primer pair, ITS-3p62p1F1 & ITS-4unR1, amplifying the ITS2 region in plants, generated significantly higher numbers of reads and ASVs in sample PCR replicates than in negative control PCR replicates and resulted in ASV communities that differed between sample and negative control PCR replicates. The data from this dataset was analysed to answer the research questions. As this dataset also had reads and ASVs assigned to negative controls, including the negative tagging controls, this dataset was cleaned with to two different methods, the Romahn and MetabaR cleaning methods, before analysis. Furthermore, the bioinformatics pipeline was adapted to 1) perform demultiplexing (i.e. assignment to the reaction it came from) before paired-end pairing, with the aim to test if something went wrong in the bioinformatics and 2) to cluster ASVs into OTUs, which would make the detection of contaminants easier. The adaptation in the bioinformatics pipeline did not result in major changes in the number of taxa detected (J. Romahn, unpubl. dat), but strangely enough increased the number of reads and ASVs/OTUs resulting from the positive control PCR replicates relative to the other PCR replicate types (*Fig. 5.5*, *Fig. 5.6*, *Fig. 5.7*).

5.4.1. ITS2 Detected plant species

For the nine study ponds, the Romahn-cleaned dataset consisted of 257 OTUs assigned to 77 plant taxa, of which 23 at species and 21 at genus level, and the MetabaR-cleaned dataset contained 853 OTUs assigned to 183 taxa, of which 75 at species and 55 at genus level. Most detected plant species were terrestrial (61% of species in the Romahn- and 65% of species in the MetabaR-cleaned dataset). Some of these detected species are trees, such as Betula pendula, which could have grown around the ponds and whose leaves could have fallen in the ponds. The DNA of terrestrial plants growing in the pond catchment could also, as plant fragments or DNA bound to particles, have been transported by ground water or over land flow (Sjögren et al., 2017). Terrestrial plant DNA might also have originated from pollen, but for lake sediments, pollen contribute minimally to the extracted plant DNA (Alsos et al., 2018; Sjögren et al., 2017).

Other freshwater eDNA plant studies also detected terrestrial species. For example, 64% of species detected in pond water by Robertson (2024), 27% of species detected in lake water by Drummond et al. (2021), 84% of species in headwater streams and rivers detected by Espinosa Prieto et al. (2024) and 82% of species detected in a large river by Espinosa Prieto et al. (2025) were terrestrial. It could be that the higher circumference to surface area ratio of ponds and rivers compared to lakes may result in a higher proportion of the plant eDNA in pond and river water originating from terrestrial species than in lake water.

One of the terrestrial plants detected with eDNA metabarcoding, both with the Romahn and MetabaR cleaning method, was hemp (*Cannabis sativa*). The detected eDNA may have originated from plants grown in the region, or from hemp fibre products such as rope (Dunbar &

Murphy, 2009) that may have been used on nearby farms or by the operators handling the eDNA samples in this study. In the MetabaR-cleaned dataset, also other crop species were present (Appendix 5.E, *Table S.5.7*). These detections may have resulted from plants that could have been present on farms or vegetable gardens near the studied ponds, or from nutrition consumed by the operators. In the study of Robertson (2024), more than 30% of the ITS2 plant amplicons assigned to species level were assigned to crop species. Drummond et al. (2021) and Armando Espinosa Prieto et al. (2025) also detected crop plants in their water samples.

Both the Romahn- and MetabaR-cleaned dataset contained an OTU assigned to *Carex stipata*, a species that is native to the far east of Russia to central China, Japan and North America (Royal Botanic Gardens, 2017) and has no observations in Europe or middle and western Russia on the Global Biodiversity Information Facility (GBIF) (*Global Biodiversity Information Facility*, z.d.). This OTU had a similarity of 97.3% to a GenBank accession MF669178 from a plant from Canada (Appendix 5.E, *Table S.5.10*, Benson et al., 2002). Eight other species of *Carex* were observed with conventional surveys in the studied ponds (Appendix 5.E, *Table S.5.4*), and the Romahn- and MetabaR-cleaned datasets contained respectively three and four other OTUs assigned to the genus *Carex*. I think it is likely that the OTU assigned to *Carex stipata* actually originated from a different *Carex* species. If I had chosen a higher threshold for species assignment, 98% for example, as used by Coghlan et al. (2021) for multiple plant markers, this OTU would have been assigned to the genus *Carex*. It would be interesting to test different thresholds of the minimum similarity with the best match reference sequence for adjusting the levels of taxonomic assignment.

The MetabaR cleaned dataset also contained OTUs assigned to ten additional “exotic” species without known observation in Latvia (Appendix 5.E, *Table S.5.7*). These OTUs had similarities between 97.1 and 100% to GenBank accessions. It cannot be excluded that the exotic species were present on the site, but it could also be that the detections were false positives. Reference databases are not perfect and the GenBank sequences in our database may have been mislabelled and may contain sequencing errors (Keck et al., 2022). As with the *Carex*, it could be that these OTUs originate from plants of the same genus or family as the assigned species. To verify the correctness of exotic detections, data from the other plant markers would have been useful (Sepulveda et al., 2020).

5.4.2. Comparison with conventional macrophyte surveys

The Romahn-cleaned eDNA metabarcoding dataset contained three macrophyte species not detected with conventional methods, and the MetabaR-cleaned dataset another five. It may be that during the conventional surveys, some of these species had been wrongly identified, for example as *Juncus acutiflorus* while it was in fact *J. articulatus*. The conventional surveys may also have overlooked species. *Cicuta virosa* might for instance have grown on a pond bank. However, it could also be that some of the species in the MetabaR-cleaned dataset were false positives. According to the MetabaR-cleaned dataset, OTUs assigned to *Myriophyllum spicatum*, *Potamogeton nodosus* and *Utricularia australis* were present in every pond and to *Hippuris vulgaris* in most ponds. It cannot be excluded that (fragments of) these plants were present in the Silene ponds and that we overlooked them during both the 2022 and 2023 sampling campaigns. However, these plants were detected with conventional surveys on the Neu-Woerr site in 2023 **(Chapter 3),** and samples from the Neu-Woerr site from 2023 were amplified on the same PCR plates as the Silene samples (Appendix 5.C, *Table S.5.1*). These OTUs were also present in the negative controls (Appendix 5.F, *Table S.5.11*). The OTUs assigned to *Myriophyllum spicatum* and *Potamogeton nodosus* were for example present in 100% of the negative field, extraction, PCR and negative tagging PCR controls. These detections might therefore be false positives, and the Romahn cleaning method effectively removed them.

The conventional surveys detected 58 macrophyte species in the nine ponds. Only five of these were present in the Romahn- and seven in the MetabaR-cleaned dataset (*Fig. 5.11*). Genera of some of the species not detected with eDNA, for example *Stellaria*, were detected with eDNA (Appendix 5.E, *Table S.5.8*). However, 33 of the 43 macrophyte genera observed in the conventional surveys were not present in the Romahn-cleaned dataset, and 29 of these not in the metabaR-cleaned dataset. Several causes could have led to false negatives detections with eDNA metabarcoding. These causes include filtering out during the bioinformatics and data cleaning steps, insufficient taxonomic resolution of the markers and incomplete reference databases (Espinosa Prieto et al., 2024). Plant occurring in ponds may also go undetected if their DNA is not captured in a sample, for example if plants are low abundant (Espinosa Prieto et al., 2024), if their DNA is heterogeneously distributed, or if the sample volume is insufficient.

Similar to our results, Robertson (2024) detected with eDNA metabarcoding of sediment and water samples, only eight of the 78 macrophyte species known to be present in a pond complex. Espinosa Prieto et al. (2024) detected a larger proportion of species observed with conventional surveys in streams and rivers with eDNA metabarcoding: 16 out of 24. It could be that they had larger overlap between conventional and eDNA detections because 2) eDNA is more homogeneously distributed in rivers than in ponds, 3) they used five plant markers, 4) they had a reference database that only contained species occurring in the region, and/or 5) they took 24 litre samples.

Marker choice is a major consideration in eDNA metabarcoding (Coghlan et al., 2021; Espinosa Prieto et al., 2024). The ITS2 primers used in our study may not have amplified all plant eDNA present in the samples. Coghlan et al. (2021) tested five primer pairs for aquatic vascular plant metabarcoding on a mock community of DNA from 25 Canadian macrophytes. Their oITS2 primer pair, targeting the ITS2 region, allowed detection of 15 of these macrophytes at genus level and nine at species level. All taxa in the mock community could be detected at genus level with another primer pair, orbcL2, which targets the rbcL gene. However, this primer pair only detected seven taxa at species level. Also, from water samples of Canadian lakes and rivers, the orbcL2 primer detected more taxa than the oITS2 primer, but less taxa at species level.

For vascular plants in general, Espinosa Prieto et al. (2024) tested 15 metabarcoding primers on a mock community. They found that ITS2 primers provided better species level detection than primers amplifying the rbcL, trnL or ITS1 region. However, ITS2 primers they tested exhibited poor amplification of vascular plants other than seed plants, such as horsetails, mosses and ferns. Their rbcL primers amplified these groups, and a combination of ITS2 and rbcL primers improved detection compared to ITS2 alone. The use of multiple markers can also reduce the problems associated with incomplete reference databases, because reference sequences missing for a species for one marker may be available for another marker (Espinosa Prieto et al., 2024). It is very unfortunate that we could not use the data from the rbcL and trnL markers.

The completeness, quality and scope of the reference database used for taxonomic assignment are of importance for metabarcoding outcomes (Blackman et al., 2024a; Gold et al., 2021; Keck et al., 2023; Weigand et al., 2019). Incomplete reference databases can lead to false negatives, erroneous assignment of species without reference sequences to closely related species with reference sequences or assignment to higher taxonomic levels. Reference sequences that are mislabelled or have PCR or sequencing errors can lead to wrong assignment (Gold et al., 2021; Keck et al., 2023; Weigand et al., 2019). The scope of a reference database can range from global, for instance when the entire GenBank is used, to regional, when only sequences of species expected to occur in the study region are included (Gold et al., 2021). Although it is clear that the scope of a reference database, especially when incomplete, affects taxonomic assignment, it is not clear whether global or curated regional reference databases provide more accurate assignments (Gold et al., 2021).

Our reference database represented a compromise between a regional and global database, as it contained available sequences of genera of plants known to occur in Europe, including the known invasive alien species. A smaller reference database containing only species known to occur in the study region, as for example used in Espinosa Prieto et al. (2024), could improve the resolution of taxonomic assignment. This is because reference databases with fewer species increase the probability that the lowest common ancestor algorithm assigns an ASV or OTU to species level. However, especially when incomplete, such a smaller reference database could increase the number of taxa not detected at all (i.e. not at genus or higher taxonomic levels). Small reference databases may be preferred when the species that could be expected in the study system are well known, sequences for these species are available, and species level assignment is important, and large reference databases when this is not the case.

5.4.3. Plant eDNA heterogeneity

For both cleaning methods, samples from the same pond generated significantly more similar OTU communities than samples from different ponds (*Table 5.5*). However, more OTUs were detected when multiple mesohabitats were sampled (Appendix 5.E, *S.5.8*), and plant species were often not detected in all mesohabitats in a pond (*Fig. 5.12*). This suggests that plant eDNA was heterogeneously distributed in the ponds and that multiple mesohabitats should be sampled. This aligns with the recommendation of Harper et al. (2019) to sample multiple locations within a pond, including around barriers, at different depths and with consideration for the ecology of the target organisms.

Only for the Romahn-cleaned and not for the MetabaR-cleaned dataset, samples from the same mesohabitat within a pond resulted in significantly more similar OTU communities than samples from different mesohabitats within a pond (*Table 5.5*). A considerable proportion of the aquatic and amphibious plants detected with eDNA metabarcoding in our study was only recorded in one of the duplicate samples from the same mesohabitat. This could indicate that the 250 mL samples taken did not include enough eDNA to detect all species. Research is still needed to determine the required water volumes and sample numbers to effectively sample ponds (Harper et al., 2019). Studies testing different filtering volumes and sampling locations and the effect of pooling samples before filtering on the detection of macrophytes in ponds (such as performed by Mayne et al. (2024) and Peixoto et al. (2023) for amphibians) would be beneficial. Evidence from aquatic systems in general, suggests that larger sample volumes (more than five, even up to 60 L) may improve detections of macroorganisms (Blackman et al., 2024a).

We used Sterivex capsules to limit contamination, but the volume of water, especially of turbid water, that can be filtered with this type of capsules is limited (Sepulveda et al., 2020; Spens et al., 2017). For example, in the Neu-Woerr sampling campaign we took 1 L samples, but most samples could not be entirely filtered through a Sterivex capsule (*Fig. 5.3*). High capacity enclosed filters would allow larger volumes to be filtered without increasing the risk of contamination that comes with filter handling (Peixoto et al., 2023). Instead of filtering water, passive samplers could be a solution for turbid ponds. For example, filter membranes incubated in the pond water, can be used to capture environmental DNA and could easily be deployed in many locations within a pond (Bessey et al., 2021; Werner et al., Submitted). Furthermore, instead of water, sediment samples could be taken for aquatic plant metabarcoding (Alsos et al., 2018; Robertson, 2024). In ponds, more plant species were detected from sediment samples than from water samples (Roberston, 2024). However, sediment samples do provide other information than water samples, because eDNA in water degrades within days or weeks, while even the top few centimetres of sediment may contain eDNA accumulated over tens of years (Alsos et al., 2018; Espinosa Prieto et al., 2023; Gantz et al., 2018).

5.4.4. Multiplexing and amplification of negative controls

In this study, sets of one plant and one invertebrate primer pair were combined in multiplex PCRs to minimize PCR reagent and consumable costs. In all three multiplexes, the plant primer pair generated more reads than the invertebrate primer pair (*Fig. 5.4*). It could be that our samples contained more plant than invertebrate eDNA, or that the plant primers led to more effective amplification than the invertebrate primers. While we added all primer pairs at the same concentration, De Barba et al. (2014) added in their multiplexing setup the primer pairs at different concentrations, in order to adjust for the differences in amplification yield among the primer pairs. Determining the right relative concentrations of the primers combined in a multiplex PCR can be complex and time consuming, and, even when combining primers that do not form primer dimers and have comparable sizes and melting temperatures, as we did (Werner et al., Submitted) multiplexing can cause artifacts. Therefore, it may be preferable to avoid the multiplexing of multiple primer pairs (Taberlet et al. 2018).

The high number of reads and ASVs assigned to the negative control PCR replicates, and the similarity of ASV communities in the sample and control PCR replicates, prevented me from analysing five of the datasets. As these include the dataset from the 12S-V5 (fwd) & 12S-V5 (rev) primer pair, which had not been multiplexed with another primer pair, this problem could not have been caused by multiplex metabarcoding. Even the tagging negative control PCR replicates, that is unused tag combinations, had similar numbers of reads and ASVs as the sample PCR replicates. The only explanation I have for this is that the tags used to label PCR replicates got mixed. As we used tags of eight nucleotides with five differences between them and the sequencing quality was good (Appendix 5.C, *Table S.5.2*), it is highly unlikely that sequencing errors could have caused the mixing of tags. Furthermore, since the problem is present in five datasets, it is also unlikely that the mixing is a consequence of contamination with the tagged primers. The tagged primers would not have systematically ended up in the wrong wells for five primer pairs. Therefore, I think it is most likely that a tag jump issue (Esling et al., 2015; Schnell et al., 2015) caused the high numbers of reads and ASVs in the negative control PCR replicates.

Tag jumps, or tag switches, are changes through which amplicons carry different tags than originally applied (Esling et al., 2015; Schnell et al., 2015). Since in our study PCR products were assigned to the sample or control PCR replicate they came from by the combination of the forward and reverse tag, a change in one of the tags could lead to the assignment of a sequence to the PCR replicate of another sample or control. Tag jumps may occur during two library preparation steps: during T4 DNA polymerase blunt ending and during post ligation PCR amplification of libraries (Carøe & Bohmann, 2020; Schnell et al., 2015). Our libraries were constructed without post ligation PCR amplification, but blunt ending was performed and could have caused tag jumps as follows. Some of the pooled tagged amplicons could have been single stranded and could have formed duplexes with amplicons from other PCRs. In case T4 DNA polymerase was used for the blunt ending, this polymerase would have removed the tags at the 3' ends of the duplexes that could have been formed between amplicons originating from different PCRs, because the unmatching tags would have formed overhangs. Subsequently, the T4 DNA polymerase would have extended the 3' end using the opposite tag, resulting in a tag jump (Schnell et al. 2015).

Some of the few studies assessing the occurrence of tag jumps found that reads with jumped tags are not very abundant (e.g. 2.1 and 2.6% of the reads from a library, Schnell et al, 2015), but others found that up to 55% of reads from an Illumina run can carry jumped tags (Esling et al., 2015). To avoid assignment of sequences to the wrong sample or control PCR replicate, the same forward and reverse tag could be used (Schnell et al., 2015). However, for our study, using the same forward and reverse tag would have required 1536 tags, which would have been prohibitively expensive and would have required tags that are either longer or have less differences between them. If our libraries suffered from tag jumps because of the blunt ending, a

library preparation protocol without blunt ending, such as the Tagsteady protocol (Carøe & Bohmann, 2020), could have prevented tag jumps.

5.4.5. Data cleaning with information from negative controls

After OBITools bioinformatic processing, the ITS2 OTU dataset had significantly more reads in the sample PCR replicates than in the control PCR replicates (*Fig. 5.8*). In the ITS2 dataset, OTUs were nevertheless present in output from the negative control PCR replicates, including the tagging negative control replicates. I therefore think that tag jumps were less present in this dataset than in the five datasets I did not analyse, but they were not absent either. To clean the ITS2 dataset, two methods that use information from the negative controls were employed. The Romahn method was harsher than the MetabaR method. The Romahn cleaning method, followed by removal of OTUs not present in two out of four PCR replicates, resulted in a reduction of 62% of reads and 33% of samples with data. The MetabaR cleaning method, followed by the same two out of four PCR replicates filtering step, resulted in a reduction of 18% of reads and less than 1% of samples (Appendix 5.F, *S.5.5*).

Cleaning metabarcoding data entails a balance between loosing false positive detections and keeping true detections (Alsos et al., 2018). The Romahn cleaning method was probably more effective than the MetabaR cleaning method at reducing false positive detections in the ITS2 OTU dataset. Unlike the MetabaR-cleaned dataset, the Romahn-cleaned dataset did, for instance, not contain species that were not observed by conventional methods in the Silene site but on the Neu-Woerr site. However, the harsher cleaning could also have induced false negatives. For example, *Potamogeton natans,* detected with conventional methods and present in the MetabaR-cleaned dataset, was absent from the Romahn-cleaned dataset.

The Romahn cleaning included a step where for each OTU the highest number of reads in any of the negative control replicates is removed from all PCR replicates. This type of correction for contamination is used in other studies (e.g. Zizka et al., 2025). For our data the correction caused a large reduction in data because many OTUs were amplified in negative control replicates. The MetabaR cleaning removed OTUs whose relative abundance across the whole dataset was highest in the negative control replicates. This correction is based on the assumption that these OTUs are contaminants, as contaminants would preferentially be amplified in negative control PCRs because there is no sample DNA. The MetabaR method also removed OTUs from PCR replicates in which they were low abundant compared to the rest of the dataset. This step should correct for tag jumps, because the total abundance of an OTU in the entire dataset determines the number of tag jumps that occur for this OTU (Zinger et al. 2019). A similar filtering based on the relative abundance of each ASV in a PCR replicate with respect to the total abundance of this ASV in the dataset is implemented in the VTAM pipeline (González et al., 2023).

The MetabaR tagjump correction probably did not work well for our dataset. It did not lower the number of reads and OTUs in the PCR replicates from the tagging negative controls compared to the samples (K. van der Zon, unpubl. data), as it was supposed to do (Zinger et al., 2021). It could be that the MetabaR cleaning did not remove all false positives from our dataset because the cleaning method requires all data from a sequencing library to be treated together, while we used it on a selection of samples only. The use of information from negative controls to correct metabarcoding data is still a relatively undeveloped practice. Bioinformatic pipelines generally only correct for PCR and sequencing errors, and do not use the information provided by multiple PCR replicates of the same sample and negative controls to correct for other sources of error in metabarcoding data, such as cross-contamination and tag jumps (González et al., 2023; Hakimzadeh et al., 2024; Zinger et al., 2021). Comparisons among strategies to correct for all known possible biases in metabarcoding data would be very useful.

5.4.6. Outlook

Environmental DNA metabarcoding has great potential to overcome the challenges of monitoring numerous ponds at multiple points in time (Harper et al., 2019; Hill et al., 2021). In ponds, eDNA metabarcoding has shown to provide similar detection probabilities of fish and amphibians as conventional methods (Bálint et al., 2018; Li et al., 2019; Peixoto et al., 2023; Schwesig et al., 2025; Skelton et al., 2023). However, for pond macrophytes and macroinvertebrates such congruence has not been achieved yet. The only other pond macrophyte eDNA metabarcoding study (Roberston 2024) and the few pond invertebrate studies (Harper et al., 2021; Krol et al., 2019; Robertson, 2024; Schwesig et al., 2025) have shown poor or no overlap between water eDNA metabarcoding and conventional surveys.

For macrophytes, pond metabarcoding could be improved with the use of multiple markers and improved reference databases (Coghlan et al., 2021; Espinosa Prieto et al., 2024). Systematic studies on the best primers for European macrophytes, sampling volumes and sampling designs would be highly beneficial. High-capacity enclosed filters, sediment sampling and passive eDNA sampling may also enhance macrophyte detection (Alsos et al., 2018; Bessey et al., 2021; Peixoto et al., 2023; Robertson, 2024; Werner et al., Submitted).

To monitor the entire polyphyletic group of macroinvertebrates, eDNA metabarcoding may not be a sensible choice. Generic primers for all macroinvertebrates can lead to high levels of false negatives and to nontarget amplification (Blackman et al., 2024a). The use of primer pairs designed for specific orders (e.g. Ephemeroptera, Plecoptera and Trichoptera) or families (e.g. *Culicidae*), as well as the use of multiple primer pairs enhance agreement with conventional methods (Brantschen et al., 2022; Krol et al., 2019; Leese et al., 2021; Meyer et al., 2021). The extension of macroinvertebrate reference databases would also improve detection (Blackman 2024b; Weigand et al., 2019). Metabarcoding of DNA extracted from bulk macroinvertebrate samples results in inventories that resemble conventional surveys more closely than eDNA metabarcoding of water samples (Harper et al., 2021; Macher et al., 2018). This method is, however, as invasive as conventional sampling. Incubating net sampled macroinvertebrates or substrates that had been placed in the study system, followed by extraction of DNA from the incubation water, are also promising methods to improve invertebrate detections (Sander et al., 2025a; Sander et al., 2025b).

The inclusion of negative controls at every step of the metabarcoding process and the use of PCR replicates (Zinger et al., 2019) did allow us to assess the quality of our data, and to correct for erroneous data in the ITS2 dataset. It is common practice for metabarcoding studies to include negative controls (Blackman et al., 2024a). However, often only PCR negative controls are taken and often it is not reported whether negative control PCRs amplify. Of the aquatic metabarcoding studies published between 2008 and 2020, 71% used PCR negative controls, 44% extraction negative controls and 20% field negative controls. Between 2016 and 2020, 44% of the studies with negative controls did not report the outcomes, 30% reported no amplification and 26% reported amplification of the negative controls (Sepulveda et al., 2020). Even in the last two months, August and September 2025, metabarcoding studies got published that do not mention negative controls at all (e.g. Dong et al., 2025; Nhlengethwa et al., 2025; Viollaz et al., 2025; Wu et al., 2025; Zeng et al., 2025). While low levels of negative control amplification are seen as unavoidable in metabarcoding studies (Sepulveda et al., 2020), outcomes of negative control PCRs should be reported and used to correct metabarcoding data. At the moment, it is possible that studies with high levels of amplification in the negative controls do not report this, or do not get published (Sepulveda et al., 2020). This is problematic because information on negative control amplification is required for the optimization of metabarcoding protocols. To provide sound ecological conclusions, to improve confidence in eDNA metabarcoding and to allow its use as a standard application in biomonitoring, metabarcoding studies should always report PCR results

of negative field, extraction, PCR and tagging controls (Jerde, 2021; Schenekar, 2023; Sepulveda et al., 2020; Zinger et al., 2019).

Chapter 6

Jana and Aija using a Secchi tube. The water of pond 308 was pretty clear

Chapter 6: General discussion

The importance of ponds, especially when placed in networks, has been increasingly recognized over the last three decades. Collectively, ponds can contribute more to regional freshwater biodiversity than lakes or rivers (Williams et al, 2004, Davies 2008). In addition, they provide habitat to rare and threatened species and deliver other ecosystem services including pollution control, carbon cycling and water supply (**Chapter 1**, section 1.2.2, Biggs et al., 2017; Fehlinger et al., 2023; Hill et al., 2021; Williams et al., 2008). In 2014, ponds were called “a practical conservation solution waiting to happen” (Céréghino et al., 2014), and pond restoration and creation projects have been initiated in Europe (Bartrons et al., 2024; De Necker et al., 2025; Hill et al., 2025). Although pond creation is not ecological restoration in the strict sense (as defined in Gann et al., 2019; SER, 2004), it is part of the continuum of restorative activities that can contribute to ecosystem recovery (as in Gann et al., 2019). With the implementation of the EU Nature Restoration Regulation, the number of pond network creation projects in Europe can be expected to increase (**Chapter 1**, section 1.2.3).

To ensure that future pond network creation projects are effective, evidence-based practices should be used. However, current knowledge on how to best design and locate ponds largely stems from pond research in the United Kingdom (e.g. Biggs et al., 2018; Biggs & Williams, 2024). The limited knowledge available for other parts of Europe may be related to the infrequent and narrowly focused monitoring of pond creation projects in the EU (De Necker et al., 2025). Furthermore, guidance on how to monitor and evaluate pond creation projects is lacking. In this PhD thesis I studied how environmental and spatial variables influence macrophyte and macroinvertebrate communities in two human-made pond networks: Neu-Woerr on the French German border, and Silene in Latvia. Furthermore, I explored macrophyte surveys, the S_3i macroinvertebrate sampling protocol (Labat et al., 2022) and environmental DNA metabarcoding as methods to monitor human-made ponds. The results and limitations of the study are discussed in sections 6.1 and 6.2, the implications for pond creation, and for the monitoring, evaluation and management of human-made ponds in section 6.3 and perspectives for future research in section 6.4.

6.1. Variables structuring macrophyte and macroinvertebrate communities in human-made pond networks

For the effective creation and management of human-made pond networks, it is necessary to understand which factors could influence the biological communities in ponds. Below I provide a synthesis of the results of **Chapter 3** and **4**. Based on this synthesis I discuss how environmental and spatial variation within pond networks can influence macrophyte and macroinvertebrate communities (research question A, **Chapter 1**, section 1.5.2). Furthermore, I address how environmental variables that were not measured or not measured correctly on the study sites, and other factors could influence communities in human-made pond networks as well. These other factors relate to time, vertebrate herbivores and ecosystem engineers, and differences between pond networks.

6.1.1. Environmental variables structuring macrophyte and macroinvertebrate communities within pond networks

In **Chapter 3**, for pond networks Neu-Woerr and Silene separately, I analysed the coinertia between environmental variables and the cover of macrophyte taxa, as well as between environmental variables and macrophyte community metrics. In **Chapter 4,** for the Silene site, I additionally analysed the coinertia between environmental variables and densities of macroinvertebrate taxa, as well as between the cover of macrophyte taxa and densities of macroinvertebrate taxa. Moreover, I investigated how macroinvertebrate communities varied between different pond mesohabitat types. Although macroinvertebrates were sampled on the Neu-Woerr site as well (**Chapter 2,** *Table 2.4*), I only analysed macroinvertebrate data from the Silene site from 2022 because the sorting and identification of the other samples was not finished in time to include the data in my PhD. In **Chapter 3** data from 2023 and in **Chapter 4** data from 2022 were used.

The environmental variables I included in the coinertia analyses comprise water quality variables (pH, transparency, chlorophyll-a concentration, specific conductivity, and only in **Chapter 4** concentrations of dissolved oxygen, total phosphorus and total nitrogen), pond construction variables (age, shade from surrounding trees, and only in **Chapter 3** depth, surface area and distance to the nearest waterbody), and for Neu-Woerr the crayfish abundance and for Silene in **Chapter 4** the presence of fish as well. I did not include nutrient concentrations in **Chapter 3**, because the nutrient data I obtained were not compatible between Neu-Woerr and Silene, and I aimed to include the same environmental variables in the analyses for the two sites. In this chapter I did not include the concentration of dissolved oxygen neither, because I did not expect dissolved oxygen concentration to influence macrophyte communities. In **Chapter 3** I did not include fish presence because I did not have reliable data on the presence of fish in the Neu-Woerr ponds at the time of the macrophyte surveys. In **Chapter 4**, I did not include depth, surface area and distance to the nearest waterbodies. However, when I add them to the analyses, these variables have low contributions to the coinertia, so their omission did not have a large influence on the outcomes (K. van der Zon, unpub. data).

The macrophytes in Neu-Woerr and Silene and the macroinvertebrates in Silene were all primarily structured along a gradient with relatively high levels of shade from surrounding trees and chlorophyll-a concentrations on one end (the “low light” end) to relatively high levels of water transparency on the other end (the “clear water” end) (*Fig. 6.1a*, **Chapter 3**, *Fig. 3.3*, *Fig. 3.4*, **Chapter 4**, *Fig. 4.6*). In Neu-Woerr, ponds with relatively high crayfish abundance were also located at the “low light” end of the gradient. In Silene, ponds at the “low light” end had relatively high levels of total nitrogen and total phosphorus, whereas the ponds at the “clear water” end were relatively deep, had relatively high pH and had relatively high concentrations of dissolved oxygen.

In both sites, the macrophyte taxonomic distinctness and the relative cover of anchored submerged macrophytes were highest in the “clear water” ponds (Fig 6.1b, **Chapter 3**, Fig. 3.3). In Neu-Woerr, “low light” ponds had the highest relative cover of emergent macrophytes while “clear water” ponds were characterized by a relatively high total macrophyte cover. In Silene, the relative cover of free-floating macrophytes was highest in “low light” ponds. The abundances of some species were also structured along this gradient (**Chapter 3**). The density of most macroinvertebrate taxa was highest in the “clear water” ponds but some taxa, for example several species of ramshorn snails *(Planorbidae),* were more abundant in the “low light” ponds (**Chapter 4**).

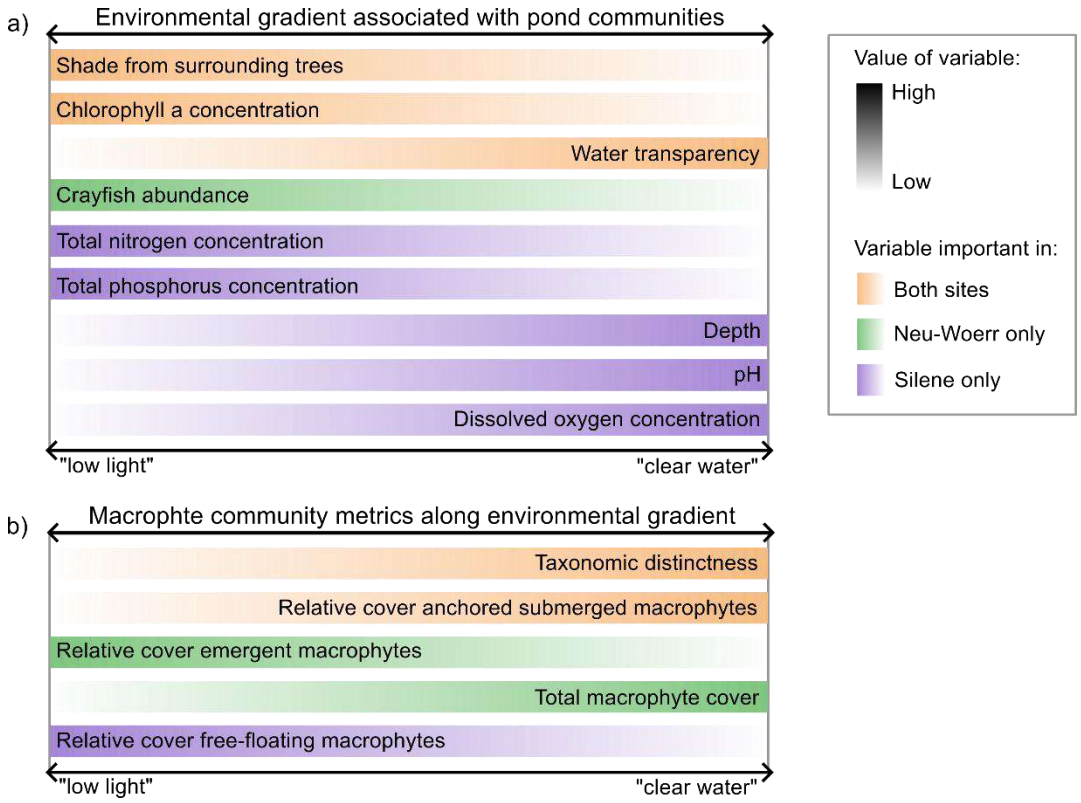


Fig. 6.1. Schematic gradients within the two studied pond networks. Each bar indicates a variable that had a relatively large contribution to the first coinertia axes of Figs. 3.3, 3.4 and 4.6, with the colour indicating whether the variable was important in Neu-Woerr, Silene or both sites, and the colour intensity schematically representing the variable value along the main gradient (see legend). "Low light" and "clear water" are names given to the ends of the gradient in the text. a) Main environmental gradient along which macrophyte and macroinvertebrate communities were structured. b) Main macrophyte community metrics along the environmental gradient.

In **Chapter 3** I evoked the shallow lakes theory of alternative stable states (**Chapter 1**, section 1.3.3, Scheffer & Van Nes, 2007) to explain how crayfish, phytoplankton (with biomass measured as chlorophyll a concentration) and free-floating macrophytes could induce feedback mechanisms that reduce the underwater light conditions and thereby the cover of submerged macrophytes. The lower cover of submerged macrophytes and the higher abundance of *Planorbidae* in the "low light" ponds could be explained with the environmental filtering metaphor (**Chapter 1**, section 1.3.2, Keddy, 1992). In this metaphor, macrophytes with the trait "submerged life form" cannot pass the abiotic filter of "low underwater light condition" because they would not be able to photosynthesize under this condition (Meyer et al., 1943). The *Planorbidae* belong to the few macroinvertebrate taxa that can pass the abiotic filter of "low oxygen concentration" because they can take oxygen at the surface, have lungs and carry haemoglobins with high affinity for oxygen (Jones, 1961; Lieb et al., 2006). In conclusion, the community composition of macrophytes and macroinvertebrates was likely influenced by environmental variables that were stabilised by positive feedback loops.

In **Chapter 4**, I found that the Silene macroinvertebrate communities differed significantly between ponds, but not between mesohabitat types (*Fig. 4.5*). I therefore suggested that pond wide environmental variables may have been more important than the mesohabitat types in structuring the macroinvertebrate community composition. In theory, the different mesohabitat types provide different levels of habitat complexity (Jeffries, 1993; Walker et al., 2013) and I expected that this would result in distinct macroinvertebrate community compositions. However, the communities in

the same mesohabitat type in different ponds were not similar. It could be that the same mesohabitat type (i.e. same dominant macrophyte growth form) may have contained different plants or different densities of plants in the different ponds. Per pond, there was a strong and significative correlation between macrophyte and macroinvertebrate community composition. However, I could not show if this was related to the complexity of the structural habitat the macrophytes provide.

Based on my findings and the literature cited in **Chapters 3** and **4,** as well as Jeffries (1993) and Messyasz et al. (2009), I propose that the main relationships between environmental variables, macrophyte and macroinvertebrate communities in the Neu-Woerr ponds were those depicted in *Fig. 6.2*. The relationships are hypothetical, and have not yet been evaluated using approaches such as Structural Equation Modelling (Grace et al., 2010) or manipulative experiments (Karban et al., 2014). I did not include feedback mechanisms nor fish or other potentially influencing biological compartments (e.g. zooplankton, herpetofauna) in the model.

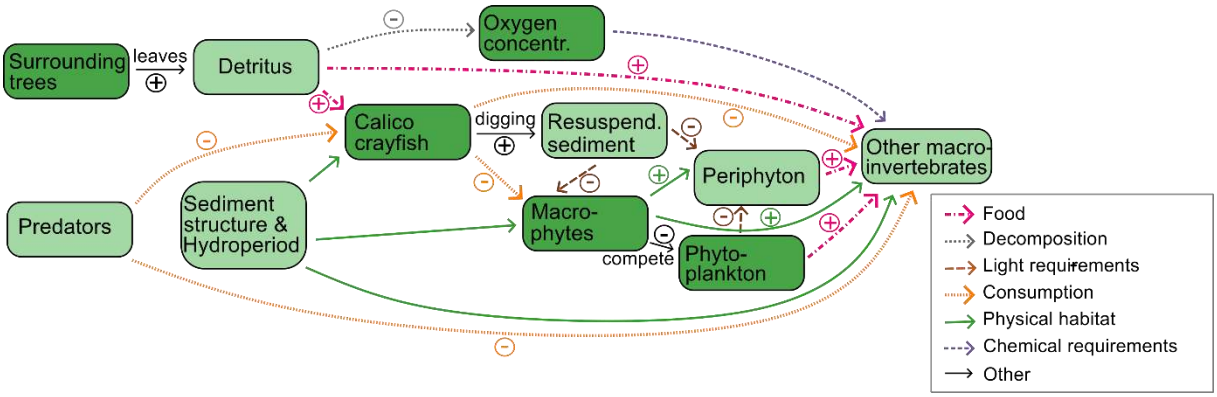


Fig. 6.2. Hypothetical main relations between environmental and biological components in the Neu-Woerr ponds, without feedback loops. Boxes indicate the quantity and/or diversity of components. Boxes in dark green relate to components for which I had some measure, and boxes in light green to components which I did not measure. The colour and line type of arrows indicate the type of relation (see legend, and for "other relations" the text next to the arrows). Relations that are generally positive are indicated with +, negative with - and impacts that do not relate to quantity but to diversity have no sign. For clarity some relations were omitted, for example the potential effects of macrophytes, periphyton and phytoplankton on dissolved oxygen concentration and pH.

Calico crayfish play a central role in the hypothetical model for Neu-Woerr (*Fig 6.2*). In the Neu-Woerr ponds, the density of calico crayfish could be very high. We captured up to 67 individuals in a single a sweep net sample covering one square metre for pond surface (Appendix 3.A, *Table S.3.1*). Our sweep netting method was biased towards juveniles, but other studies also found that calico crayfish can reach remarkably high densities in ponds. In German ponds close to the Neu-Woerr site, Herrmann et al. (2022), for example, found calico crayfish densities higher than ten individuals per square metre using mark-recapture methods. As discussed in **Chapter 3**, Calico crayfish can have very strong negative effects on macrophyte biomass, which affects the other biological compartments.

The abundance of calico crayfish in a pond could be influenced by several variables. These include the sediment structure and hydroperiod of the pond. This is because calico crayfish have a strong preference for muddy sediments where they can dig burrows, and are well adapted to periods of drought (Herrmann et al., 2022; Herrmann & Martens, 2024; Tack, 1941), which could give them a competitive advantage in ponds that dry up. Although they also feed on macrophytes and macroinvertebrates, detritus is one of their main food resources (Chucholl, 2012) and it could

be that the amount of leaf litter falling into ponds may affect their densities. Furthermore, it is likely that calico crayfish can reach high abundances in Neu-Woerr because of a lack of predators that could reduce their numbers, such as eel (*Anguilla Anguilla* L., 1758) and otters (*Lutra Lutra* L., 1758) (Gherardi et al., 2011; Lacombe et al., 2025).

Similarly as above, I constructed a hypothetical model for Silene (*Fig. 6.3*). In this model, macroinvertebrates are directly influenced by macrophytes because of the physical habitat they provide. It has been suggested that macroinvertebrates of different sizes could live on macrophyte leaves and stems with different sizes and shapes providing different sizes and shapes of the interstitial spaces (Jeffries, 1993). Macrophytes also provide surfaces for periphyton communities (algae, bacteria, fungi, protists and detritus growing on surfaces), which serve as food for many macroinvertebrate species. Macroinvertebrate community composition and abundance can therefore directly and indirectly be influenced by the architecture of the macrophytes. Furthermore, the macrophyte and macroinvertebrate communities could be influenced by the same variables, such as sediment structure or water acidity.

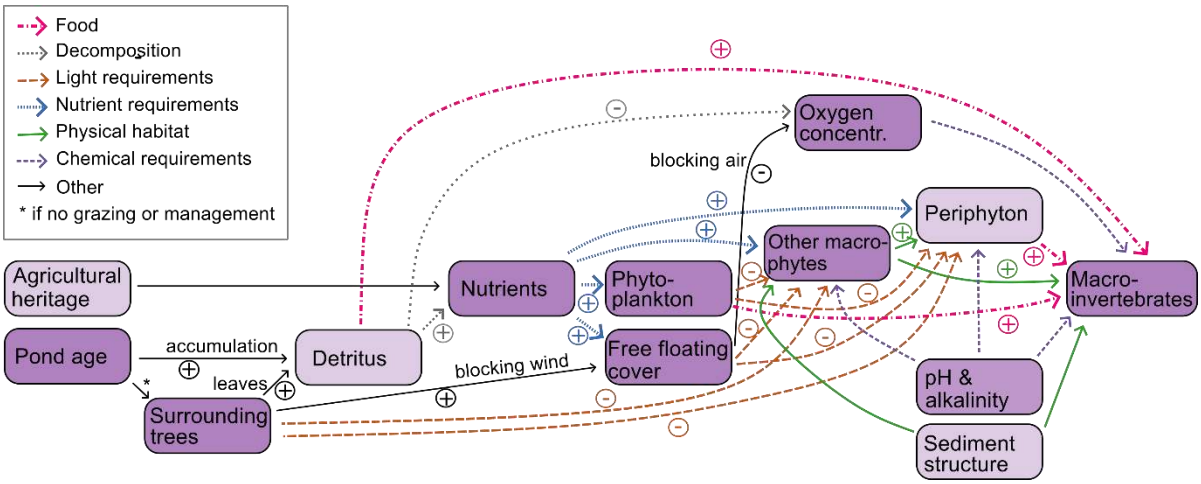


Fig. 6.3. Hypothetical main relations between environmental and biological components, in the Silene ponds, without feedback loops. Boxes indicate the quantity and/or diversity of components. Boxes in dark purple relate to components for which I had some measure, and boxes in light purple to components which I did not measure. Since I had data on pH but not on alkalinity the pH & alkalinity box is in medium purple. The colour and line type of arrows indicate the type of relation (see legend, and for "other relations" the text next to the arrows). Relations that are generally positive are indicated with +, negative with – and impacts that do not relate to quantity but to diversity have no sign. For clarity some relations were omitted, for example the potential effects of macrophytes, periphyton and phytoplankton on dissolved oxygen concentration and pH.

6.1.2. Environmental and spatial structuring of macrophyte beta diversity within pond networks

In **Chapter 3**, I used non-linear regression and generalized dissimilarity modelling (GDM) to study the Bray-Curtis dissimilarity (calculated on the log(x+1) transformed percentage cover) between the macrophyte communities in the pond networks. For the Silene pond network, non-linear regression showed that the similarity between macrophyte communities decayed significantly with the geographic distance between ponds (*Fig 3.5*). However, the GDM did not show a significant effect of the geographic distance between ponds, but did show a significant effect of the difference in shade from surrounding trees and water transparency between ponds on the macrophyte dissimilarity (*Fig. 3.6*). For Neu-Woerr I obtained no significant relationships with non-linear regression, nor with GDM.

Based on these findings, I concluded that the distance between ponds in itself was not an important variable for the macrophyte beta diversity in the studied pond networks. In Appendix 6.A I show some more analyses on the macrophyte beta diversity in Silene that were not included in the published article of **Chapter 3**. These show, for example, that in Silene, ponds close to each other (separated by less than 300 m) had similar macrophyte communities, but similar environmental conditions as well (Appendix 6.A, *Fig S.6.1*, *Table S.6.1*). In Neu-Woerr, ponds close to each other were not similar in the environmental variables I measured. This could explain why I found no significant relation between macrophyte dissimilarity and spatial distance in Neu-Woerr. I expect that on the small scale of the studied pond networks, the macrophyte communities were not influenced by dispersal limitation, but mainly assembled by species sorting along environmental gradients (**Chapter 1**, section 1.3.5)

In Appendix 6.A, to further explore if dispersal could have influenced macrophyte community assembly in the pond networks, for macrophytes dispersed by air, water and animals separately, I tested Mantel correlations between their community dissimilarity and the geographical distance between ponds. For Neu-Woerr, none of the Mantel correlations were significant (Appendix 6.A, *Fig S.6.1*, *Table S.6.1*). For Silene, the result was not significant for wind dispersed taxa either, but the dissimilarity between macrophyte communities was significantly correlated with the distance between ponds for the water and animal dispersed taxa. However, I cannot conclude that in Silene wind dispersed taxa were not dispersal limited while water and animal dispersed taxa were. This is because the wind dispersed taxa were generally emergent macrophytes and the submerged macrophytes did generally not disperse by wind (Appendix 6.A, *Fig. S.6.3*). It could be that the emergent taxa were less influenced by spatially structured environmental variables than the submerged macrophytes and that this is the reason wind dispersed taxa seemed dispersal limited and water and animal dispersed taxa seemed.

In conclusion, I did not find convincing evidence for dispersal limitation of macrophytes in the pond networks I studied. However, studies of lakes and temporary ponds have shown that dispersal limitation can be important for macrophyte community composition (Capers et al., 2010; García-Girón et al., 2019). The difference between my results and these studies could be that I studied ponds that were close to each other (all less than 3 km apart), these studies included waterbodies up to a few hundred kilometres apart (Capers et al., 2010; García-Girón et al., 2019). Capers et al. (2010), for example, found a significant decay of community similarity with spatial distance in lakes up to 170 apart, but they found no significant relation in a subset of lakes less than 40 km apart. To further investigate dispersal limitation in pond macrophytes, it would be interesting to include ponds with a wide range of geographic distances between them.

6.1.3. Unmeasured variables

Besides the variables discussed above, other factors, including environmental variables that I did not measure or not measure correctly, ecological succession over time, vertebrate herbivores and ecosystem engineers and differences between pond networks can influence macrophyte and macroinvertebrate communities in human-made pond networks as well. Some of the variables in *Figs. 6.2* and *6.3* were not measured, such as alkalinity and sediment structure, or not measured correctly, such as nutrient and oxygen concentrations. It would have been preferable to measure water nutrient concentrations in winter, when they tend to be highest (Linton & Goulder, 2000; Sager & Lachavanne, 2009). Since sediments are important sources of nitrogen and phosphorus for rooted macrophytes (Barko & Smart, 1981), it would have been interesting to measure sediment nutrient concentrations as well. As discussed in **Chapter 4**, it would have been preferable to measure oxygen concentrations early in the morning and at different depths. Furthermore, the results of **Chapter 4** may indicate that for some ponds a higher number of macroinvertebrate samples would have improved the completeness of the inventories. With regards to Neu-Woerr, in **Chapter 3** I suggested that the unmeasured variables water level

fluctuations and pesticide levels could have influenced the macrophyte communities, and that overnight trapping with artificial refuge traps could have improved the crayfish abundance estimations.

One of the original PhD goals was to investigate ecological succession in created ponds by studying differently aged ponds (chronosequences, or space-for-time substitutions, **Chapter 1**, section 1.3.1, section 1.5.1). However, I could not fully address this aim because the ages of the studied ponds had a limited range in both sites, and in Neu-Woerr an uneven distribution. In Neu-Woerr, eight ponds dated from 2011/2012, three from 2013 and two from 2015 and in Silene six dated from 2013 and seven from 2018 (**Chapter 3**, *Table 3.1*). The coinertia analyses in **Chapter 3** and **Chapter 4** for Silene indicated that macrophyte and macroinvertebrate communities could have been influenced by pond age, but for Neu-Woerr I found no contribution of pond age to the coinertia with macrophyte community composition nor with the macrophyte community metrics (*Fig 3.3*, *Fig 3.4* and *Fig. 4.6*). In Silene, pond age was negatively correlated with depth, while the newer ponds were not constructed to be deeper. I suggested that the older ponds might have lost some depth to sedimentation (**Chapter 3**). However, with the low number of ponds studied it is not possible to differentiate between the effect of age and other variables. Because of their placement on the site, the 2018 ponds could, for example, also have had a different water level regime than the 2013 ponds, and this could have resulted in a correlation between measured depth and age as well.

To study succession, the ponds should be monitored for more years. Macrophyte data were collected in 2022 and 2023. However, pond macrophyte communities can portray interannual variability for reasons other than succession (Marlene et al., 2020). It would be more interesting to study the Silene ponds again in 2028 or 2029, when the 2018 ponds will be about ten years old, the same age as the 2013 ponds at the time of study. In general, it is more informative to study succession in the same set of ponds over time than to perform chronosequences. This is because chronosequences can be misleading (e.g. Kreyling, 2025) and would require large numbers of ponds that experienced similar conditions and only differ in age. Long term (>10 year) studies of ponds are rare but are needed to create knowledge about the timescales and pathways of succession in ponds. These have been hypothesised to be influenced by pond origin, climatic and geological settings, and by natural and anthropogenic disturbances. However, the long-term datasets required to study these factors are lacking (Hill et al., 2021).

Besides the fish mentioned before, other vertebrates could have influenced the macrophyte and macroinvertebrate communities in the study sites. In the study site Silene, beavers (*Castor fiber*, L. 1758) and elk (*Alces alces*, L. 1758) are active. Beavers are known to increase macrophyte and macroinvertebrate alpha and beta diversity through selective feeding on macrophytes and alterations to the environment (Law et al., 2014, 2019; Nummi et al., 2021). Elk also feed selectively on macrophytes and can have a large effect on them (Fraser & Hristienko, 1983). Both sites are reintroduction locations for *Emys orbicularis* (**Chapter 1**, section 1.5.1), an opportunistic omnivorous turtle that feeds primarily on macroinvertebrates and macrophytes. However, based on the finding that the number of *E. orbicularis* did not influence the macroinvertebrate community composition in their acclimatisation basins (Meyer et al., 2025), I do not expect that *E. orbicularis* exerted an important influence on the macroinvertebrate communities in the study sites.

Pond network creation outcomes differed largely between Neu-Woerr and Silene. Whereas the macrophyte diversity in the Silene ponds was high (11 to 30 taxa per pond) and the ponds varied in their relative cover of different life forms (*Fig 3.2*), the macrophyte diversity in Neu-Woerr was low (5 to 17 taxa per pond) and consisted mainly of emergent plants and charophytes. Outcomes of restorative activities can be context dependent (Dickens et al., 2016; Perring et al., 2015). They could, for example, depend on the history, regional species pool and environmental conditions of the site where they are implemented. I only studied two pond networks and only performed

analyses within the networks, so I have no results on the context variables that could have caused the macrophyte communities to be richer in Silene than in Neu-Woerr. In **Chapter 3** I suggested that the low macrophyte diversity and cover in Neu-Woerr may have been caused by the calico crayfish, but I cannot exclude other factors. To study the effect of crayfish, the macrophyte communities in the Neu-Woerr ponds could be compared to those in nearby ponds without crayfish. To investigate other variables that could cause macrophyte communities to differ between human-made pond networks, a large number of human-made pond networks across Europe should be investigated. The development of an open-access global pond database of biotic and environmental data proposed by Hill et al. (2021) would be highly beneficial for this purpose.

6.2. Pond monitoring

The monitoring of ponds created as restorative activity is indispensable for the evaluation of the effect of the restorative activity, to improve pond creation techniques and to inform adaptive management. However, monitoring of pond creation projects is generally lacking or limited (De Necker et al., 2025). As ponds are rarely monitored, it may not be evident what protocol to use. In this PhD I studied macrophytes and macroinvertebrates. Like amphibians, these groups are seen as suitable indicator groups for pond biodiversity assessment (**Chapter 1**, section 1.4.2**,** (Oertli et al., 2005b). In this section I discuss the suitability of the methods applied in this PhD research, namely macrophyte surveying, macroinvertebrate sampling following the S_3i protocol and eDNA metabarcoding for monitoring human-made pond networks (research question B, **Chapter 1,** section 1.5.2).

Macrophytes are central to the functioning of pond ecosystems (**Chapter 3**). Pond macrophyte surveys, as performed in **Chapter 3,** do not require expensive equipment and have a relatively low impact on the environment (limited to disturbing sediment and sometimes harvesting a few individuals for observation in the lab). Furthermore, of the pond monitoring methods used in this PhD, macrophyte surveys are probably the least time consuming (Appendix 6.B). As with macroinvertebrate identification, they do require taxonomic expertise. In the rest of this section, I will discuss the suitability of the S_3i macroinvertebrate protocol and eDNA metabarcoding for pond monitoring.

6.2.1. Macroinvertebrate sampling with the S_3i protocol

In **Chapter 4**, I applied the S_3i macroinvertebrate sampling protocol (**Chapter 1**, section 1.4.3), which was recently developed in France (Labat et al., 2022), to sample invertebrates in Latvian ponds. As far as I am aware, there are no published studies in English language on macroinvertebrates in Latvian ponds, and no macroinvertebrate sampling methods suitable for ponds had been tested in this country yet. The S_3i protocol prescribes one sample per mesohabitat type, and I aimed to assess to what extent this protocol would capture the richness of macroinvertebrates inhabiting the studied ponds. I resolved taxonomic ambiguity in the data by assigning higher level “parent” identifications to their lower level “children” in the same sample and subsequently in the rest of the dataset (Meredith et al., 2019). I then transformed the sample-based abundance data into sample-based incidence data, as is recommended (Chiu, 2023), to calculate sample-based rarefaction curves.

For the ten ponds combined, the sample-based rarefaction curve and the Chao2 estimator (*Fig. 4.4*) indicated that the 116 observed taxa constituted a considerable proportion (88% of the Chao2 estimator) of the macroinvertebrate taxa that were likely to be present in the ponds. However, for the individual ponds, sample-based rarefaction curves did not saturate for ponds where only two or three samples had been taken (*Fig. 4.3*). I therefore suggested in **Chapter 4** that for ponds with sizes and macrophyte diversities similar to the ones I studied, at least five or six samples

should be taken. However, I would have needed more samples from the same pond to observe at which point the rarefaction curves would saturate. Furthermore, I did not calculate Chao2 estimators for individual ponds because the estimator is calculated based on the number of taxa that are observed in only one or two samples (Chao, 1984), and we took only two to six samples per pond, and the mesohabitat sampling probably reduced the probability to find the same taxon in multiple samples. All in all, I concluded that the S_3i macroinvertebrate protocol is suitable, but that tests with more samples per pond should be performed to determine the required number of samples per pond.

6.2.2. Environmental DNA metabarcoding

Environmental DNA (eDNA) metabarcoding is non-invasive and has the potential to upscale pond monitoring, but has been rarely studied in ponds for macroorganisms other than amphibians and fish (Harper et al., 2019, **Chapter 1**, section 1.4.4). In **Chapter 5**, I applied eDNA metabarcoding to identify the macrophytes, macroinvertebrates, amphibians and fish in the Silene pond network. Duplicate water samples were taken in each of the mesohabitats where afterwards macroinvertebrates were sampled. I adapted a high salt DNA extraction protocol (Kusanke et al., 2020, Appendix 5.B) so that it could be used to extract DNA from Sterivex filters stored in Longmire's buffer. Polymerase Chain Reaction (PCR) was performed in four replicates on samples, negative field, negative extraction, negative PCR, positive PCR and negative tagging controls. Primer pairs amplifying regions of a) plant and b) invertebrate DNA were combined in the same PCR. This resulted in more DNA sequence reads for plants than for invertebrates. Optimisation of the ratio between the concentrations of the plant and invertebrate primer pairs could resolve these uneven outcomes, but since this would be time consuming and since combining multiple metabarcodes in the same PCR could still cause artifacts, it is preferable to not combine multiple metabarcodes in the same PCR reaction (Taberlet et al., 2018).

Seven primer pairs were used and the one targeting *Unionidae* bivalves did not result in DNA sequence reads of the target group, probably because they were absent from the Silene ponds. Five primer pairs did result in numbers of DNA sequence reads and Amplicon Sequence Variants (ASVs) that did not differ significantly between the PCRs from the samples and negative controls. Only for the primer pair amplifying the ITS2 region in plants the numbers of reads and ASVs resulting from the sample PCRs were higher than the numbers from the negative controls. I decided to only analyse the plant ITS2 dataset. Since this dataset still had some reads and ASVs in the negative controls, I used two different post-bioinformatic processing cleaning methods, "Romahn" and "MetabaR" (Zinger et al., 2021), to clean the data. Furthermore, we adapted the bioinformatic pipeline to produce Operational Taxonomic Units (OTUs), even though this probably did not have an effect on the results.

The Romahn-cleaned ITS2 plant dataset contained 77 taxa assigned to 23 species and 36 genera, and the MetabaR-cleaned dataset 183 taxa assigned to 75 species and 97 genera. Most detected species were terrestrial. The Romahn- and MetabaR-cleaned datasets contained respectively eight and 15 macrophyte species. With the conventional macrophyte surveys 58 macrophyte species (43 genera) were detected. The overlap between eDNA metabarcoding and conventional surveys was small: five species and ten genera for the Romahn-cleaned and seven species and 14 genera for the MetabaR-cleaned dataset. The only other pond macrophyte study I am aware of, a recent PhD thesis (Robertson, 2024), also found little overlap between plant species detected with eDNA metabarcoding and conventional surveys: eight species detected by both methods, 70 only detected by conventional and 39 only by eDNA.

In both the Romahn- and MetabaR-cleaned datasets, OTUs detected in samples from the same pond were more similar than in samples from different ponds. In the Romahn-cleaned dataset, samples from the same mesohabitat had also more similar OTU communities than samples from

different mesohabitats within the same pond. The distribution of eDNA in ponds is patchy (Harper et al., 2019; Mayne et al., 2024). This suggests that sampling multiple mesohabitats in a pond would result in more complete plant eDNA inventories than taking only one sample. The detection of macrophyte species in some but not all samples from the same pond (*Fig. 5.12*) suggests the same. However, it could also be that the sample volume we used (250 mL), was too small to capture all eDNA in a mesohabitat. The effects of the sample volume, number of samples and sample locations on macrophyte detection in ponds should be further investigated.

A low level of negative control amplification is probably unavoidable in eDNA metabarcoding (Sepulveda et al., 2020). However, finding as many reads and ASVs in PCRs of negative controls as in sample PCRs in five datasets, points at an error in the metabarcoding process. In our study a combination of a forward and reverse tags (molecular labels) was used to indicate from which sample or control reaction the sequences originated. One of the negative control types we used, the negative tagging control, consisted of not using several combinations of forward and reverse tags. It was therefore highly surprising to find in five datasets similar numbers of reads and ASVs in the negative tagging controls (i.e. sequences carrying tag combinations we did not use, *Fig. 5.5* and *Fig. 5.6*) as in the samples. I concluded that something had gone wrong with the tags. Switching of tags ("tagjumps") can occur during library preparation (preparation for sequencing) when T4 DNA polymerase is used (Schnell et al., 2015). I do not know if this polymerase was used for our amplicons, but if this was the case, it could have caused the high numbers of reads and ASVs in the negative controls.

To control for potential tagjumps and contamination in the ITS2 dataset, I applied the Romahn and MetabaR cleaning methods. With the Romahn cleaning method I lost more data (56% of reads) than with the MetabaR cleaning method (13% of reads), but it was probably more effective at removing false positive detections. The Romahn cleaning method removed, for example, all the species from the Silene dataset that had been observed by conventional methods on the Neu-Woerr site but not on the Silene site, and the MetabaR method did not do this. The MetabaR cleaning method may be more effective when used on the entire dataset resulting from an amplicon library (i.e. not only on the Silene samples). The development of post-bioinformatic processing metabarcoding data cleaning methods started only recently (e.g. González et al., 2023; Zinger et al., 2021) and their application is not standardized. Future research should compare different data cleaning methods and evaluate their effectiveness at removing false positive detections without removing real detections.

6.3. Implications for pond network creation, monitoring, evaluation and management

Pond networks have been created for biodiversity enhancement and species conservation in Europe (Bartrons et al., 2024; De Necker et al., 2025; Hill et al., 2025). As pond network creation can contribute to the achievement of the Kunming-Montreal Global Biodiversity Framework and EU Nature Restoration Regulation targets (**Chapter 1**, section 1.2.3), I expect that pond network creation projects will be increasingly implemented in the coming years. In this section I will discuss how the results from this PhD contribute knowledge on how to improve the design of pond networks for biodiversity and conservation, and on how to monitor and evaluate them.

6.3.1. Implications for pond network design and management

This PhD highlights environmental variables that can be important for the assembly of macrophyte and macroinvertebrate communities in human-made pond networks. Shade from surrounding trees, water transparency and chlorophyll-a concentration, which can influence water transparency, were important for the community composition, taxonomic distinctness and relative

cover of submerged macrophytes in both pond networks. In the Neu-Woerr, calico crayfish abundance, and in Silene pH were important as well. In Silene these variables, perhaps mediated through their effect on the macrophyte communities, as well as dissolved oxygen concentration, were important for the macroinvertebrate community composition as well. In Silene, macrophyte beta diversity was higher between ponds that differed more in their level of shade from surrounding trees and water transparency.

Shade from surrounding trees can relatively easily be influenced by design; by placing ponds on a location without trees and by management; by cutting riparian trees. Water transparency, which can be related to nutrient concentrations and bioturbating organisms like calico crayfish, is more difficult to influence. Ponds could be placed in a location without bioturbating crayfish and with a water source that has low nutrient levels. For management of water transparency in created ponds, the drivers of water transparency should be diagnosed. If turbidity is caused by external nutrient loads, these should be addressed on the scale of the catchment. If turbidity is caused by internal nutrient loads, sediment removal may be a management option. Invasive crayfish are notoriously difficult to manage (Gherardi et al., 2011), and work package 3 of the EMYS-R project (**Chapter 1,** *Fig. 1.18*) is addressing this issue. Depletion of oxygen in ponds can be caused by decomposition of organic matter, for example leaf litter from riparian vegetation, and by cover of free-floating macrophytes (**Chapter 4**, section 4.4.2). Management of riparian trees could therefore influence pond oxygen concentrations (Sayer et al., 2012).

If in Silene it would be aimed to maintain a high level of macrophyte and macroinvertebrate diversity, it would be important to maintain ponds at different levels of shading from surrounding trees and water transparency. With the decline of agricultural activity in the area (**Chapter 2***, Fig. 2.5*), some of the ponds that are bordering old fields are becoming overgrown by riparian shrubs and trees. To prevent these ponds from becoming increasingly shaded by surrounding trees, and from potentially forming mats of free-floating macrophytes and becoming hypoxic, periodic deshading some of the ponds may be beneficial (Sayer et al., 2012). For example, every three to five years, riparian vegetation may be removed around three to five of the 27 ponds, and some of the ponds may never be managed. It is likely that the beavers, and perhaps the elk, also contribute to keeping the ponds at different levels of succession. Their effect on the ponds could be studied and considered in management.

Although this PhD highlighted some variables that can relate to the macrophyte and macroinvertebrate communities assembling in human-made ponds, it is hard to generalise the findings to other pond projects. It is likely that pond macrophytes and macroinvertebrates sort along environmental gradients that were not present in the two case studies. The importance and effects of variables may differ between human-made pond networks. For example, macrophytes in two regions in Slovakia were structured by different variables (Hrivnák et al., 2013). Another example is that I found no effect of pond isolation on the macrophyte community composition and metrics, but that this may be different in networks where ponds are further apart (for example as in Linton & Goulder, 2000). More networks of pond created for biodiversity should be studied to provide general advice on their design and management.

In any case, pond network design and management should be adapted to clearly stated goals. Pond networks designed for the conservation of specific target species may not have the same requirements as for enhancing macrophyte beta diversity (**Chapter 3**). Defining goals and quantifiable targets for restorative activities is not straightforward (Thorpe & Stanley, 2011). For pond creation to be a restorative activity it should reduce the degradation of the ecosystem. Therefore, an assessment of the degradation and the causes should be made to set goals and plan the restorative activity. Furthermore, an assessment of potential negative effects of the restorative activity should be performed as well. For example, damage by the machinery should be minimized and the absence of rare or protected species on the location where a pond will be

constructed should be ensured. There is also the case described by Calhoun et al. (2014), where temporary ponds were made for temporary pond amphibians. The hydroperiods in these created ponds were, however, too long and attracted amphibians that use permanent ponds. The permanent pond amphibians then predated on the larvae and eggs of the temporary pond amphibians, rendering the reproductive effort of the target temporary pond amphibians futile.

6.3.2. Implications for pond monitoring and evaluation

In **Chapter 3,** I used multiple community metrics to investigate how environmental variables could influence macrophyte communities. These metrics could also be used as indicators for restorative projects that aim to increase freshwater biodiversity. Species richness is the most common metric used in biodiversity conservation planning. However, this metric does not carry information about ecological functioning of the ecosystem or the conservation value of the species concerned (Fleishman et al., 2006; Rosset et al., 2013). Total cover of macrophytes and relative cover of emergent, submerged, floating anchored and free-floating leaved life forms may be easy to measure functional metrics that could be used as indicators if they match the project target. One could, for example, aim to create a pond network with a total macrophyte cover higher than a certain percentage and a Simpson diversity of life forms higher than a certain value.

When no funds are available to let a taxonomic expert perform identifications, pond creation projects with objectives for total macrophyte cover and relative cover of macrophyte life forms could be monitored according to the minimal monitoring protocol I propose in Appendix 6.C. This protocol quantifies the total macrophyte cover and relative cover of macrophyte life forms, as well as two variables that can be important for the achievement of these targets: shade form surrounding trees and water quality. It requires minimal time, equipment and taxonomic expertise. It could, for example, be performed one, two, five and ten years after pond creation and then be repeated every ten years.

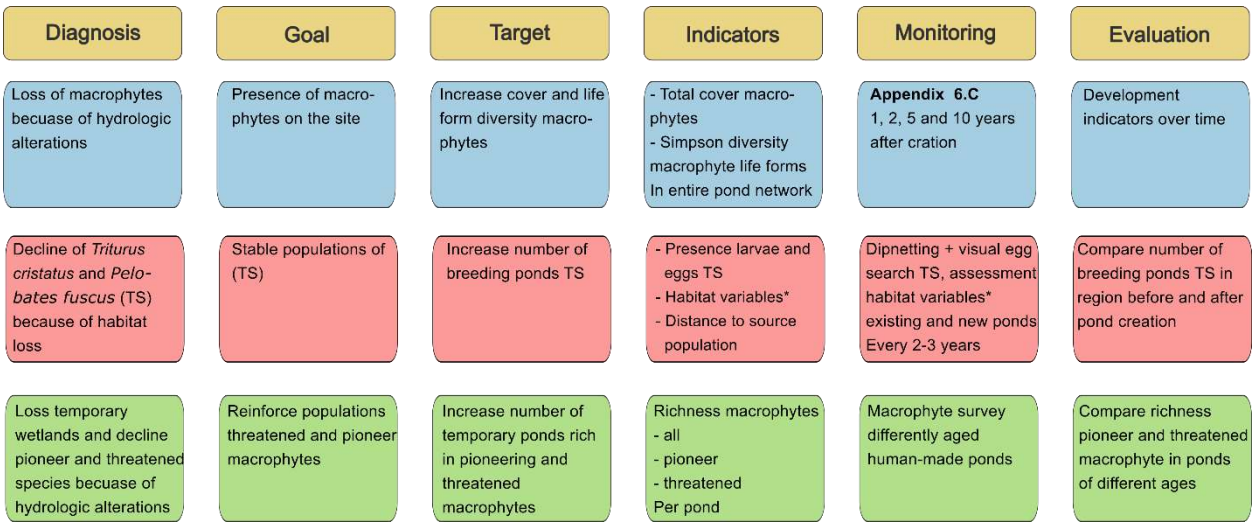


*Fig. 6.4. Examples of monitoring and evaluation strategies for networks of pond created for biodiversity or conservation. In blue the example described in this section, in red an example based on Rannap et al., 2009 and in green an example based on Fleury & Strehler Perrin, 2004. TS stands for target species (Triturus cristatus and Pelobates fuscus). * Habitat variables measured were presence of fish, pond surface area, width of zone < 30 cm deep, bank slope, water colour and transparency, mean land cover within 50 m, percentage of pond area occupied by floating and submerged macrophytes.*

As described in **Chapter 1** (Section 1.4, Fig 1.10) the monitoring and evaluation of created pond networks should be performed in relation to the stated goals and objectives. The example with the minimal monitoring protocol is only applicable for projects aiming to have increase macrophyte presence on the site in general (Fig. 6.4). It does not provide information on species identity, richness, conservation value and other macrophyte metrics, nor on other biological groups. This information is needed when pond networks are created for other objectives, for example to provide habitat to target species, such as in the example in Fig. 6.4 in red, or for species with specific characteristics, such as being pioneers or threatened as in the example in Fig. 6.4 in green.

If it is required to know the identity and abundance of macrophyte and macroinvertebrate taxa to determine values of indicators relevant to a pond creation project, the macrophyte survey methods and S_3i macroinvertebrate sampling protocol used in this thesis could be suitable (section 6.2). Environmental DNA metabarcoding could be used to identify macrophyte and macroinvertebrate taxa, but probably not their abundance (Blackman et al., 2024a; Elbrecht & Leese, 2015), and still has to be optimised for ponds. In **Chapter 5,** I attempted to use eDNA metabarcoding to detect macrophyte, macroinvertebrate, amphibian and fish taxa. As discussed in **Chapter 5**, high levels of tagjumps could have occurred. I therefore recommended the use of library preparation protocols that avoid tagjumps, like the tagsteady protocol (Carøe & Bohmann, 2020). Furthermore, I stressed the importance of PCR replicates and controls and advised to not multiplex multiple metabarcodes in the same PCR. The book by Taberlet et al. (2018) is still very useful as a guide, but future research should still elucidate how to best sample ponds and what bioinformatics pipelines (cf. Hakimzadeh et al., 2024) and post-bioinformatics cleaning procedures should be used. For amphibians and fish, eDNA metabarcoding can be as reliable for species detection as conventional methods (Li et al., 2019; Schwesig et al., 2025). Section 6.4.1 includes perspectives for studies on pond eDNA metabarcoding of macrophytes, macroinvertebrates and other taxonomic groups.

6.4. Perspectives for future research

As described in section 6.1.3, it would be interesting to keep following the study sites over time to study succession. Beaver and elk activity could be quantified and related to macrophyte patterns. The development of the calico crayfish populations can be followed. Population monitoring of the European pond turtles and the tests of adaptive management of calico crayfish are still ongoing. A larger number of pond networks could be studied to investigate how biogeographical variables and levels of anthropogenic stress could result in differences in outcomes between pond creation projects. Furthermore, there are exciting scientific advances that are relevant for the study of pond networks created for biodiversity and conservation. In the remainder of this section, I will mention some novel tools that could be developed for pond monitoring and evaluation and describe how landscape approaches could be used to improve placement of new ponds.

6.4.1. Pond monitoring and evaluation

Environmental DNA metabarcoding is a rapidly developing research field and the development of eDNA metabarcoding tools for ponds could be highly beneficial (Blackman et al., 2024a; Hill et al., 2021). As discussed in **Chapter 5**, section 5.4.6, future studies on eDNA metabarcoding of pond macrophytes could aim to identify the most effective primer pairs and combinations of primer pairs to use for macrophytes in Europe, and develop clearer guidance on the number, location and volume of samples to take in ponds. Furthermore, eDNA sampling in ponds could benefit from passive sampling. Pond water can be turbid, and this can impact the volume of water that can be filtered. In Neu-Woerr, we took 1 L samples, but the volume of water that could be filtered through a Sterivex capsule was highly variable (*Fig. 5.3*). Environmental DNA may also be

patchily distributed in ponds. Passive eDNA collection (Bessey 2021) may a very promising strategy to overcome the issues of turbidity and patchy distributions.

In a study that is currently under review (Werner et al., Submitted), we compared passive eDNA collection to water sampling of eDNA following a protocol similar to Appendix 5.A, but with 1 L samples. This study was performed in a laguna that was created as a rehabilitation measure of the Neu-Woerr gravel pit (**Chapter 2,** "NW_LED" in *Fig 2.4*). For the passive eDNA collection, we incubated filter membranes of cellulose nitrate and glass fibre, as well as household coffee filters, for six hours in the laguna. The passive filter membranes were stored in Longmire's buffer, and DNA extraction and subsequent steps were performed as for the actively filtered samples. These steps were similar to the methods describe in **Chapter 5**, with the only difference that concentrations of DNA input for the PCRs had been normalized in this study and not in **Chapter 5**. Macrophytes, macroinvertebrates and vertebrates could be detected from the passive samples. As filter membranes could be incubated in many pond locations and do not present the issue of filter clogging, it is a very interesting method to optimise in further studies.

In **Chapter 5**, section 5.4.6, I mentioned two methods that have been recently developed for streams and can considerably improve macroinvertebrate eDNA metabarcoding (Sander et al., 2025a; Sander et al., 2025b). The incubate-capture-release method consists of sampling macroinvertebrates, incubating them in water from the stream, releasing the macroinvertebrates, filtering the water and extracting DNA from the filters. The second strategy consists of preparing net bags with dried substrates from the stream, placing these for four weeks in the stream, incubating them in stream water and filtering the water and extracting DNA from the filters (Sander et al., 2025a). These methods have only been tested for streams but could be highly beneficial for ponds as well. I think that it would be highly interesting to try to develop capture-incubate-release eDNA detection methods for pond beetles (Coleoptera). As described **Chapter 4**, water beetles are sensitive indicators of pond ecological quality, and ponds are important for water beetle conservation (Della Bella et al., 2005; Fairchild et al., 2000; Menetrey et al., 2011). They could be sampled with minimally invasive activity traps (Elmberg et al., 1992; Law et al., 2019). Caught beetles could then be incubated in water from their pond and then released again. Incubation water could be filtered, and DNA could be extracted from the filters and amplified with specific coleoptera primers. Coleoptera primers have been developed (Cole01 in Taberlet et al., 2018) but no published data on the application exists yet.

Currently, taxonomic groups suitable for bioindication have to meet the requirements of being accessible to sampling or surveying and identification (Blackman et al., 2024b; Oertli et al., 2005b). However, eDNA metabarcoding would enable the detection of species from groups that are difficult to identify. Pawlowski et al. (2021) distinguish three main research areas for the implementation of eDNA-based biomonitoring. These are 1) renovate: using eDNA to detect conventional bioindicator taxa, 2) rebuild: use eDNA to detect new bioindicator taxa and 3) revolutionize: using taxonomy free metrics that relate to anthropogenic stressors. As pond monitoring is still rare and protocols still need to be developed, it may be interesting to already "rebuild" pond monitoring by including, for example, microorganisms or Diptera as indicator groups (Pawlowski et al., 2021; Zizka et al., 2025). Water mites are ubiquitous and sensitive to environmental conditions and could be a promising indicator group (Stenger et al., 2024). I noticed a large variety of water mites in the samples from Silene, but I lacked the skill to identify them. Metabarcoding methods may be useful for their identification.

Besides molecular tools, other novel technologies could be developed for pond monitoring. Ecoacoustics, sound-based monitoring techniques, are largely unexplored in freshwaters. However, since beetles, bugs (Hemiptera), other arthropods, fishes, amphibians, plants and even decomposing organic matter produce sound in ponds, ecoacoustics could be developed as easy to apply non-invasive passive pond monitoring tools (Greenhalgh et al., 2021). Remote sensing

techniques can be applied for the detection of ponds and other aquatic habitats and can be used to study their seasonality and connectivity (Borthagaray et al., 2023; Hill et al., 2021). Furthermore, images collected with unmanned aerial vehicles can be used to map macrophytes (Cvijanović et al., 2025). These techniques could be used to increase the spatial and temporal scale of pond research.

Apart from monitoring tools, future research could develop and calibrate indicators for pond assessment. Several multimetric pond indices have been developed in Europe, but their application is restricted to the region they were developed (**Chapter 1**, *Table 1.4*). The BECOME index (Labat & Usseglio-Polatera, 2023), which can be calculated from macroinvertebrate data collected with the S_3i protocol and macrophyte data, was for example calibrated on ponds in France. This index could be expanded to other parts of Europe, if data from impacted and least impacted ponds in other parts of Europe are collected, and if it is investigated how the metrics in the index respond to anthropogenic stressors in these regions. Application of standardized pond monitoring protocols and indices would be highly beneficial for the assessment of pond biodiversity trends across Europe.

Since species pools may not be the same in different parts of Europe, Europe-wide trait-based indices could be developed. The development of metrics of functional diversity, the trait variation or multivariate trait differences within a community, has been proposed as highly beneficial for conservation and restoration planning (Cadotte et al., 2011; Gallardo et al., 2011). Functional diversity metrics have already been applied to the evaluation of human-made ponds (Coccia et al., 2021). However, it is not yet clear which traits should be included in such metrics. If traits are selected only based on their availability, the interpretation of the metrics may be complicated. It should be noted that traits can be response traits, which describe how organisms respond to their environment, and effect traits, that describe how organisms influence their ecosystem (Lavorel & Garnier, 2002; Perring et al., 2015; Suding et al., 2008; Violle et al., 2007). Functional diversity metrics based on response traits could indicate the resilience of communities to environmental changes. On the other side, functional diversity metrics based on effect traits could be related to ecosystem functioning. However, studies are needed to investigate these relationships and to study which traits could be relevant for the development of interpretable pond diversity indices. Furthermore, since functional diversity has many facets, and there are many ways to calculate functional diversity metrics (Magneville et al., 2022), future work could investigate what kind of metrics could be relevant for ponds.

6.3.2. Placement of ponds in the landscape

In **Chapter 4,** I started to compare macroinvertebrate communities in human-made ponds to those in other aquatic habitats, lakes in this case. To fully assess the potential benefits of human-made ponds, their biodiversity should be studied with respect to other freshwater habitats (Sayer et al., 2012). Metapopulations of aquatic species may use multiple waterbody types. The few studies comparing the macrophyte and macroinvertebrate diversity between ditches, lakes, ponds, rivers and streams have shown that some species are shared between waterbody types, and that some species are unique to one type. These studies have also shown that ponds contribute more species and also more unique species to the landscape than the other waterbody types (Davies et al., 2008; Williams et al., 2004). Future studies could perform similar comparisons in other regions of Europe and could include other waterbody types like pools, mires, and backwaters. For the evaluation of human-made ponds created to improve landscape scale biodiversity, the communities in these ponds could be compared to those in other waterbodies. This would allow assessment of the complementarity of the human-made ponds, and therefore their contribution to landscape scale biodiversity.

It would also be interesting to investigate how the addition of ponds to a landscape could alter its freshwater connectivity. In the two pond networks I studied, I observed no effect of the distance to the nearest waterbody on the pond communities. It would be interesting to investigate which distance to the nearest waterbody could matter for the diversity of different groups of organisms. In light of climate change, landscape connectivity analysis could be interesting to predict if species can migrate northwards or into the mountains. Ponds can be important stepping stones that may allow this migration (Céréghino et al., 2014). Analysis of landscape connectivity can be used to determine if there are locations with insufficient connectivity, and where ponds could be created to allow this migration.

Landscape connectivity can be expressed in structural metrics, which only consider the spatial arrangement of ponds, but also in potential population connectivity metrics, which also account for the presence of populations (Hill et al., 2021; Moor et al., 2024). Analysis of potential population connectivity could be used to determine the location of source populations where new ponds could be placed. They should be located near declining populations of species of conservation interest (Moor et al., 2024). An issue for many freshwater species is, however, that their distributions and conservation status may not be known. European Red lists, for example, exist for amphibians, freshwater fishes, freshwater molluscs, selected vascular plants, mosses, liverworts and hornworts, ferns and lycopods, and for dragonflies, but not for any other group of aquatic invertebrates (IUCN, 2025). Europe-wide distributions of aquatic plants, Trichoptera and Odonata have been analysed (Kalkman et al., 2018; Schmidt-Kloiber et al., 2017; Schou et al., 2023). For the effective conservation of freshwater organisms, such analyses should also be preformed for other groups.

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Appendices

Appendix 3.A. Pond environmental conditions per site

The Neu-Woerr ponds were characterized by a larger surface area, older age, higher pH, and higher specific conductivity compared to the Silene ponds (*Table 3.S.1*). In Neu-Woerr, all crayfish caught were *Faxonius immunis.* With a body length between 14 and 58 mm (median 29 mm for N=544 individuals caught), they were likely young-of-the-year.

Table S.3.1. Local biotic values with abbreviations (Abbrev) used in the CoIA figures and their ranges for the Neu-Woerr and Silene study sites, with median values between brackets. CPUE stands for Catch Per Unit Effort, the number of crayfish per sweep net sample averaged over the 4 samples taken per pond. The last two columns display the Wilcoxon rank-sum statistic (W) and Benjamini-Hochberg adjusted for multiple testing p-value (Adj. p-value) from comparison of the variables between the study sites. Significant differences (adj. p<0.05) in bold.

Variable	Abbrev	Neu-Woerr	Silene	W	Adj. p-value
Age (year)	Age	8-12 (11)	4-10 (10)	141	0.007
Distance to nearest waterbody (m)	DistNWb	20-170 (40)	0-240 (30)	107	0.4
Surface area (m^2)	Area	120-4990 (1300)	70-1050 (170)	156	0.001
Depth (m)	Depth	0.5-1.3 (1)	0.3-1.5 (0.8)	96.5	0.6
Pond surface shaded at midday (%)	Shade	0-30 (0)	0-50 (5)	63	0.4
pH	pH	7.3-8.7 (8)	6.6-8.3 (7.1)	146	0.007
Transparency (cm)	Trans	20-120 (70)	10-180 (80)	70.5	0.5
Specific conductivity (µs cm^{-1})	SpCond	120-439 (223)	72-470 (214)	138	0.01
Chl-a concentrations (µg L^{-1})	Chl-a	1-16 (2.8)	0.8-4.2 (1.7)	106	0.4
Crayfish mean CPUE	Cray	0-67 (4)	-		

Appendix 3.B. Macrophyte communities

Table S.3.2. Taxa observed in Neu-Woerr and Silene study sites, with the abbreviation used in Fig. 3.4, attribution of emergent (Em), anchored floating (Fl), free-floating (Fr) and anchored submerged (Su) life forms, and the number of ponds per network in which it is observed (13 ponds per network were surveyed).

	Species	Abbreviation	Life form	Neu-Woerr	Silene
1	*Acorus calamus*	AcorCala	Em	-	1
2	*Agrostis stolonifera*	AgroStol	Em	4	-
3	*Alisma lanceolatum*	AlisLanc	Em	5	-
4	*Alisma plantago-aquatica*	AlisPlan	Em	-	11
5	*Calla palustris*	CallPalu	Em	-	1
6	*Carex acutiformis*	CareAcut	Em	4	11
7	*Carex dioica*	CareDioi	Em	-	1
8	*Carex echinata*	CareEchi	Em	-	1
9	*Carex lasiocarpa*	CareLasi	Em	-	7
10	*Carex nigra*	CareNigr	Em	-	2
11	*Carex riparia*	CareRipa	Em	-	1
12	*Carex rostrata*	CareRost	Em	1	5
13	*Carex vesicaria*	CareVers	Em	2	-
14	*Carex viridula*	CareViri	Em	-	2
15	*Carex vulpina*	CareVulp	Em	-	1
16	*Ceratophyllum submersum*	CeraSubm	Su	-	3
17	*Chara globularis*	CharGlob	Su	-	4
18	*Chara vulgaris*	CharVulg	Su	7	-
19	*Comarum palustre*	ComaPalu	Em, Fl	-	4
20	*Eleocharis palustris*	EleoPalu	Em	-	5
21	*Eleocharis uniglumis*	EleoUnig	Em	3	-
22	*Epilobium hirsutum*	EpilHirs	Em	-	1
23	*Epilobium palustre*	EpilPalu	Em	-	2
24	*Equisetum fluviatile*	EquiFluv	Em	-	9
25	*Equisetum palustre*	EquiPalu	Em	7	2
26	*Eriophorum latifolium*	ErioLati	Em	-	1
27	*Galium palustre*	GaliPalu	Em	2	8
28	*Glyceria fluitans*	GlycFlui	Em, Fl	-	8
29	*Hippuris vulgaris*	HippVulg	Em, Su	2	-
30	*Hottonia palustris*	HottPalu	Em, Su	-	6
31	*Hydrocharis morsus-ranae*	HydrMors	Fr	-	4
32	*Iris pseudacorus*	IrisPseu	Em	3	-
33	*Juncus acutiflorus*	JuncAcut	Em	10	5
34	*Juncus conglomeratus*	JuncCong	Em	1	4
35	*Juncus effusus*	JuncEffu	Em	1	10
36	*Lemna minor*	LemnMino	Fr	1	11
37	*Lemna trisulca*	LemnTris	Fr	-	4
38	*Lycopus europaeus*	LycoEuro	Em	4	7
39	*Lysimachia vulgaris*	LysiVulg	Em	1	7

	Species	Abbreviation	Life form	Neu-Woerr	Silene
40	*Lythrum salicaria*	LythSali	Em	4	1
41	*Mentha aquatica*	MentAqua	Em, Su	2	4
42	*Menyanthes trifoliata*	MenyTrif	Em	-	1
43	*Myosotis scorpioides*	MyosScor	Em, Su	2	1
44	*Myriophyllum spicatum*	MyriSpic	Su	1	-
45	*Nasturtium officinale*	NastOffi	Em, Fl	-	3
46	*Nitella gracilis*	NiteGrac	Su	-	2
47	*Nitella tenuissima*	NiteTenu	Su	-	1
48	*Nitellopsis obtusa*	NiteObtu	Su	-	1
49	*Persicaria amphibia*	PersAmph		2	-
50	*Phalaris arundinacea*	PhalArun	Em	6	1
51	*Phragmites australis*	PhraAust	Em	13	4
52	*Potamogeton alpinus*	PotaAlpi	Fl, Su	-	1
53	*Potamogeton berchtoldii*	PotaBerc	Su	-	9
54	*Potamogeton friesii*	PotaFrie	Su	-	1
55	*Potamogeton lucens*	PotaLuce	Su	1	-
56	*Potamogeton natans*	PotaNata	Fl, Su	-	2
57	*Potamogeton nodosus*	PotaNodo	Fl, Su	1	-
58	*Potamogeton pusillus*	PotaPusi	Su	-	1
59	*Ranunculus circinatus*	RanuCirc	Su	4	-
60	*Ranunculus flammula*	RanuFlam	Em, Fl	-	5
61	*Ranunculus sceleratus*	RanuScel	Em, Fl	2	-
62	*Riccia fluitans*	RiccFlui	Fr	-	4
63	*Ricciocarpos natans*	RiccNata	Fr	2	1
64	*Rorippa amphibia*	RoriAmph	Em, Fl, Su	5	-
65	*Schoenoplectus lacustris*	SchoLaco	Em, Su	4	3
66	*Scirpus sylvaticus*	ScirSylv	Em	-	6
67	*Scutellaria galericulata*	ScutGale	Em	-	4
68	*Solanum dulcamara*	SolaDulc	Em, Fl	-	8
69	*Sparganium emersum*	SparEmer	Em, Fl, Su	-	1
70	*Sparganium natans*	SparNata	Em, Fl, Su	-	5
71	*Spirodela polyrhiza*	SpirPoly	Fr	-	10
72	*Stellaria palustris*	StelPalu	Em	-	1
73	*Stuckenia pectinata*	StucPect	Su	3	-
74	*Typha angustifolia*	TyphAngu	Em	1	-
75	*Typha latifolia*	TyphLati	Em	-	11
76	*Utricularia australis*	UtriAust	Fr	3	-
77	*Utricularia minor*	UtriMino	Fr	-	3
78	*Utricularia vulgaris*	UtriVulg	Fr	-	4
79	*Veronica anagallis-aquatica*	VeroAnag	Em, Su	11	-
80	*Veronica beccabunga*	VeroBecc	Em, Su	-	1
81	*Veronica scutellata*	VeroScut	Em, Su	-	1
	Carex sp.	CareSpec	Em	3	2

Table S.3.3. Macrophyte metrics with abbreviations (Abbrev) used in the CoIA figures and their ranges for Neu-Woerr and Silene study sites with median values between brackets. Rel. cov. stands for relative cover and macr. for macrophytes. The last two columns display the Wilcoxon rank-sum statistic (W) and Benjamini-Hochberg adjusted for multiple testing p-value (Adj. p-value) from comparison of the metrics between the sites. Significant differences (Adj. p<0.05) in bold.

Variable	Abbrev	Neu-Woerr	Silene	W	Adj. p-value
Taxonomic richness	**Rich**	**5-17 (9)**	**11-30 (19)**	**12**	**0.001**
Hill Shannon diversity	**Shan**	**2-6 (3)**	**3-11 (6)**	**22**	**0.002**
Simpson evenness	Simp	0.1-0.7 (0.3)	0.1-0.3 (0.2)	116	0.15
Taxonomic distinctness	Dstar	55-99 (86)	55-99 (86)	101	0.42
Total cover	Cove	1-99 (47)	29-99 (86)	49	0.12
Rel. cov. emergent macr.	Emer	7-99 (58)	11-82 (36)	118	0.14
Rel. cov. anchored floating macr.	**Floa**	**0-1 (0)**	**0-40 (11)**	**10**	**0.001**
Rel. cov. anchored submerged macr.	Subm	1-93 (38)	0-71 (31)	101	0.4
Rel. cov. free-floating macr.	**Free**	**0-39 (0)**	**0-86 (15)**	**27**	**0.006**

Appendix 3.C. Coinertia land cover environmental variables

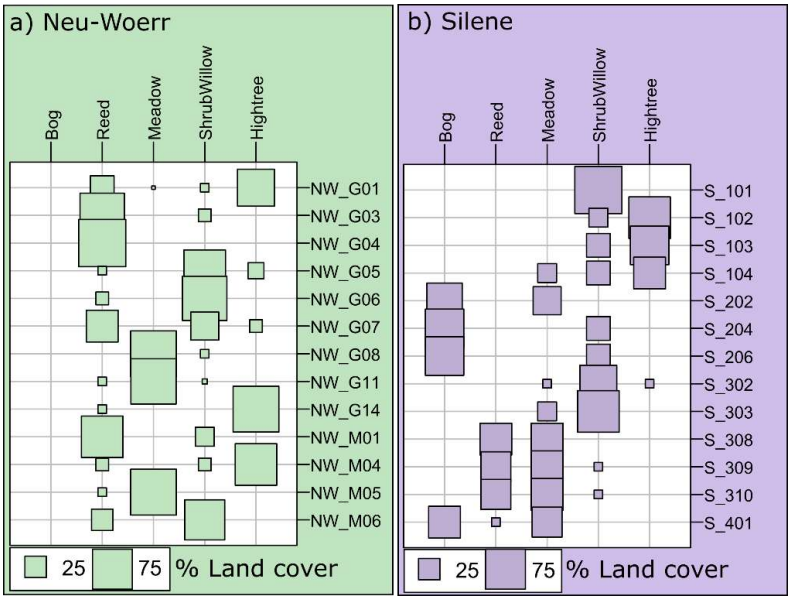


Fig. S.3.1. Land cover around ponds. This size of the squares indicates the percentage of the 5 m buffer around the pond covered by each land cover type (scale at the bottom left of each panel). a) Neu-Woerr. b) Silene.

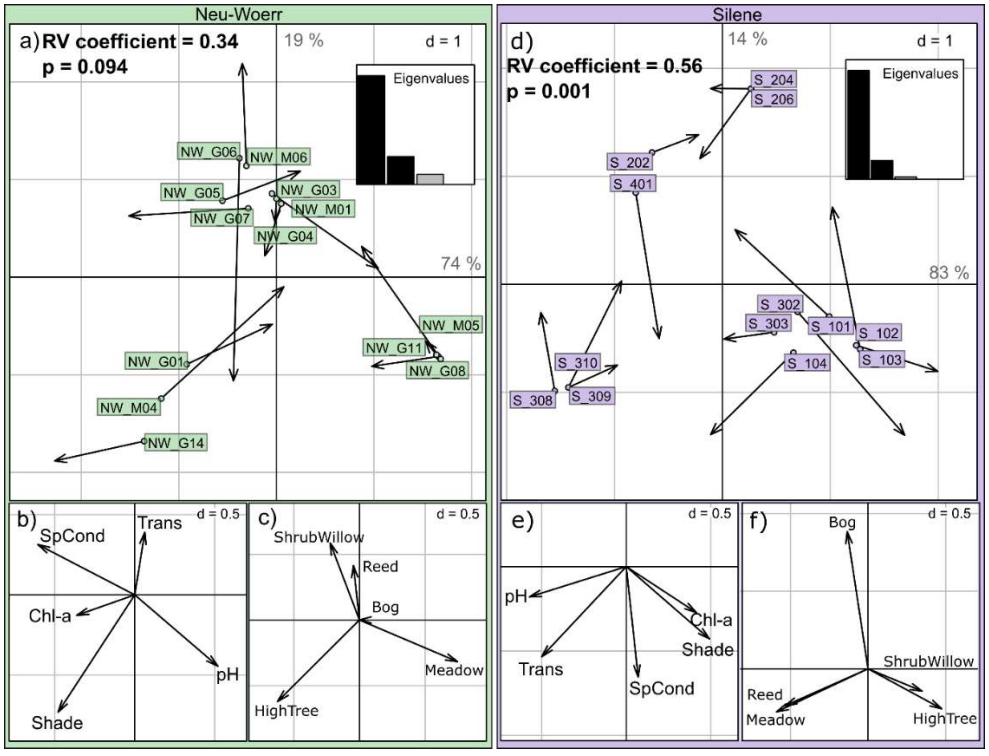


Fig. S.3.2. CoIA Land cover – environmental variables. a - c) Neu-Woerr. d – f) Silene. a, d) Positions of the ponds on the plane of the first two coinertia axes (% inertia projected by each axis in grey) according to the environmental variables (dots and labels) and according to land cover in the 5 m buffer around the pond (arrowheads). RV coefficients and p-values are in the top left corner in bold and the eigenvalue bar plots in the insets. b, e) Positions of environmental variables transparency (Trans), chlorophyll-a concentration (Chl-a), Specific conductivity (SpCond), pH and % of pond surface area shaded by surrounding trees (Shade) on the coinertia plane. c, f) Positions of land cover on the coinertia plane.

Appendix 4.A. Environmental variables and macroinvertebrate taxa

Table S.4.1. Environmental variables per pond with abbreviations (Abbrev.) used in figure 4.6.

Variable	Abbrev	S_101	S_102	S_104	S_202	S_204	S_206	S_302	S_303	S_308	S_401
Pond surface shaded at midday (%)	Shade	15	30	50	0	0	5	65	20	0	0
Water transparency (cm)	Trans	85	10	175	175	40	55	10	10	175	60
Specific conductivity (µs cm^{-1})	SpCond	194	133	346	78	79	82	388	462	244	258
Dissolved oxygen concentration (mg L^{-1})	DO	3.21	1.65	4.31	2.77	4.66	3.28	0.69	6.56	8.01	8.48
pH	pH	6.8	6.3	7.4	6.4	6.4	6.4	6.7	7	7.9	7.6
Chl-a concentration (µg L^{-1})	Chl-a	2.9	4.7	1.1	1.6	1.5	2.7	4.4	2.8	1.1	1.5
Total Nitrogen (mg L^{-1})	TN	4.9	4.4	1.7	2.1	1.9	2.5	7.1	4	2.7	4.3
Total Phosphorus (mg L^{-1})	TP	0.28	0.32	0.13	0.05	0.19	0.24	0.54	0.24	0.18	0.15

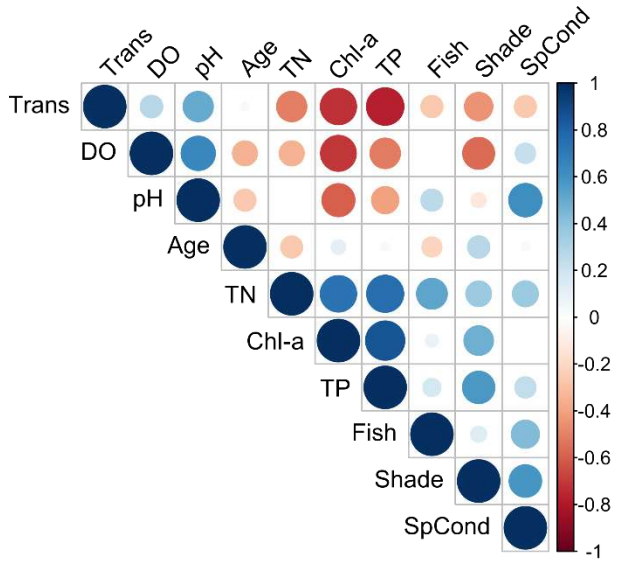


Fig. 4.S.1. Spearman rank correlation coefficients among environmental variables

Table S.4.2. APTC-SG resolved macroinvertebrate taxa in studied ponds, with the number (Nb.) of ponds in which each taxon was found, the original identifications that could have been assigned to the resolved taxon and the higher level used in Fig. 4.2. (a) stands for adult, (l) for larva (p) for pupa, EPT for Ephemeroptera, Plecoptera and Trichoptera, arth. for Arthropoda and phyl. for phylum.

	Resolved taxon	Nb. of ponds	Original assignments	Higher level
1	*Acilius canaliculatus*	2	*Acilius canaliculatus* (a), *Aciliini* (a)	Coleoptera
2	*Acilius sulcatus*	1	*Acilius sulcatus* (a), *Aciliini* (a)	Coleoptera
3	*Agabus*	4	*Agabus* (a), *Agabus* (l)	Coleoptera
4	*Berosus*	1	*Berosus* (l)	Coleoptera
5	*Coelostoma*	1	*Coelostoma* (a)	Coleoptera
6	*Cybister*	2	*Cybister* (l)	Coleoptera
7	*Cymbiodyta*	1	*Cymbiodyta* (l)	Coleoptera
8	*Dryops*	3	*Dryops* (l)	Coleoptera
9	*Enochrus*	9	*Enochrus* (a), *Enochrus* (l)	Coleoptera
10	*Eretes*	1	*Eretes* (a)	Coleoptera
11	*Graptodytes*	2	*Graptodytes* (l)	Coleoptera
12	*Gyrinus*	2	*Gyrinus* (l)	Coleoptera
13	*Haliplus*	10	*Haliplus* (a), *Haliplus* (l)	Coleoptera
14	*Helochares*	8	*Helochares* (a), *Helochares* (l)	Coleoptera
15	*Hydaticus transversalis*	6	*Hydaticus transversalis* (a), *Hydaticus* (l)	Coleoptera
16	*Hydrobius fuscipes*	2	*Hydrobius fuscipes* (a), *Hydrobius* (l)	Coleoptera
17	*Hydrochara*	1	*Hydrochara* (a), *Hydrochara* (l)	Coleoptera
18	*Hydrochus*	2	*Hydrochus* (a)	Coleoptera
19	*Hydroglyphus*	1	*Hydroglyphus* (a)	Coleoptera
20	*Hydroporus*	7	*Hydroporus* (a), *Hydroporus* (l)	Coleoptera
21	*Hydrovatus*	1	*Hydrovatus* (l)	Coleoptera
22	*Hygrotus*	2	*Hygrotus* (a)	Coleoptera
23	*Hyphydrus*	9	*Hyphydrus* (a), *Hyphydrus* (l)	Coleoptera
24	*Ilybius*	3	*Ilybius* (a), *Ilybius* (l)	Coleoptera
25	*Laccobius*	5	*Laccobius* (a), *Laccobius* (l)	Coleoptera
26	*Laccophilus*	5	*Laccophilus* (a), *Laccophilus* (l)	Coleoptera
27	*Limnebius*	4	*Limnebius* (a)	Coleoptera
28	*Nebrioporus*	1	*Nebrioporus* (a)	Coleoptera
29	*Noterus*	6	*Noterus* (a), *Noterus* (l)	Coleoptera
30	*Porhydrus*	2	*Porhydrus* (l)	Coleoptera
31	*Spercheus*	2	*Spercheus* (a), *Spercheus* (l)	Coleoptera
32	*Suphrodytes*	5	*Suphrodytes* (l)	Coleoptera
33	*Anophelinae*	3	*Anophelinae* (l), *Anophelinae* (p), *Culicidae* (l)	Diptera
34	*Ceratopogonidae*	10	*Ceratopogonidae* (l), *Ceratopogonidae* (p)	Diptera
35	*Chaoborus*	9	*Chaoborus* (l), *Chaoborus* (p)	Diptera
36	Chironomini	10	Chironomini (l), Chironomini (p), *Chironomidae* (p)	Diptera
37	*Corynoneura*	3	*Corynoneura, Chironomidae* (p)	Diptera
38	*Culicoidea*	2	*Culicoidea* (p)	Diptera

	Resolved taxon	Nb. of ponds	Original assignments	Higher level
39	*Dixella*	8	*Dixella* (l), *Dixidae* (l), *Dixidae* (p)	Diptera
40	*Limoniidae*	3	*Limoniidae* (l), Limoniidae (p)	Diptera
41	*Orthocladiinae*	8	*Orthocladiinae* (l), *Orthocladiinae* (p), *Chironomidae* (p)	Diptera
42	*Psychodidae*	1	Psychodidae (p)	Diptera
43	*Sciomyzidae*	3	*Sciomyzidae* (l), *Sciomyzidae* (p)	Diptera
44	*Stratiomyidae*	8	*Stratiomyidae* (l), *Stratiomyidae* (p)	Diptera
45	*Tabanidae*	2	*Tabanidae* (l)	Diptera
46	*Tanypodinae*	10	*Tanypodinae* (l), *Tanypodinae* (p), *Chironomidae* (p)	Diptera
47	Tanytarsini	6	Tanytarsini (l), *Chironomidae* (p)	Diptera
48	*Caenis*	2	*Caenis*	EPT
49	*Cloeon dipterum*	10	*Cloeon dipterum*	EPT
50	*Holocentropus stagnalis*	4	*Holocentropus stagnalis*	EPT
51	Limnephilini	2	Limnephilini	EPT
52	*Limnephilus*	1	*Limnephilus* (p)	EPT
53	*Oligotricha striata*	2	*Oligotricha striata*	EPT
54	*Triaenodes bicolor*	5	*Triaenodes bicolor, Leptoceridae*	EPT
55	*Tricholeiochiton fagesii*	2	*Tricholeiochiton fagesii, Hydroptilidae*	EPT
56	*Callicorixa wollastoni*	1	*Callicorixa wollastoni, Corixidae*	Hemiptera
57	*Gerris*	9	*Gerris, Gerridae*	Hemiptera
58	*Hesperocorixa sahlbergi*	5	*Hesperocorixa sahlbergi, Corixidae*	Hemiptera
59	*Hydrometra gracilenta*	8	*Hydrometra gracilenta, Hydrometra*	Hemiptera
60	*Ilyocoris cimicoides*	6	*Ilyocoris cimicoides*	Hemiptera
61	*Limnoporus rufoscutellatus*	1	*Limnoporus rufoscutellatus, Gerridae*	Hemiptera
62	*Mesovelia*	2	*Mesovelia*	Hemiptera
63	*Microvelia reticulata*	8	*Microvelia reticulata, Microvelia*	Hemiptera
64	*Notonecta glauca*	6	*Notonecta glauca, Notonecta*	Hemiptera
65	*Plea minutissima*	9	*Plea minutissima*	Hemiptera
66	*Ranatra linearis*	3	*Ranatra linearis*	Hemiptera
67	*Aeshna*	1	*Aeshna*	Odonata
68	*Aeshna caerulea*	1	*Aeshna caerulea*	Odonata
69	*Aeshna cyanea*	2	*Aeshna cyanea*	Odonata
70	*Chalcolestes viridis*	2	*Chalcolestes viridis*	Odonata
71	*Coenagrion hastelatum*	10	*Coenagrion hastelatum, Coenagrion, Coenagrionidae*	Odonata
72	*Coenagrion lunulatum*	1	*Coenagrion lunulatum, Coenagrion, Coenagrionidae*	Odonata
73	*Cordulia aenea*	2	*Cordulia aenea*	Odonata
74	*Enallagma cyathigerum*	1	*Enallagma cyathigerum, Coenagrionidae*	Odonata
75	*Erythromma*	1	*Erythromma, Coenagrionidae*	Odonata
76	*Lestes sponsa*	1	*Lestes sponsa*	Odonata

	Resolved taxon	Nb. of ponds	Original assignments	Higher level
77	*Lestes virens*	1	*Lestes virens*	Odonata
78	*Leucorrhinia albifrons*	2	*Leucorrhinia albifrons, Leucorrhinia, Libellulidae*	Odonata
79	*Leucorrhinia pectoralis*	2	*Leucorrhinia pectoralis, Leucorrhinia, Libellulidae*	Odonata
80	*Libellula depressa*	2	*Libellula depressa, Libellulidae*	Odonata
81	*Pyrrhosoma nymphula*	1	*Pyrrhosoma nymphula, Coenagrionidae*	Odonata
82	*Somatochlora*	1	*Somatochlora*	Odonata
83	*Sympecma*	1	*Sympecma*	Odonata
84	*Sympetrum flaveolum*	2	*Sympetrum flaveolum, Sympetrum, Libellulidae*	Odonata
85	*Sympetrum vulgatum*	4	*Sympetrum vulgatum, Sympetrum, Libellulidae*	Odonata
86	*Asellidae*	8	*Asellidae*	Other arth.
87	*Cataclysta lemnata*	7	*Cataclysta lemnata*	Other arth.
88	*Elophila*	1	*Elophila*	Other arth.
89	Hydracarina	10	*Hydracarina*	Other arth.
90	Neuroptera	1	*Neuroptera*	Other arth.
91	*Synurella ambulans*	2	*Synurella ambulans*	Other arth.
92	*Anisus*	3	*Anisus*	Mollusca
93	*Bathyomphalus contortus*	5	*Bathyomphalus contortus*	Mollusca
94	*Galba truncatula*	4	*Galba truncatula*	Mollusca
95	*Gyraulus crista*	4	*Gyraulus crista, Gyraulus*	Mollusca
96	*Hippeutis complanatus*	4	*Hippeutis complanatus*	Mollusca
97	*Musculium*	5	*Musculium*	Mollusca
98	*Pisidium*	4	*Pisidium*	Mollusca
99	*Planorbarius*	2	*Planorbarius*	Mollusca
100	*Planorbis carinatus*	1	*Planorbis planorbis*	Mollusca
101	*Planorbis planorbis*	9	*Planorbis carinatus*	Mollusca
102	*Radix balthica*	7	*Radix balthica*	Mollusca
103	*Segmentina nitida*	7	*Segmentina nitida*	Mollusca
104	*Sphaerium*	7	*Sphaerium*	Mollusca
105	*Stagnicola*	3	*Stagnicola*	Mollusca
106	*Valvata cristata*	7	*Valvata cristata*	Mollusca
107	*Viviparus contectus*	3	*Viviparus contectus*	Mollusca
108	*Alboglossiphonia*	1	*Alboglossiphonia, Glossiphoniidae*	Other phyl.
109	*Erpobdella*	4	*Erpobdella, Erpobdellidae*	Other phyl.
110	*Glossiphonia*	4	*Glossiphonia, Glossiphoniidae*	Other phyl.
111	*Hyalinella*	1	*Hyalinella*	Other phyl.
112	*Hydra*	1	*Hydra*	Other phyl.
113	Nematoda	2	Nematoda	Other phyl.
114	Nemertea	2	Nemertea	Other phyl.
115	Oligochaeta	6	Oligochaeta	Other phyl.
116	*Placobdella costata*	1	*Placobdella costata, Glossiphoniidae*	Other phyl.

Appendix 4.B. Community Weighted Means

Fuzzy coded traits in *Table S.4.3*. were obtained from Tachet et al. (2010) for 100 of the 126 original taxonomic assignments. Traits were not obtained for assignments at family level or higher, neither for *Limnephilus, Limnoporus rufoscutellatus, Musculium, Suphrodytes, Synurella ambulans, Hydrobius fuscipes* and *Alboglossiphonia.* Trait Community Weighted Means (CWM) were calculated per sample using the densities per m^2 of the taxa for which traits were available. This was done with the cmw() function of the BAT R package. Resulting CWMs were ordinated in a centred non standardized covariance PCA (*Fig. S.4.2*).

It was also attempted to perform RLQ analysis on a FCA of the traits, a centred and standardized correlation PCA of the environmental variables and a CA of the taxa (Thioulouse et al., 2018) but this was not significant (p-value model 2 = 0.158, p-value model 4 = 0.473). RLQ on only the traits food type, feeding mode and respiration and only the environmental variables dissolved oxygen concentration, chlorophyll-a concentration, Total Nitrogen concentration, Total Phosphorus concentration, percentage shade from surrounding trees and water transparency was not significant either (p-value model 2 = 0.355, p-value model 4 = 0.235).

Table S.4.3. Traits for which Community Weighted Means were calculated

Biological trait	Trait modality	Code
Adult body size	< 2.5 mm	Size1
	2.5 – 5 mm	Size2
	5 – 10 mm	Size3
	10 – 20 mm	Size4
	20 – 40 mm	Size5
	40 – 80 mm	Size6
	> 80 mm	Size7
Life cycle duration	≤ 1 year	Dura1
	> 1 year	Dura2
Number of generations per year	<1	Gene1
	1	Gene2
	>1	Gene3
Aquatic life stages	Egg	Egg
	Larva	Larva
	Nymph	Nymph
	Adult	Adult
Reproduction	Ovoviviparity	Ovovi
	Isolated eggs, free	EggFr
	Isolated eggs, cemented	EggCe
	Clutches, cemented or fixed	CluCe
	Clutches, free	CluFr
	Clutches, in vegetation	CluVe
	Clutches, terrestrial	CluTe
	Asexual reproduction	Asex
Dispersal mode	Aquatic, passive	AqPas
	Aquatic, active	AqAct
	Aerial, passive	AePas
	Aerial, active	AeAct
Resistance form	Eggs, statoblasts	EggSta
	Cocoon	Cocoon
	Housing against desiccation	HouDes

Biological trait	Trait modality	Code
	Diapause or dormancy	DiaDor
	None	None
Food type	Microorganisms	MicSed
	Fine detritus (< 1mm)	DetFin
	Death plant (>= 1mm)	DetVeg
	Live microphytes	MicPhy
	Live macrophytes	MacPhy
	Dead animals (>= 1mm)	DeadAn
	Live microinvertebrates	MicInv
	Live macroinvertebrates	MacInv
	Vertebrates	Vert
Feeding mode	Deposit feeders	Depos
	Shredder	Shred
	Grazer – scraper	GraSc
	Filter feeders	Filte
	Piercer	Pierc
	Predator	Preda
	Parasite	Paras
Respiration	Tegument	Tegu
	Gill	Gill
	Plastron	Plas
	Spiracle	Spir
	Hydrostatic vesicles	HyVe
Locomotion	Flier	Flie
	Surface swimmer	SwiSur
	Full water swimmer	SwiFul
	Crawler	Craw
	Burrower	Burr
	Interstitial	Inter
	Temporary attached	AttTem
	Permanently attached	AttPer

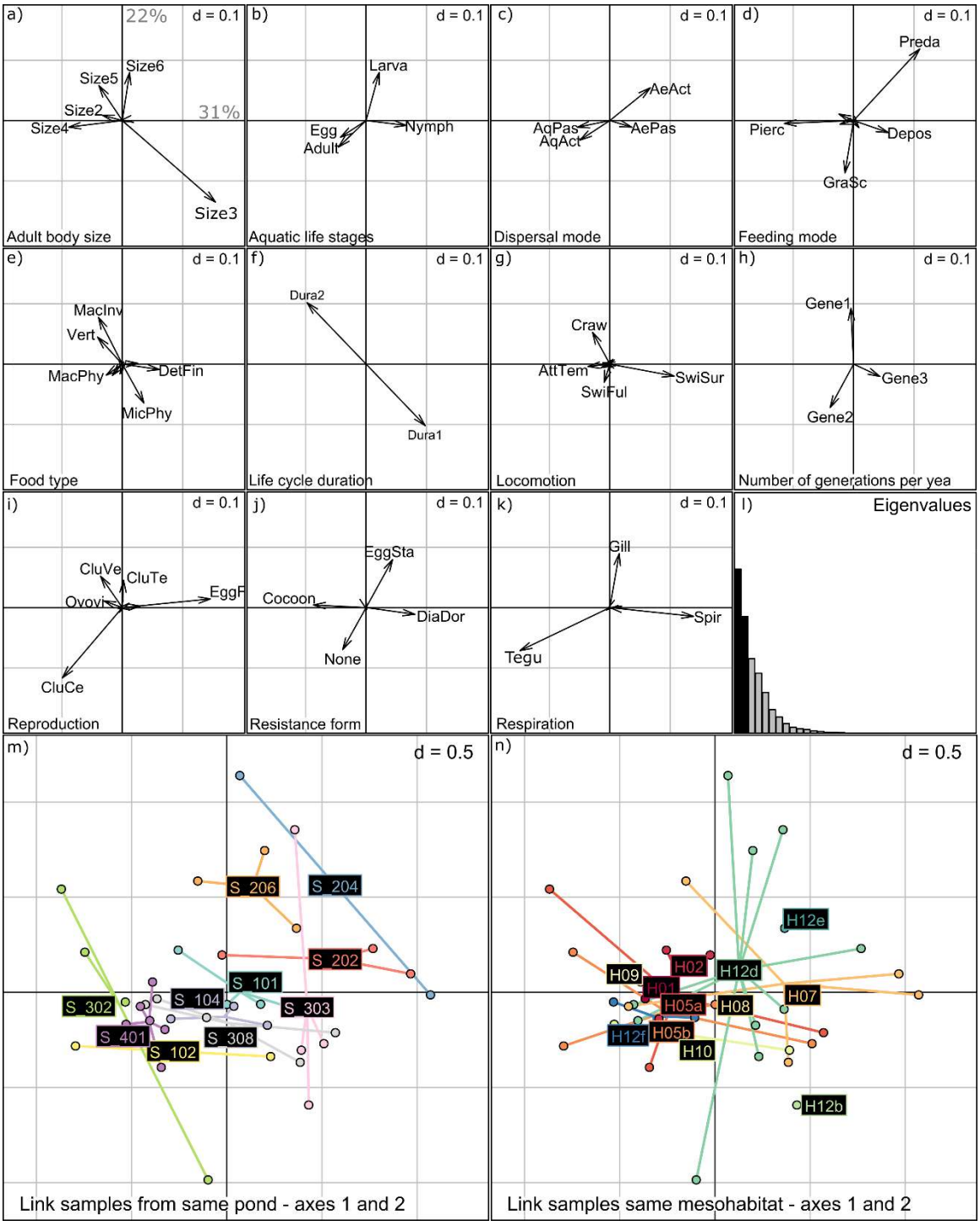


Fig. S.4.2. Community Weighted Means PCA. % inertia projected by each axis in grey. a-k) Trait modalities organised by trait with abbreviations in Table S.4.3. l) Eigenvalue barplot m) Spiders connect samples from the same pond. n) Spiders connect samples from the same mesohabitat type.

Appendix 5.A. Environmental DNA field protocol Latvia July 2022

Important! eDNA samples should be taken before the waterbodies are entered for other samplings and surveys! It is very important to not stir up sediment because this will clog the filters. It is also very important to not contaminate a waterbody with water or sediment from another waterbody. Therefore, the eDNA samples should be taken before the macrophyte survey and macroinvertebrate sampling can be done.

1. Before going to the field

- Charge the vampire sampler (Bürkle 5327-100) batteries.
- Place Luer connectors (Drifton, LM61), Tygon tubes (Saint Gobain, AZT00012, cut in pieces of 0.5 m), 250 mL Nalgene bottles (Nalgene, style 2104), 2 mL syringes (Braun Petzold, Luer lock solo) and waders in 10% solution of bleach (from 3% sodium hypochlorite) for at least 15 min. Rinse with tap water. Collect a sample of this tap water. Reuse the 10% bleach solution.

2. Items to bring to the field

General
Vampire pump and extra batteries
Longmire's buffer (2 mL per sample)
Gloves (1 per sample)
Liquid waste bottle
50 mL syringes as back-up
Measuring beaker
Paper labels
Marker pen, paper and pencil
Secchi disk and water quality sonde
GPS and fieldwork forms

Bring 2 per microhabitat to sample
Nalgene bottle
Tube with connector
0.45 µm Sterivex PVDF filter
2 mL syringe
Parafilm strip
Zip-lock bag

3. Limiting contamination in the field

- Wear gloves. Change gloves between each sample
- Only use new or bleached materials
- Do not enter waterbodies before sampling
- Do not stir up sediment when sampling

4. Sampling

2 replicate samples (labelled "a" and "b") are taken from each microhabitat sampled. The microhabitats sampled are the same as the invertebrate microhabitats sampled.

1) Put on clean gloves
2) Take two clean Nalgene bottles, fill the bottles with 250 mL surface water and close the bottles
3) Attach a clean tube with connector to a Sterivex 0.45 um pore size PVDF membrane filter
4) Place the tube in the pump, open the first Nalgene bottle, put the tube in the bottle and pump the sample through the Sterivex filter

5) Stop filtering in case less than 1 drop per second comes out of the filter. If less than 250 mL of water is filtered, write down how much water is filtered (measure with the beaker).
6) Fill the 2 mL syringe with Longmire's buffer
7) Detach the Sterivex filter from the tube and push the buffer into the filter
8) Label the Sterivex filter, close with a Luer inlet stopper and seal the Sterivex filter with the parafilm strip
9) Place the Sterivex filter in the plastic bag, together with a paper label
10) Repeat steps 3 to 9 with sample "b" from the second Nalgene bottle
11) Keep the Sterivex filters at or below room temperature and freeze within 2 weeks

5. Negative controls

- Take two negative controls per day: 1 air sample and 1 water sample
- A negative control is taken in the same way as a normal sample but instead of pond water air or DNA-free water is filtered.
    - Air sample: run the pump for 5 min
    - Water sample: filter 250 mL of the water that is used for rinsing

6. Field forms

- Record GPS coordinates for each sample
- Measure pH, temperature, conductivity, dissolved oxygen concentration and Secchi depth at the surface of each waterbody that is sampled
- Complete the field form (*Fig. S.5.1.)*

eDNA field form		
Waterbody:	Date:	Team initials:

Water quality and water body characteristics	
Time:	Conductivity (µS/cm):
Temperature (°C):	O_2 concentration (mg/L):
pH:	O_2 saturation (%):
Secchi depth (cm):	Water depth (cm):
% surface with shade at midday:	

eDNA sample	
Microhabitat:	GPS coordinates:
Code sample a:	Code sample b:
Volume filtered sample a (mL):	Volume filtered sample b (mL):
Remarks:	

eDNA sample	
Microhabitat:	GPS coordinates:
Code sample a:	Code sample b:
Volume filtered sample a (mL):	Volume filtered sample b (mL):
Remarks:	

eDNA sample	
Microhabitat:	GPS coordinates:
Code sample a:	Code sample b:
Volume filtered sample a (mL):	Volume filtered sample b (mL):
Remarks:	

eDNA sample	
Microhabitat:	GPS coordinates:
Code sample a:	Code sample b:
Volume filtered sample a (mL):	Volume filtered sample b (mL):
Remarks:	

eDNA sample	
Microhabitat:	GPS coordinates:
Code sample a:	Code sample b:
Volume filtered sample a (mL):	Volume filtered sample b (mL):
Remarks:	

eDNA sample	
Microhabitat:	GPS coordinates:
Code sample a:	Code sample b:
Volume filtered sample a (mL):	Volume filtered sample b (mL):
Remarks:	

Fig. S.5.1. Environmental DNA sampling field form

Appendix 5.B. High Salt DNA Extraction Protocol for Sterivex filters stored in Longmire's buffer

Protocol modified following Kusanke et al. (2020) and Longmire et al. (1997)

Materials needed for 7 samples and 1 negative control:

- UV cabinet (VWR PCR workstation)
- Rotation incubator (Thermo Mixer) with blocks for 1,5 mL Eppendorf tubes, 2 mL Eppendorf tubes and 15 mL falcon tubes
- Cooling centrifuge
- Vortex mixer with adapter for 15 mL falcon tubes
- 8 2 mL Eppendorf LoBind microcentrifuge tubes
- 32 1.5 mL Eppendorf LoBind microcentrifuge tubes
- 8 Luer outlet stoppers and 1 Luer inlet stopper
- 8 0.7 cm pieces of sterile catheter tube (Sendal Perfusend)
- 8 2 mL syringes (Braun Petzold, Luer lock solo)
- 1 clean 0.45 µm PVDF Sterivex filter capsule (SX)
- Pipettes and tips
- Tissue for wiping
- Gloves (about 50)
- DNA exitus
- 70% ethanol for cleaning

Chemicals needed for 7 samples and 1 negative control:

- 2 mL of Longmire's buffer as negative control
- 0.4 mL proteinase K 20 mg/mL (enzyme that cleaves proteins). Prepare 2 mL Eppendorf tubes and keep them at -20 (should be good for 2 years) and take out one every time and keep it in the fridge (should be good for a month or two). Don't freeze thaw freeze thaw
- 5.6 mL 5M NaCl (to precipitate proteins out)
- 9.6 mL ice-cold isopropanol (to precipitate the DNA)
- 6.4 mL ice-cold 70% ethanol (to wash the DNA). You can prepare 50 mL (35 mL of >99.8% ethanol and 15 mL H_2O) and keep this at -20 degrees
- 1.6 mL H_2O

Longmire's buffer :

- 0,1 M Tris-HCl pH 8 (buffer to keep pH stable)
- 0,1 M EDTA (chelating agent that binds magnesium, and in this way inactivates nucleases that require magnesium)
- 0,01 M NaCl
- 0,5 % SDS (detergent, lyses cells)

Decontamination :

- Change gloves between every step, and whenever you touched the inside of an Eppendorf tube or get a drop on it.
- Change labcoat every week. Have a different labcoat for the pre and post-PCR rooms
- Clean holders for eppis with DNA-exitus and water and crosslink them
- Crosslink all tips

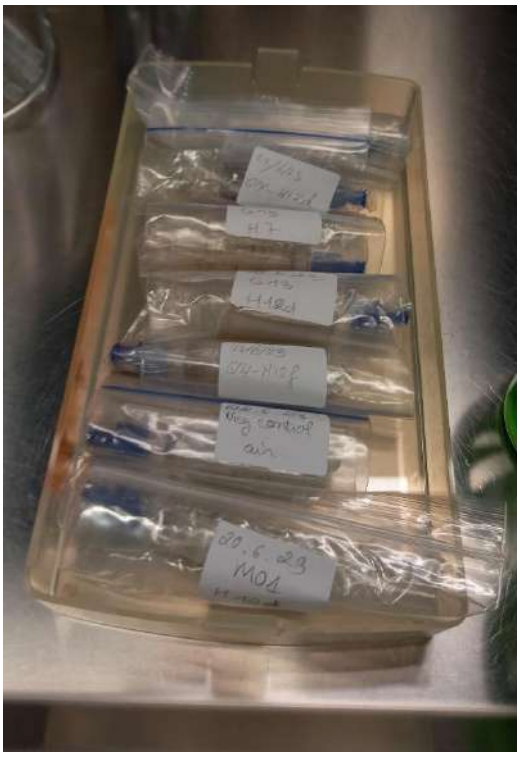
Higher temperature Longmire lysis: (takes about 1 hour to prepare + 2 hours of lysis + 0.5 hours after lysis)

a) Clean UV hood and tools with DNA-exitus and ethanol, turn on UV for 30 min in UV hood

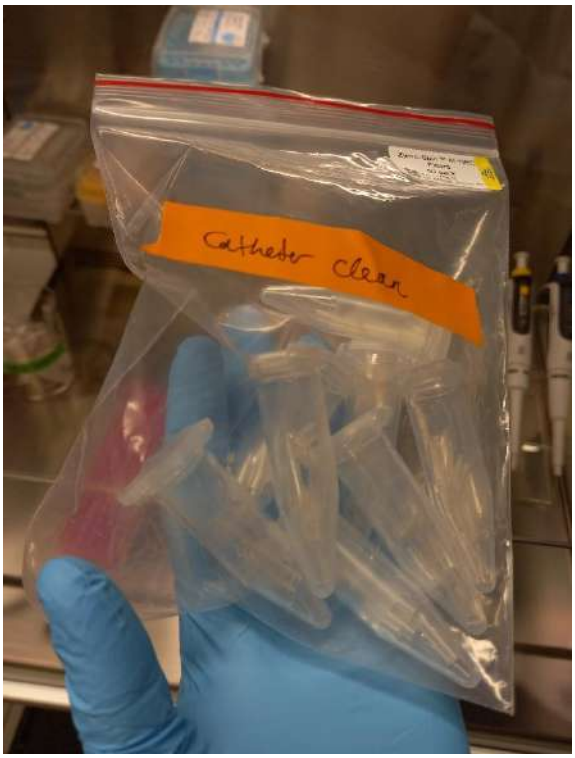
b) Clean rotation incubator, benches and crosslinker with ethanol
c) Crosslink rack for SX, and tray to place SX in while defrosting
d) Take 7 **Sterivex capsules** out of freezer and let defrost (*Fig. S.5.2a*, takes about 5 min from -20 freezer) (If I do two batches at the same time, I defrost 15)
e) Clean **15 mL falcon tube adapter for vortex** with DNA exitus and distilled water
f) Crosslink Longmire's, holder for proteinase K tube
g) Clean the **15 mL falcon tube incubator block** for the rotation incubator with DNA exitus (not too much) and ethanol (not too much)
h) Remove parafilm from the SX, close the and outlet with 0.7 cm of sterile **catheter tube** and a **stopper** and place in the vortex adapter (*Fig. S.5.2b,c*).
i) Close the outlet of the **negative control** SX with catheter tube and a stopper, add 2 mL of Longmire's, close the inlet with a stopper and place in the vortex adapter as well
j) **Vortex** the SXs for 10 min (*Fig. S.5.2d*)
k) Add 50 µL of 20 mg/mL **Proteinase K** to each SX
l) Vortex
m) Incubate samples **for 2 hours at 60** °C at 350 rpm in the rotation incubator
n) Label **8 2 mL** and **16 1.5 mL Lobind** tubes. Label the 1.5 mL tubes "**a" and "b"**. Open them and put them 10 min in the crosslinker
o) Transfer the lysate into a clean **2 mL LoBind** using a clean 2 mL syringe (*Fig. S.5.2e*)
p) Pipette 2 times 555 µL **of lysate** into clean 1.5 Lobinds and freeze **tubes b** as a backup. Continue with the high salt extraction or freeze **tubes a** to extract them later.

High salt Kusanke extraction: (takes about 3 hours)

a) Clean UV hood and tools with DNA-exitus and ethanol, turn on UV for 30 min in UV hood
b) Clean centrifuge, cooling centrifuge, rotation incubator, benches and crosslinker with ethanol
c) If the lysate is frozen, let defrost at 37 degrees in the Thermomix
d) Add 350 µL of 5 M **NaCl** to the lysate
e) Vortex
f) **Centrifuge** for 30 min at 16,200 x g (room temperature). Crosslink rack for after centrifugation. After centrifugation programme the centrifuge to cool to 4°C
g) Label 24 **1.5 mL LoBind** tubes, open them and put them 10 min in the crosslinker
h) **Transfer 600 µL supernatant** from each tube into a clean 1.5 mL LoBind
i) **Add** 600 µL of ice-cold **isopropanol** to each LoBind
j) **Mix** the content by inverting 3 times
k) **Incubate** at room temperature for 10 min
l) **Centrifuge** for 10 min at 4°C at 16,200 x g
m) **Pipette** out supernatant
n) **Add** 200 µL ice-cold 70% **ethanol**
o) **Centrifuge** for 10 min at 4°C at 16,200 x g
p) Preheat Thermomix 1.5 mL block to 60 °C
q) Pipette out supernatant
r) **Add** 200 µL ice-cold 70% **ethanol**
s) **Centrifuge** for 10 min at 4°C at 16,200 x g
t) **Pipette out supernatant**. Remove droplets carefully
u) **Dry pellet** in the adapter of the rotation incubator for 5 to 20 min in the UV hood until all ethanol is evaporated (not too long, then it will be hard to get DNA in solution later, but not too short because EtOH inhibits PCR)
v) Place water to dissolve DNA in for 10 min in crosslinker
w) **Dissolve pellet** in 100 µL of H_2O in the rotation incubator (37°C, 700 rpm) – this can take a long time (e.g. overnight)



a) Letting SX defrost



b) Sterile catheter tubes in 0.7 cm pieces

c) Capping SX with catheter tube and stopper

e) Preparing to remove lysate using 2 mL syringes

d) Vortexing SX

Fig. S.5.2. Performing the higher temperature Longmire lysis

Appendix 5.C. All samples on PCR plates and overall sequencing quality

Table S.5.1. All samples, negative field and negative extraction controls on the PCR plates with the type (NW for Neu-Woerr, LA for Silene, KAZ for samples, ASN for air field negative, SWN for rinsing water field negative, CEN for extraction negative, JMP for samples from another project), the volume of water filtered (mL) and concentration of extracted DNA in ng/µL (ltb is lower than blank and hts higher than standard). These were cycles in four PCR replicates on 16 plates that all had negative PCR, multiplex negative PCR and positive controls.

Order	Sample ID	Eppi code	Type	Date sampled	Waterbody	Mesohabitat	Vol.filt.	Conc.
1	A_GRA_04	4.GRA.4	NWKAZ	19/04/2023	W_GRA	H12b	1000	87
2	L_306_01	36.1a	LAKAZ	19/07/2022	S_306	H12	250	25
3	A_GAE_04	4.GAE.4	NWKAZ	18/04/2023	N_GAE	H12d	520	18
4	L_204_02	24.2a	LAKAZ	21/07/2022	S_204	H12d, H12f	250	12
5	A_L98_01	4.L98.1	NWKAZ	19/04/2023	W_L98	H14	950	0.85
6	L_101_16	11.16a	LAKAZ	22/07/2022	S_101	H2	250	1.18
7	L_202_07	22.7a	LAKAZ	21/07/2022	S_202	H2	250	9.1
8	S_G14_02	G14.2a	NWKAZ	14/09/2022	N_G14	H13b	160	5.4
9	L_303_07	33.7a	LAKAZ	19/07/2022	S_303	H5	250	12
10	J_G08_01	6.G08.1	NWKAZ	19/06/2023	N_G08	H13c	100	13
11	J_PGB_05	6.PGB.5	NWKAZ	22/06/2023	N_PGB	H12f	400	52
12	L_302_06	32.6a	LAKAZ	19/07/2022	S_302	H5	175	34
13	L_NFA_06	NF.6a	LAASN	20/07/2022	Control	Control	0	0.0586
14	J_G14_04	6.G14.4	NWKAZ	22/06/2023	N_G14	H5	400	35
15	J_G07_03	6.G07.3	NWKAZ	22/06/2023	N_G07	H13c		26
16	J_PGB_04	6.PGB.4	NWKAZ	22/06/2023	N_PGB	H5	450	15
17	S_G13_04	G13.4a	NWKAZ	14/09/2022	N_G13	H12f	17	1.38
18	A_GRA_03	4.GRA.3	NWKAZ	19/04/2023	W_GRA	H12f	1000	36
19	L_202_06	22.6a	LAKAZ	21/07/2022	S_202	H7	250	
20	J_BAS_03	6.BAS.3	NWKAZ	19/06/2023	W_BAS	H7	950	60
21	J_G14_01	6.G14.1	NWKAZ	22/06/2023	N_G14	H10	720	12
22	A_PGB_06	4.PGB.6	NWKAZ	18/04/2023	N_PGB	H12f	980	46
23	J_PGB_01	6.PGB.1	NWKAZ	22/06/2023	N_PGB	H14	700	15
24	J_M05_04	6.M05.4	NWKAZ	20/06/2023	W_M05	H8	170	14
25	A_LED_04	4.LED.4 JM527	NWKAZ	19/04/2023	W_LED	H7	950	6.3
26	A_PGB_07	4.PGB.7	NWKAZ	18/04/2023	N_PGB	H12b	400	14
27	A_GRA_02	4.GRA.2 JM537	NWKAZ	19/04/2023	W_GRA	H7	1000	6.2
28	L_101_07	11.7a	LAKAZ	22/07/2022	S_101	H5	250	
29	S_PGB_03	PG.3a	NWKAZ	14/09/2022	N_PGB	H12g	350	9.6
30	N_NEC_02	NE.2	NWCEN		Control	Control		0.0041
31	J_M06_01	6.M06.1	NWKAZ	20/06/2023	W_M06	H12f	100	4.12
32	J_LFC_05	6.LFC.5	NWKAZ	21/06/2023	W_LFC	H9	1000	28
33	J_M05_02	6.M05.2	NWKAZ	20/06/2023	W_M05	H12d	90	5.8
34	N_NEC_30	NE.30a	LACEN		Control	Control		0.0135
35	L_302_03	32.3a	LAKAZ	19/07/2022	S_302	H12f	150	29
36	N_NEC_21	NE.21a	LACEN		Control	Control		0.0333
37	L_302_01	32.1a	LAKAZ	19/07/2022	S_302	H12d	220	33
38	S_LED_04	ED.4a	NWKAZ	13/09/2022	W_LED	H5	900	12
39	N_NEC_17	NE.17a	NWCEN		Control	Control		
40	N_NEC_42	NE.42a	NWCEN		Control	Control		0.042
41	L_NFW_02	NF.2a	LAWSN	20/07/2022	Control	Control	250	0.052
42	J_LED_05	6.LED.5	NWKAZ	20/06/2023	W_LED	H12c	500	25
43	A_G14_07	4.G14.7	NWKAZ	18/04/2023	N_G14	H10	310	71
44	S_LED_13	ED.13a	NWKAZ	13/09/2022	W_LED	H5	100	11
45	J_GRA_01	6.GRA.1	NWKAZ	20/06/2023	W_GRA	H12e	1000	14
46	S_BAS_03	BS.3a	NWKAZ	13/09/2022	W_BAS	H5	225	10
47	S_G14_05	G14.5a	NWKAZ	14/09/2022	N_G14	H2	150	24
48	J_G13_01	6.G13.1	NWKAZ	22/06/2023	N_G13	H14	150	20
49	L_206_06	26.6a	LAKAZ	21/07/2022	S_206	H7	250	
50	N_NEC_07	NE.7.a	NWCEN		Control	Control		0.206
51	J_G01_01	6.G01.1	NWKAZ	19/06/2023	N_G01	H10	180	31
52	J_LED_01	6.LED.1	NWKAZ	20/06/2023	W_LED	H12f	700	59

Order	Sample ID	Eppi code	Type	Date sampled	Waterbody	Mesohabitat	Vol.filt.	Conc.
53	N_NEC_24	NE.24a	LACEN		Control	Control		0.0161
54	L_101_18	11.18a	LAKAZ	22/07/2022	S_101	H8	250	2.79
55	J_G11_03	6.G11.3	NWKAZ	21/06/2023	N_G11	H7	600	5.6
56	J_L98_03	6.L98.3	NWKAZ	21/06/2023	W_L98	H12f	200	58
57	L_306_04	36.4a	LAKAZ	20/07/2022	S_306		250	ltb
58	L_204_04	24.4a	LAKAZ	21/07/2022	S_204	H7	250	
59	A_NFA_03	4.NF.3 JM536	NWASN	19/04/2023	Control	Control	0	115
60	S_GAE_09	AE.9a	NWKAZ	15/09/2022	N_GAE	H123c	400	99
61	J_LFC_04	6.LFC.4	NWKAZ	21/06/2023	W_LFC	H7	1000	11
62	J_PGB_02	6.PGB.2	NWKAZ	22/06/2023	N_PGB	H7	600	76
63	J_G04_03	6.G04.3	NWKAZ	21/06/2023	N_G04	H12f	30	3.51
64	J_GAE_02	6.GAE.2	NWKAZ	23/06/2023	N_GAE	H12b	200	36
65	L_303_02	33.2a	LAKAZ	19/07/2022	S_303	H12d	250	9.4
66	S_G13_02	G13.2a	NWKAZ	14/09/2022	N_G13	H12d	40	0.789
67	J_L98_04	6.L98.4	NWKAZ	21/06/2023	W_L98	H5	900	97
68	A_LEC_03	4.LEC.3	NWKAZ	19/04/2023	W_LEC	H12d	900	35
69	S_G14_06	G14.6a	NWKAZ	14/09/2022	N_G14	H2	120	1.12
70	J_LEC_04	6.LEC.4	NWKAZ	20/06/2023	W_LEC	H7	1000	22
71	J_G04_02	6.G04.2	NWKAZ	21/06/2023	N_G04	H7	70	7.6
72	J_M01_03	6.M01.3	NWKAZ	20/06/2023	W_M01	H12f	250	8.4
73	N_NEC_37	NE.37a	NWCEN		Control	Control		0.0069
74	A_GAE_05	4.GAE.5	NWKAZ	18/04/2023	N_GAE	H14	700	71
75	N_NEC_26	NE.26a	LACEN		Control	Control		0.017
76	J_GRA_05	6.GRA.5	NWKAZ	20/06/2023	W_GRA	H12d	650	61
77	L_204_01	24.1a	LAKAZ	21/07/2022	S_204	H12d, H12f	250	18
78	J_GRA_08	6.GRA.8	NWKAZ	20/06/2023	W_GRA	H9	1000	8.5
79	J_L98_02	6.L98.2	NWKAZ	21/06/2023	W_L98	H12d	800	32
80	S_BAS_08	BS.8a	NWKAZ	13/09/2022	W_BAS	H9	190	1.49
81	N_NEC_32	NE.32a	LACEN		Control	Control		0.022
82	S_G14_04	G14.4a	NWKAZ	14/09/2022	N_G14	H12f	120	14
83	S_LED_09	ED.9a	NWKAZ	13/09/2022	W_LED	H5	500	44
84	A_GAE_06	4.GAE.6	NWCEN	18/04/2023	N_GAE	H5b	350	24
85	S_BAS_02	BS.2a	NWKAZ	13/09/2022	W_BAS	H12f	150	60
86	A_GRA_05	4.GRA.5 JM538	NWKAZ	19/04/2023	W_GRA	H12d	1000	13
87	J_G07_04	6.G07.4	NWKAZ	22/06/2023	N_G07	H12h		16
88	A_BAS_04	505	NWKAZ	20/04/2023	W_BAS	H12f	690	3.46
89	S_LED_06	ED.6a	NWKAZ	13/09/2022	W_LED	H12f	800	14
90	J_M05_05	6.M05.5	NWKAZ	20/06/2023	W_M05	H5	190	23
91	S_NFA_15	NF.15.a	NWASN	16/09/2023	Control	Control	0	0.0077
92	A_LEC_02	4.LEC.2	NWKAZ	19/04/2023	W_LEC	H10	1000	2.02
93	A_G14_02	4.G14.2 JM514	NWKAZ	18/04/2023	N_G14	H12b	420	41
94	N_NEC_08	NE.8a	NWCEN		Control	Control		0.0007
95	L_303_05	33.5a	LAKAZ	19/07/2022	S_303	H10	175	10
96	J_GRA_07	6.GRA.7	NWKAZ	20/06/2023	W_GRA	H5	1000	14
97	L_308_06	38.6a	LAKAZ	20/07/2022	S_308	H1	250	3.42
98	J_VLA_01	6.VLA.1	NWKAZ	21/06/2023	W_VLA	H1	250	13
99	J_G04_04	6.G04.4	NWKAZ	21/06/2023	N_G04	H10	50	5.4
100	S_G13_05	G13.5a	NWKAZ	14/09/2022	N_G13	H13b	20	2.61
101	S_G13_06	G13.6a	NWKAZ	14/09/2022	N_G13	H13b	23	2.3
102	L_101_03	11.3a	LAKAZ	22/07/2022	S_101	H8	250	2.53
103	S_LED_07	ED.7a	NWKAZ	13/09/2022	W_LED	H12f	300	69
104	S_LED_01	ED.1a	NWKAZ	13/09/2022	W_LED	H5	400	42
105	J_GAE_05	6.GAE.5	NWKAZ	23/06/2023	N_GAE	H13c	500	15
106	S_PGB_12	PG.12a	NWKAZ	14/09/2022	N_PGB	H12d	180	23
107	J_BAS_01	6.BAS.1	NWKAZ	19/06/2023	W_BAS	H8	450	72
108	J_G14_02	6.G14.2	NWKAZ	22/06/2023	N_G14	H12f	430	51
109	S_GRA_12	GP.12a	NWKAZ	16/09/2022	W_GRA	H3	280	19
110	L_104_01	14.1a	LAKAZ	22/07/2022	S_104	H12d, H12f	250	6.7
111	L_104_05	14.5a	LAKAZ	22/07/2022	S_104	H2	250	5.1
112	J_NFW_02	6.NF.2	NWWSN	19/06/2023	Control	Control	250	0.0425
113	L_308_05	38.5a	LAKAZ	20/07/2022	S_308	H1	250	11
114	S_GAE_13	AE.13a	NWKAZ	15/09/2022	N_GAE	H12d	220	10
115	A_L98_02	4.L98.2 JM519	NWKAZ	19/04/2023	W_L98	H10	1000	7

Order	Sample ID	Eppi code	Type	Date sampled	Waterbody	Mesohabitat	Vol.filt.	Conc.
116	J_M03_01	6.M03.1	NWKAZ	21/06/2023	W_M03	H12f	400	43
117	S_GAE_14	AE.14a	NWKAZ	15/09/2022	N_GAE	H12d	200	0.0619
118	L_NFW_04	NF.4a	LAWSN	22/07/2022	Control	Control	250	0.0258
119	J_M01_01	6.M01.1	NWKAZ	20/06/2023	W_M01	H12a	200	16
120	J_L98_06	6.L98.6	NWKAZ	21/06/2023	W_L98	H10	600	7
121	J_PGB_03	6.PGB.3	NWKAZ	22/06/2023	N_PGB	H12d	500	39
122	L_202_03	22.3a	LAKAZ	21/07/2022	S_202	H12d, H12f	250	3.86
123	L_NFA_05	NF.5a	LAASN	19/07/2022	Control	Control	0	ltb
124	J_G01_02	6.G01.2	NWKAZ	19/06/2023	N_G01	H12f	180	30
125	S_LED_03	ED.3a	NWKAZ	13/09/2022	W_LED	H5	900	3.21
126	A_LFC_03	4.LFC.3 JM528	NWKAZ	19/04/2023	W_LFC	H5b	1000	1.81
127	L_206_03	26.3a	LAKAZ	21/07/2022	S_206	H12e	250	9.8
128	J_LED_04	6.LED.4	NWKAZ	20/06/2023	W_LED	H12d	450	18
129	N_NEC_16	NE.16a	NWCEN		Control	Control		0.0236
130	J_G14_05	6.G14.5	NWKAZ	22/06/2023	N_G14	H8	200	40
131	L_302_09	32.9a	LAKAZ	19/07/2022	S_302	H12d	200	25
132	L_101_09	11.9a	LAKAZ	22/07/2022	S_101	H2	250	1.97
133	L_101_11	11.11a	LAKAZ	22/07/2022	S_101	H12d	250	6.3
134	J_G11_02	6.G11.2	NWKAZ	21/06/2023	N_G11	H12d	500	0.0123
135	A_G13_01	4.G13.1	NWKAZ	18/04/2023	N_G13	H13b	900	52
136	S_LED_12	ED.12a	NWKAZ	13/09/2022	W_LED	H12f	350	35
137	L_NFW_03	NF.3a	LAWSN	21/07/2022	Control	Control	250	0.015
138	A_BAS_06	500	NWKAZ	20/04/2023	W_BAS	H9	620	2.05
139	A_GRA_01	4.GRA.1	NWKAZ	19/04/2023	W_GRA	H14	1000	0.2
140	J_GAE_06	6.GAE.6	NWKAZ	23/06/2023	N_GAE	H5	400	36
141	S_GRA_05	GP.5a	NWKAZ	16/09/2022	W_GRA	C2	1000	1.2
142	A_G14_01	4.G14.1 JM518	NWKAZ	18/04/2023	N_G14	H12d	680	70
143	S_NFA_13	NF.13a	NWASN	15/09/2023	Control	Control	0	0.0173
144	J_GAE_01	6.GAE.1	NWKAZ	23/06/2023	N_GAE	H7	400	14
145	S_BAS_06	BS.6a	NWKAZ	13/09/2022	W_BAS	H3	210	4.54
146	N_NEC_29	NE.29a	LACEN		Control	Control		0.0641
147	S_LED_08	ED.8a	NWKAZ	13/09/2022	W_LED	H12f	400	14
148	S_GAE_03	AE.3a	NWKAZ	15/09/2022	N_GAE	H12b	280	19
149	S_GAE_11	AE.11a	NWKAZ	15/09/2022	N_GAE	H12f	500	82
150	J_G14_06	6.G14.6	NWKAZ	22/06/2023	N_G14	H12d	200	8.1
151	L_NFA_08	NF.8a	LAASN	22/07/2022	Control	Control	0	0.008
152	S_GRA_13	GP.13a	NWKAZ	16/09/2022	W_GRA	H7	1000	2.02
153	J_M05_06	6.M05.6	NWKAZ	20/06/2023	W_M05	H10	120	12
154	A_GRA_07	4.GRA.7	NWKAZ	19/04/2023	W_GRA	H5b	1000	16
155	N_NEC_09	NE.9a	NWCEN		Control	Control		0.0087
156	S_LED_11	ED.11a	NWKAZ	13/09/2022	W_LED	H12f	400	75
157	L_302_02	32.2a	LAKAZ	19/07/2022	S_302	H12f	150	98
158	S_PGB_10	PG.10a	NWKAZ	14/09/2022	N_PGB	H5a	340	1.28
159	J_LFC_07	6.LFC.7	NWKAZ	21/06/2023	W_LFC	H12d	1000	52
160	L_202_08	22.8a	LAKAZ	21/07/2022	S_202	H2	250	
161	S_LED_18	ED.18a	NWKAZ	13/09/2022	W_LED	H12f	250	22
162	S_PGB_15	PG.15a	NWKAZ	14/09/2022	N_PGB	H13c	310	22
163	J_M06_04	6.M06.4	NWKAZ	20/06/2023	W_M06	H10	100	16
164	S_PGB_07	PG.7a	NWKAZ	14/09/2022	N_PGB	H5a	280	17
165	L_101_01	11.1a	LAKAZ	22/07/2022	S_101	H12d	250	9.3
166	A_LEC_01	4.LEC.1 JM520	NWKAZ	19/04/2023	W_LEC	H12f	1000	21
167	J_M05_03	6.M05.3	NWKAZ	20/06/2023	W_M05	H12f	180	8.3
168	S_LED_15	ED.15a	NWKAZ	13/09/2022	W_LED	H12f	700	2.02
169	A_LED_05	4.LED.5	NWKAZ	19/04/2023	W_LEC	H12e	1000	8.2
170	J_VLA_02	6.VLA.2	NWKAZ	21/06/2023	W_VLA	H12	50	22
171	S_BAS_05	BS.5a	NWKAZ	13/09/2022	W_BAS	H3	220	29
172	L_101_17	11.17a	LAKAZ	22/07/2022	S_101	H2	250	
173	L_204_03	24.3a	LAKAZ	21/07/2022	S_204	H7	250	14
174	L_202_04	22.4a	LAKAZ	21/07/2022	S_202	H12d, H12f	250	9
175	L_308_03	38.3a	LAKAZ	20/07/2022	S_308	H7	250	
176	J_G11_01	6.G11.1	NWKAZ	21/06/2023	N_G11	H12f	550	18
177	A_GRA_06	4.GRA.6 JM530	NWKAZ	19/04/2023	W_GRA	H12c	1000	5.5
178	J_M05_01	6.M05.1	NWKAZ	20/06/2023	W_M05	H12e	180	16

Order	Sample ID	Eppi code	Type	Date sampled	Waterbody	Mesohabitat	Vol.filt.	Conc.
179	S_BAS_01	BS.1a	NWKAZ	13/09/2022	W_BAS	H12f	175	8.8
180	L_NFW_01	NF.1a	LAWSN	19/07/2022	Control	Control	250	0.185
181	L_308_04	38.4a	LAKAZ	20/07/2022	S_308	H7	250	11
182	L_101_10	11.10a	LAKAZ	22/07/2022	S_101	H2	250	1.39
183	J_M03_02	6.M03.2	NWKAZ	21/06/2023	W_M03	H1	400	8.3
184	A_LFC_04	4.LFC.4 JM 515	NWKAZ	19/04/2023	W_LFC	H10	1000	2.9
185	A_LFC_01	4.LFC.1	NWKAZ	19/04/2023	W_LFC	H12d	900	4.33
186	J_BAS_04	6.BAS.4	NWKAZ	19/06/2023	W_BAS	H12b	780	7.8
187	A_PGB_04	512	NWKAZ	18/04/2023	N_PGB	H12h	800	33
188	J_M02_03	6.M05.7	NWKAZ	20/06/2023	W_M02	H4	1000	12
189	L_206_02	26.2a	LAKAZ	21/07/2022	S_206	H12d, H12f	250	3.24
190	S_LED_05	ED.5a	NWKAZ	13/09/2022	W_LED	H12f	340	31
191	J_M01_04	6.M01.4	NWKAZ	20/06/2023	W_M01	H7	250	7.7
192	L_101_12	11.12a	LAKAZ	22/07/2022	S_101	H12d	250	2.63
193	A_LED_03	4.LED.3 JM524	NWKAZ	19/04/2023	W_LED	H12f	1000	7.2
194	L_104_06	14.6a	LAKAZ	22/07/2022	S_104	H2	250	3.97
195	L_102_01	12.1a	LAKAZ	21/07/2022	S_102	H12d, H12f	150	30
196	J_G01_03	6.G01.3	NWKAZ	19/06/2023	N_G01	H12d	190	12
197	J_G07_01	6.G07.1	NWKAZ	22/06/2023	N_G07	H9		53
198	J_G04_01	6.G04.1	NWKAZ	21/06/2023	N_G04	H12d	100	8.4
199	J_LEC_02	6.LEC.2	NWKAZ	20/06/2023	W_LEC	H12e	1000	74
200	L_206_05	26.5a	LAKAZ	21/07/2022	S_206	H7	250	
201	A_LFC_02	4.LFC.2	NWKAZ	19/04/2023	W_LFC	H12f	1000	6.4
202	L_101_04	11.4a	LAKAZ	22/07/2022	S_101	H8	250	6
203	L_306_02	36.2a	LAKAZ	19/07/2022	S_306	H5	250	2.07
204	S_GRA_01	GP.1a	NWKAZ	16/09/2022	W_GRA	H12f	750	9.9
205	J_GRA_03	6.GRA.3	NWKAZ	20/06/2023	W_GRA	H12f	1000	6.1
206	S_GRA_07	GP.7a	NWKAZ	16/09/2022	W_GRA	H5	1000	6.8
207	N_NEC_18	NE.18a	NWCEN		Control	Control		0.0016
208	J_L98_05	6.L98.5	NWKAZ	21/06/2023	W_L98	H7	800	21
209	S_GRA_10	GP.10a	NWKAZ	16/09/2022	W_GRA	H12e	1000	1.26
210	J_LFC_02	6.LFC.2	NWKAZ	21/06/2023	W_LFC	H12f	50	38
211	J_G13_04	6.G13.4	NWKAZ	22/06/2023	N_G13	H7	250	34
212	S_GAE_05	AE.5a	NWKAZ	15/09/2022	N_GAE	H3	370	70
213	S_NFW_12	NF.12a	NWWSN	14/09/2022	Control	Control	250	0.0314
214	J_M01_02	6.M01.2	NWKAZ	20/06/2023	W_M01	H12d	210	26
215	A_L98_06	4.L98.6	NWKAZ	19/04/2023	W_L98	H14	950	29
216	J_G07_05	6.G07.5	NWKAZ	22/06/2023	N_G07	H12f		61
217	J_GRA_06	6.GRA.6	NWKAZ	20/06/2023	W_GRA	H12b	500	9.2
218	J_BAS_02	6.BAS.2	NWKAZ	19/06/2023	W_BAS	H9	1000	52
219	A_BAS_02	4.BAS.2 JM531	NWKAZ	20/04/2023	W_BAS	H5a	550	2.26
220	J_M02_01	6.M02.1	NWKAZ	20/06/2023	W_M02	H1	1000	33
221	A_PGB_02	4.PGB.2 JM523	NWKAZ	18/04/2023	N_PGB	H12d	1000	6.7
222	S_GRA_03	GP.3a	NWKAZ	16/09/2022	W_GRA	C1	1000	7.7
223	L_308_02	38.2a	LAKAZ	20/07/2022	S_308	H5	250	
224	S_G14_03	G14.3a	NWKAZ	14/09/2022	N_G14	H12f	100	5.2
225	S_LED_14	ED.14a	NWKAZ	13/09/2022	W_LED	H5	300	35
226	J_M06_02	6.M06.2	NWKAZ	20/06/2023	W_M06	H12b	100	44
227	J_M06_05	6.M06.5	NWKAZ	20/06/2023	W_M06	H5	100	9.2
228	L_302_08	32.8a	LAKAZ	19/07/2022	S_303	H5	250	
229	J_GAE_03	6.GAE.3	NWKAZ	23/06/2023	N_GAE	H12f	200	58
230	J_M02_02	6.M02.2	NWKAZ	20/06/2023	W_M02	H13a	900	66
231	J_G13_03	6.G13.3	NWKAZ	22/06/2023	N_G13	H12d	500	27
232	N_NEC_23	NE.23a	LACEN		Control	Control		0.015
233	J_GAE_04	6.GAE.4	NWKAZ	23/06/2023	N_GAE	H12d	300	31
234	A_BAS_05	504	NWKAZ	20/04/2023	W_BAS	H5b	690	2.78
235	L_303_04	33.4a	LAKAZ	19/07/2022	S_303	H10	175	
236	S_PGB_01	PG.1a	NWKAZ	14/09/2022	N_PGB	H3	500	17
237	S_GAE_07	AE.7a	NWKAZ	15/09/2022	N_GAE	H12g	300	67
238	A_NFA_05	502	NWASN	20/04/2023	Control	Control	0	0.01
239	A_G14_05	4.G14.5	NWKAZ	18/04/2023	N_G14	H12f	300	69
240	A_PGB_05	4.PGB.5	NWKAZ	18/04/2023	N_PGB	H5a	640	ltb
241	S_PGB_11	PG.11a	NWKAZ	14/09/2022	N_PGB	H12d	240	19

Order	Sample ID	Eppi code	Type	Date sampled	Waterbody	Mesohabitat	Vol.filt.	Conc.
242	L_202_05	22.5a	LAKAZ	21/07/2022	S_202	H7	250	5.5
243	J_LEC_06	6.LEC.6	NWKAZ	20/06/2023	W_LEC	H9	1000	10
244	S_LED_16	ED.16a	NWKAZ	13/09/2022	W_LED	H12f	600	4.08
245	L_102_03	12.3a	LAKAZ	21/07/2022	S_102	H5	250	13
246	J_LED_03	6.LED.3	NWKAZ	20/06/2023	W_LED	H7	700	8.3
247	S_G13_01	G13.1a	NWKAZ	14/09/2022	N_G13	H12d	40	2.19
248	A_G14_03	4.G14.3 JM522	NWKAZ	18/04/2023	N_G14	H8	380	33
249	L_303_01	33.1a	LAKAZ	19/07/2022	S_303	H12b	250	7.4
250	A_PGB_01	4.PGB.1 JM534	NWKAZ	18/04/2023	N_PGB	H14	1000	5
251	L_308_07	38.7a	LAKAZ	20/07/2022	S_308	H12d, H12f	250	8.3
252	J_LEC_03	6.LEC.3	NWKAZ	20/06/2023	W_LEC	H12d	1000	8.6
253	A_G13_04	4.G13.4 JM516	NWKAZ	18/04/2023	N_G13	H14	350	8.4
254	S_G13_08	G13.8a	NWKAZ	14/09/2022	N_G13	H2	40	7.1
255	A_G13_02	510	NWKAZ	18/04/2023	N_G13	H12d	180	9.3
256	S_PGB_05	PG.5a	NWKAZ	14/09/2022	N_PGB	H12f	560	3.53
257	L_104_03	14.3a	LAKAZ	22/07/2022	S_104	H8, H5	250	8.5
258	L_102_04	12.4a	LAKAZ	21/07/2022	S_102	H5	150	27
259	N_NEC_20	NE.20a	LACEN		Control	Control		0.0329
260	A_G14_06	509	NWKAZ	18/04/2023	N_G14	H13b	390	50
261	N_NEC_43	NE.43a	NWKAZ		Control	Control		0.145
262	J_GRA_04	6.GRA.4	NWKAZ	20/06/2023	W_GRA	H13c	1000	32
263	J_G08_02	6.G08.2	NWKAZ	19/06/2023	N_G08	H12f	100	11
264	N_NEC_27	NE.27a	LACEN		Control	Control		0.0748
265	L_206_04	26.4a	LAKAZ	21/07/2022	S_206	H12e	250	9
266	L_303_03	33.4a	LAKAZ	19/07/2022	S_303	H12d	250	7.2
267	J_NFW_04	6.NF.4	NWWSN	20/06/2023	Control	Control	250	0.0102
268	L_303_06	33.6a	LAKAZ	19/07/2022	S_303	H5	250	13
269	S_GAE_02	AE.2a	NWKAZ	15/09/2022	N_GAE	H12c	300	51
270	A_LFC_05	4.LFC.5	NWKAZ	19/04/2023	W_LFC	H14	1000	69
271	A_L98_05	4.L98.5	NWKAZ	19/04/2023	W_L98	H5b	800	23
272	J_LFC_01	6.LFC.1	NWKAZ	21/06/2023	W_LFC	H14	800	12
273	N_NEC_33	NE.33a	LACEN		Control	Control		0.0312
274	S_LED_17	ED.17a	NWKAZ	13/09/2022	W_LED	H12f	225	92
275	J_LFC_06	6.LFC.6	NWKAZ	21/06/2023	W_LFC	H5	1000	8.4
276	S_G14_01	G14.1a	NWKAZ	14/09/2022	N_G14	H13b	140	26
277	L_104_04	14.4a	LAKAZ	22/07/2022	S_104	H8, H5	250	6.7
278	A_L98_04	4.L98.4	NWKAZ	19/04/2023	W_L98	H12c	970	5.1
279	A_LED_01	4.LED.1	NWKAZ	19/04/2023	W_LED	H12e	1000	4.32
280	S_G13_03	G13.3a	NWKAZ	14/09/2022	N_G13	H12f	17	1.65
281	J_LEC_05	6.LEC.5	NWKAZ	20/06/2023	W_LEC	H12f	1000	13
282	S_LED_10	ED.10a	NWKAZ	13/09/2022	W_LED	H5	750	22
283	J_G08_03	6.G08.3	NWKAZ	19/06/2023	N_G08	H1		47
284	S_LED_02	ED.2a	NWKAZ	13/09/2022	W_LED	H5	400	0.304
285	A_BAS_01	501	NWKAZ	20/04/2023	W_BAS	H12e	410	6.6
286	J_BAS_07	6.BAS.7	NWKAZ	19/06/2023	W_BAS	H12g	700	38
287	L_102_02	12.2a	LAKAZ	21/07/2022	S_102	H12d, H12f	250	21
288	L_NFA_07	NF.7a	LAASN	21/07/2022	Control	Control	0	0.0615
289	L_101_19	11.19a	LAKAZ	22/07/2022	S_101	H8	250	
290	A_G14_04	4.G14.4	NWKAZ	18/04/2023	N_G14	H14	300	hts
291	S_BAS_07	BS.7a	NWKAZ	13/09/2022	W_BAS	H9	215	13
292	J_G01_04	6.G01.4	NWKAZ	19/06/2023	N_G01	H13c	180	38
293	N_NEC_15	NE.15a	NWCEN		Control	Control		0.104
294	L_101_21	11.21a	LAKAZ	22/07/2022	S_101	H5	250	2.63
295	L_308_01	38.1a	LAKAZ	20/07/2022	S_308	H5	250	4.82
296	J_L98_01	6.L98.1	NWKAZ	21/06/2023	W_L98	H14	600	22
297	J_G13_02	6.G13.2	NWKAZ	22/06/2023	N_G13	H5	200	5.3
298	S_G13_07	G13.7a	NWKAZ	14/09/2022	N_G13	H2	40	2.11
299	L_101_08	11.8a	LAKAZ	22/07/2022	S_101	H5	250	
300	L_206_01	26.1a	LAKAZ	21/07/2022	S_206	H12d, H12f	250	15
301	J_GRA_02	6.GRA.2	NWKAZ	20/06/2023	W_GRA	H7	1000	3.62
302	N_NEC_28	NE.28a	LACEN		Control	Control		0.0327
303	J_LED_02	6.LED.2	NWKAZ	20/06/2023	W_LED	H12e	500	14
304	J_BAS_05	6.BAS.5	NWKAZ	19/06/2023	W_BAS	H5	1000	58

Order	Sample ID	Eppi code	Type	Date sampled	Waterbody	Mesohabitat	Vol.filt.	Conc.
305	J_BAS_06	6.BAS.6	NWKAZ	19/06/2023	W_BAS	H12f	700	37
306	L_101_20	11.20a	LAKAZ	22/07/2022	S_101	H5	250	13
307	A_GAE_07	4.GAE.7 JM526	NWKAZ	18/04/2023	N_GAE	H13b	490	14
308	N_NEC_48	513	NWCEN		Control	Control		0.0016
309	J_M06_03	6.M06.3	NWKAZ	20/06/2023	W_M06	H7	100	17
310	A_GAE_03	506	NWKAZ	18/04/2023	N_GAE	H5a	320	12
311	A_PGB_03	4.PGB.3	NWKAZ	18/04/2023	N_PGB	H13b	950	13
312	L_306_03	36.3a	LAKAZ	19/07/2022	S_306	H5	250	8.1
313	S_BAS_04	BS.4a	NWKAZ	13/09/2022	W_BAS	H5	125	5.82
314	L_302_07	32.7a	LAKAZ	19/07/2022	S_302	H5	200	12
315	J_G14_03	6.G14.3	NWKAZ	22/06/2023	N_G14	H14	350	18
316	L_101_02	11.2a	LAKAZ	22/07/2022	S_101	H12d	220	5.7
317	L_308_08	38.8a	LAKAZ	20/07/2022	S_308	H12d, H12f	250	19
318	A_BAS_03	4.BAS.3 JM533	NWKAZ	20/04/2023	W_BAS	H12g	770	3.68
319	L_104_02	14.2a	LAKAZ	22/07/2022	S_104	H12d, H12f	250	
320	J_NFA_03	6.NF.3	NWASN	20/06/2023	Control	Control	0	0.0339
321	L_302_05	32.5a	LAKAZ	19/07/2022	S_302	H5	125	21
322	J_LEC_01	6.LEC.1	NWKAZ	20/06/2023	W_LEC	H12c	950	40
323	A_GAE_02	508	NWKAZ	18/04/2023	N_GAE	H12b	640	9.3
324	A_GAE_01	4.GAE.1 JM 517	NWKAZ	18/04/2023	N_GAE	H12f	470	7.6
325	N_NEC_14	NE.14a	NWCEN		Control	Control		0.0097
326	Johannes 1		JMP24					
327	Johannes 2		JMP24					
328	Johannes 3		JMP24					
329	Johannes 4		JMP24					
330	Johannes 5		JMP24					
331	Johannes 6		JMP24					
332	Johannes 7		JMP24					
333	Johannes 8		JMP24					
334	Johannes 9		JMP24					
335	Johannes 10		JMP24					
336	Johannes 11		JMP24					
337	Johannes 12		JMP24					
338	Johannes 13		JMP24					
339	Johannes 14		JMP24					
340	Johannes 15		JMP24					

*Table S.5.2. Sequencing quality for the four multiplexes (MP) with the number of raw (sum of forward and reverse) reads, quantity of data, % effective as clean reads/raw reads*100%, the base error rate, percentage of bases with Phred values higher than 20, and 30 and the GC content.*

	Library_Flowcell_Lane	Raw reads	Raw data	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
MP1	FKDN240276917-1A_H7N77DRX5_L1	316140030	265.6 Gb	99.44	0.03	97.3	92.81	56.73
MP1	FKDN240276917-1A_H7N77DRX5_L2	310205930		99.41	0.03	97.25	92.85	56.66
MP1	FKDN240276917-1A_H7MNCDRX5_L1	218947208		99.4	0.03	97.36	93.04	56.59
MP1	FKDN240276917-1A_H7MNCDRX5_L2	217287782		99.33	0.03	97.22	92.78	56.5
MP2	FKDN240276918-1A_H7NNWDRX5_L1	166016152	204.6 Gb	99.57	0.04	91.39	81.59	42.55
MP2	FKDN240276918-1A_H7NNWDRX5_L2	175039172		99.53	0.04	93.39	84.91	42.56
MP2	FKDN240276918-1A_H7VMHDRX5_L2	211732924		99.13	0.03	96.82	92.05	42.61
MP2	FKDN240276918-1A_H7VMHDRX5_L1	265806830		99.28	0.03	96.85	91.97	42.59
MP3	FKDN240276919-1A_HKJ7WDSXC_L1	24600964	56.2 Gb	100	0.02	98.76	96.25	48.49
MP3	FKDN240276919-1A_HKHC5DSXC_L2	78410928		100	0.03	97.94	94	48.45
MP3	FKDN240276919-1A_HKJJMDSXC_L4	25438986		99.98	0.02	98.2	94.68	48.44
MP3	FKDN240276919-1A_HLY2WDSXC_L3	246286558		100	0.02	98.43	95.39	48.48
MP4	FKDN240276920-1A_22G5C3LT4_L6	413644758	592.9 Gb	100	0.01	98.82	96.39	38.33
MP4	FKDN240276920-1A_HKHJ7DSXC_L1	472408422		100	0.02	98.32	94.9	38.18
MP4	FKDN240276920-1A_22G5C3LT4_L5	359694570		100	0.01	98.94	96.69	38.28
MP4	FKDN240276920-1A_HKJJMDSXC_L2	316339216		99.98	0.02	98.08	94.31	38.12
MP4	FKDN240276920-1A_HKJJMDSXC_L4	392898928		99.98	0.02	98.22	94.65	38.12
MP4	FKDN240276920-1A_HKJJWDSXC_L3	527783098		100	0.02	98.13	94.25	38.18
MP4	FKDN240276920-1A_H7TMHDSXC_L1	288877934		100	0.03	97.13	92	38.11
MP4	FKDN240276920-1A_HLV5MDSXC_L3	513136676		100	0.02	98.12	94.54	38.13
MP4	FKDN240276920-1A_HKHC5DSXC_L2	95923616		100	0.03	97.82	93.59	38.14
MP4	FKDN240276920-1A_HKJJWDSXC_L4	572040476		100	0.03	97.97	93.88	38.21

Appendix 5.D. Positive controls

Table S.5.3. Positive control DNA mixture which was added (2 µL) instead of sample DNA extract with the target marker, concentration and taxon of origin.

Marker	Conc. (ng/µL)	Species/Genus	Family	Order	Class
COI	0.05	*Gammarus fossarum*	*Gammaridae*	Amphipoda	Malacostraca
COI	0.05	*Ephemera danica*	*Ephemeridae*	Ephemeroptera	Insecta
COI	0.05	*Epeorus assimilis*	*Heptageniidae*	Ephemeroptera	Insecta
COI	0.05	*Perlodes microcephalus*	*Perlodidae*	Plecoptera	Insecta
COI	0.05	*Ecdyonurus torrentis*	*Heptageniidae*	Ephemeroptera	Insecta
COI	0.05	*Philopotamus montanus*	*Philopotamidae*	Trichoptera	Insecta
COI	0.05	*Habroleptoides confusa*	*Leptophlebiidae*	Ephemeroptera	Insecta
COI	0.05	*Philopotamus montanus*	*Philopotamidae*	Trichoptera	Insecta
COI	0.05	*Baetis rhodani*	*Baetidae*	Ephemeroptera	Insecta
COI	0.05	*Rhithrogena semicolorata*	*Heptageniidae*	Ephemeroptera	Insecta
COI	0.05	*Rhyacophila nubila*	*Rhyacophilidae*	Trichoptera	Insecta
ITS2, rbcL, trnL	0.92	*Piper betle*	*Piperaceae*	Piperales	Magnoliopsida
ITS2, rbcL, trnL	0.32	*Vanilla planifolia*	*Orchidaceae*	Asparagales	Magnoliopsida
ITS2, rbcL, trnL	0.26	*Coffea*	*Rubiaceae*	Gentianales	Magnoliopsida
12S	0.03	*Poecilia reticulata*	*Poeciliidae*	Cyprinodontiformes	Actinopteri
COI	0.04	*Folsomia candida*	*Isotomidae*	Entomobryomorpha	Collembola
COI	0.02	*Acrotritia duplicata*	*Euphthiracaridae*	Sarcoptiformes	Arachnida

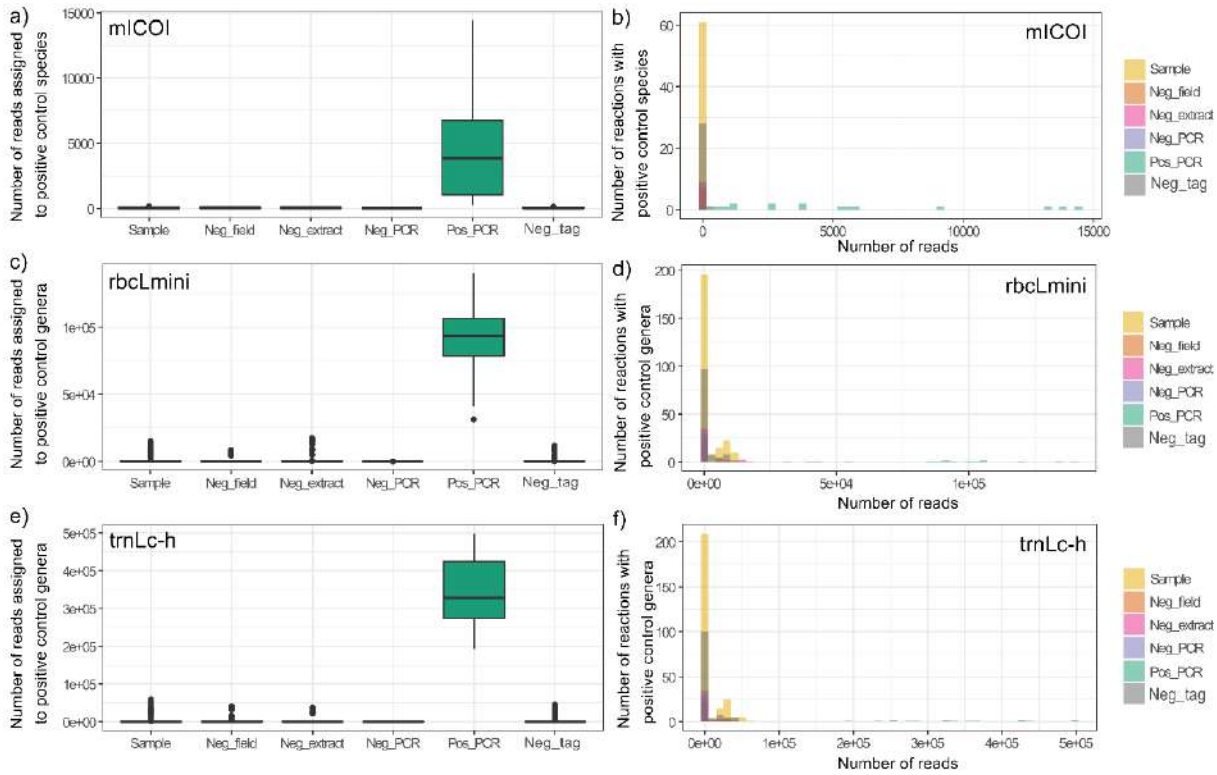


Fig. S.5.3. Detection of positive control taxa in the a,b) mlCOIintF & jgHCO2198, c,d) rbcL mini & rbcL a-R, e,f) trnL c & trnL h datasets. a,c,e) Boxplots of numbers of reads assigned to positive control taxa in PCRs of different sample and control types (negative field, negative extraction, negative PCR, positive PCR and negative tagging). b,d,f) Histograms of numbers of reads assigned to positive control taxa with colours indicating the different types of samples and controls (see legend).

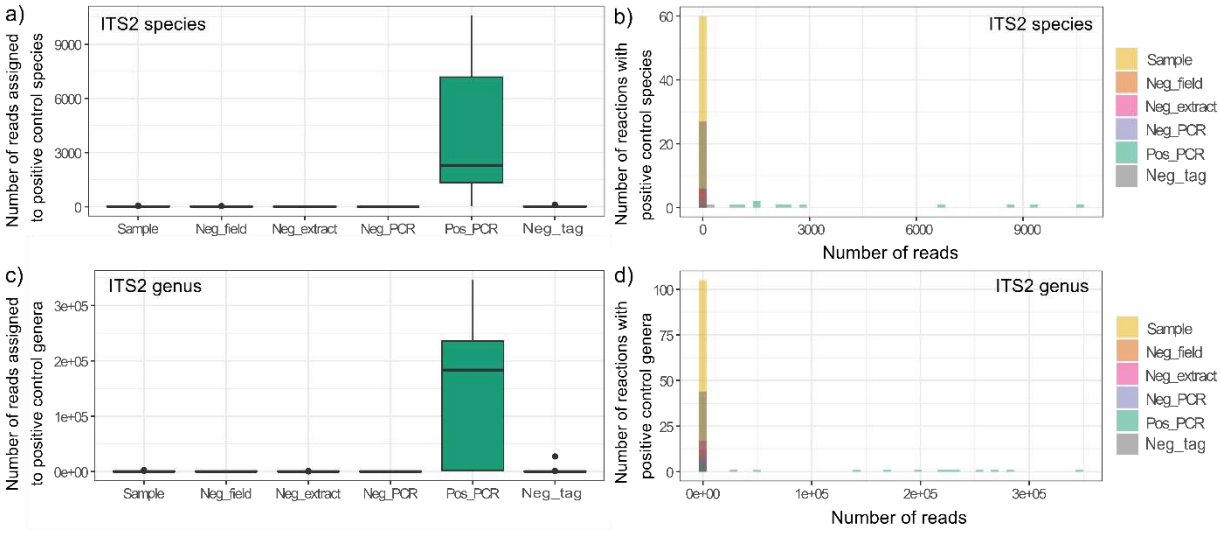


Fig. S.5.4. Detection of positive control taxa in the ITS-3p62p1F1 & ITS-4unR1 (ITS2) Operational Taxonomic Unit (OTU) Silene dataset at a,b) species, and c,d) genus level. a,c) Boxplots of numbers of reads assigned to positive control taxa in PCRs of different sample and control types (negative field, negative extraction, negative PCR, positive PCR and negative tagging). b,d) Histograms of numbers of reads assigned to positive control taxa with colours indicating the different types of samples and controls (see legend).

Appendix 5.E. Macrophytes detected with conventional methods

*Table S.5.4. Macrophyte species, genera and families detected with conventional surveys in the nine studied Silene ponds (Table 5.1). Taxa also present in the MetabaR-cleaned dataset indicated with *, and in the MetabaR- and Romahn-cleaned datasets with **.*

Species	Genus	Family
Acorus calamus	*Acorus*	*Acoraceae*
Agrostis stolonifera	*Agrostis* *	*Alismataceae* **
Alisma plantago-aquatica **	*Alisma* **	*Araceae*
Calla palustris	*Calla*	*Boraginaceae* *
Cardamine amara	*Cardamine*	*Brassicaceae* *
Carex acutiformis	*Callitriche*	*Caryophyllaceae* *
Carex dioica	*Carex* **	*Ceratophyllaceae* **
Carex lasiocarpa	*Ceratophyllum* **	*Characeae* **
Carex nigra	*Chara*	*Cyperaceae* **
Carex riparia	*Comarum* **	*Equisetaceae*
Carex rostrata	*Eleocharis*	*Hydrocharitaceae*
Carex viridula	*Epilobium* **	*Juncaceae* **
Carex vulpina	*Equisetum*	*Lamiaceae* **
Ceratophyllum submersum **	*Eriophorum*	*Lentibulariaceae* **
Chara globularis	*Galium*	*Lythraceae* *
Comarum palustre **	*Glyceria*	*Menyanthaceae*
Eleocharis palustris	*Hottonia*	*Onagraceae* **
Epilobium hirsutum	*Hydrocharis*	*Plantaginaceae* **
Epilobium palustre **	*Juncus* **	*Poaceae* **
Equisetum fluviatile	*Lemna*	*Potamogetonaceae* **
Equisetum palustre	*Lycopus* **	*Primulaceae* **
Eriophorum latifolium	*Lythrum* *	*Ranunculaceae* **
Galium palustre	*Mentha*	*Ricciaceae*
Glyceria fluitans	*Menyanthes*	*Rosaceae* **
Hottonia palustris	*Myosotis*	*Rubiaceae*
Hydrocharis morsus-ranae	*Nitella*	*Solanaceae*
Juncus acutiflorus	*Nitellopsis*	*Typhaceae*
Juncus conglomeratus	*Phalaris*	
Juncus effusus	*Phragmites*	
Lemna minor	*Potamogeton* **	
Lemna trisulca	*Ranunculus* **	
Lycopus europaeus	*Riccia*	
Lythrum salicaria *	*Schoenoplectus*	
Mentha aquatica	*Scirpus*	
Menyanthes trifoliata	*Scutellaria*	
Myosotis scorpioides	*Solanum*	
Nitella gracilis	*Sparganium*	
Nitellopsis obtusa	*Spirodela*	
Phalaris arundinacea	*Stellaria* *	
Phragmites australis	*Typha*	
Potamogeton berchtoldii	*Utricularia* **	
Potamogeton friesii	*Vallisneria*	
Potamogeton natans *	*Veronica* *	
Ranunculus flammula **		
Riccia fluitans		
Schoenoplectus lacustris		
Scirpus sylvaticus		
Scutellaria galericulata		
Solanum dulcamara		
Sparganium emersum		

Species	Genus	Family
Sparganium natans		
Spirodela polyrhiza		
Stellaria palustris		
Typha latifolia		
Utricularia minor **		
Utricularia vulgaris		
Vallisneria officinale		
Veronica beccabunga		

Appendix 5.F. ITS2 OTU cleaning and plant detection

Table S.5.5. Numbers of PCRs, total read counts, mean, minimum and maximum read counts per reaction, total OTU counts, mean, minimum and maximum read counts per reaction, in the entire dataset, after the different steps of Fig.5.2. In orange steps between OBITools pipeline and Romahn and MetabaR cleaning methods, in yellow steps in the Romahn cleaning method, in blue after the MetabaR method, in green after removal of OTUs not present in at least two PCR replicates of the same sample. From the dataset represented by the green cells the samples from Table 5.1 were selected and analysed to answer the research questions.

After step	PCR counts	Read counts				OTU counts			
		Total	Mean	Min.	Max.	Total	Mean	Min.	Max.
OBITools OTU pipeline	528	45,292,231	85,781	135	1,004,259	11,210	902	46	3,443
+ filtering taxonomy and expected length	524	29,479,120	56,258	122	953,672	3,669	282	20	1,245
+ selection Streptophyta only	524	29,357,171	56,025	115	951,051	3,555	268	20	1,198
Romahn cleaning: substr. neg. control OTU nb.	524	10,255,989	19,572	0	424,455	1,861	10	0	108
+ remove controls and rare OTUs	242	10,220,594	42,234	0	424,435	918	15	0	78
+ remove PCRs with low read numbers	178	10,217,543	57,402	1,460	424,435	901	20	2	78
+ remove PCR outliers	158	9,256,864	58,588	1,460	424,435	863	20	2	78
MetabaR cleaning: all steps	218	18,447,342	84,621	1,404	462,606	2,016	138	43	416
Romahn + removal OTUs not in 2/4 reps	145	8,003,287	55,195	12	424,412	312	11	1	49
MetabaR + removal OTUs not in 2/4 reps	215	17,272,028	80,335	242	459,297	949	72	13	173

Table S.5.6. Numbers of PCRs, total read counts, mean, minimum and maximum read counts per reaction, total OTU counts, mean, minimum and maximum read counts per reaction, in the different types of samples and controls after the OTU OBITools pipeline, and the filtering and Streptophyta selection steps.

	PCR counts	Read counts				OTU counts			
		Total	Mean	Min.	Max.	Total	Mean	Min.	Max.
Within samples	274	21,167,326	77,253	192	467,759	3,311	278	23	858
Within field neg. controls	31	389,242	12,556	4,907	41,558	1,445	218	139	479
Within extraction neg. controls	43	1,373,532	31,943	1,076	337,537	2,135	277	45	1,026
Within PCR neg. controls	32	862,486	26,953	3,500	196,643	1,594	253	137	481
Within positive controls	16	3,326,715	207,920	115	951,051	1,555	344	23	946
Within multiplex neg. controls	128	2,237,870	17,483	129	485,208	2,684	252	20	1,198

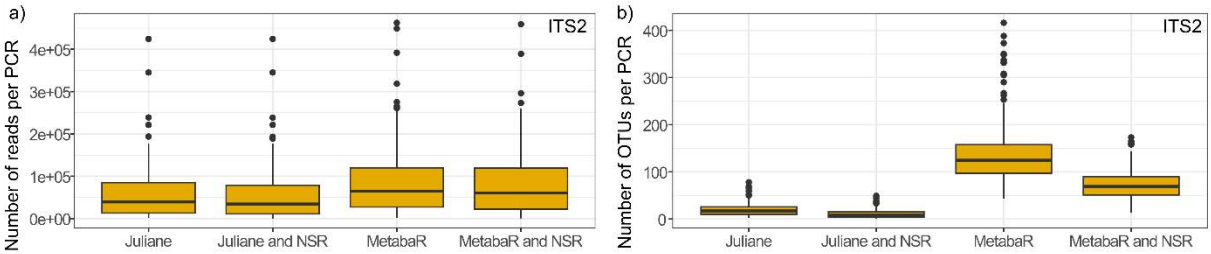


Fig. S.5.5. Boxplots of a) numbers of reads and b) Operational Taxonomic Units (OTUs) of per PCR replicates of samples in the ITS-3p62p1F1 & ITS-4unR1 (ITS2) Silene datasets before and after selecting only OTUs in at lest two PCR replicates in the Romahn and MetabaR cleaning procedures.

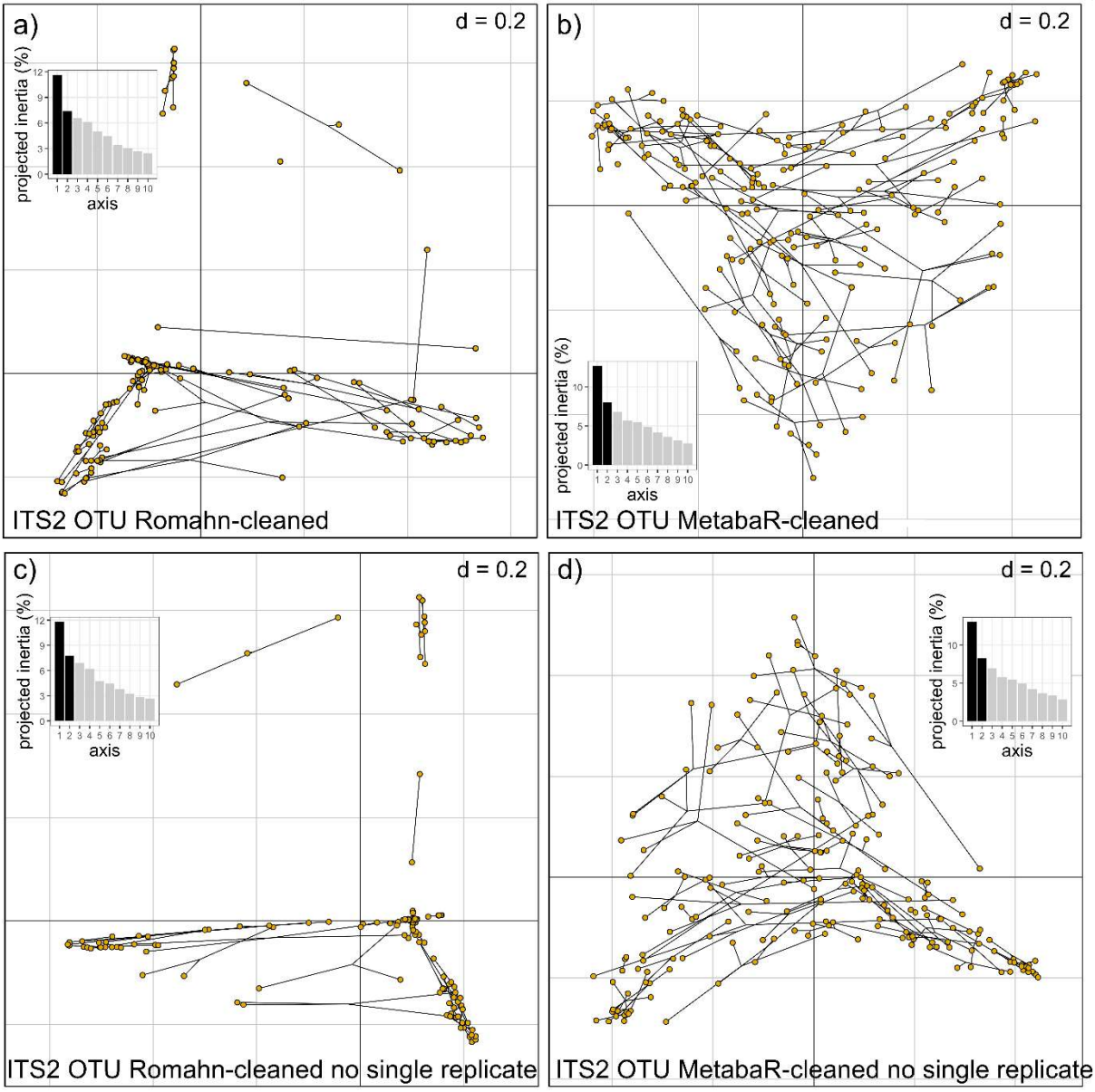


Fig. S.5.6. First two Bray-Curtis PCoA axes of ITS-3p62p1F1 & ITS-4unR1 (ITS2) Operational Taxonomic Unit (OTU) Silene communities after data cleaning using a,c) the Romahn and b,d) the MetabaR procedure. a,b) Data before and c,d) after selecting only OTUs in at lest two PCR replicates. Spider cluster PCR replicates of the same sample. Barplots of percentage projected inertia for the first ten axes in insets.

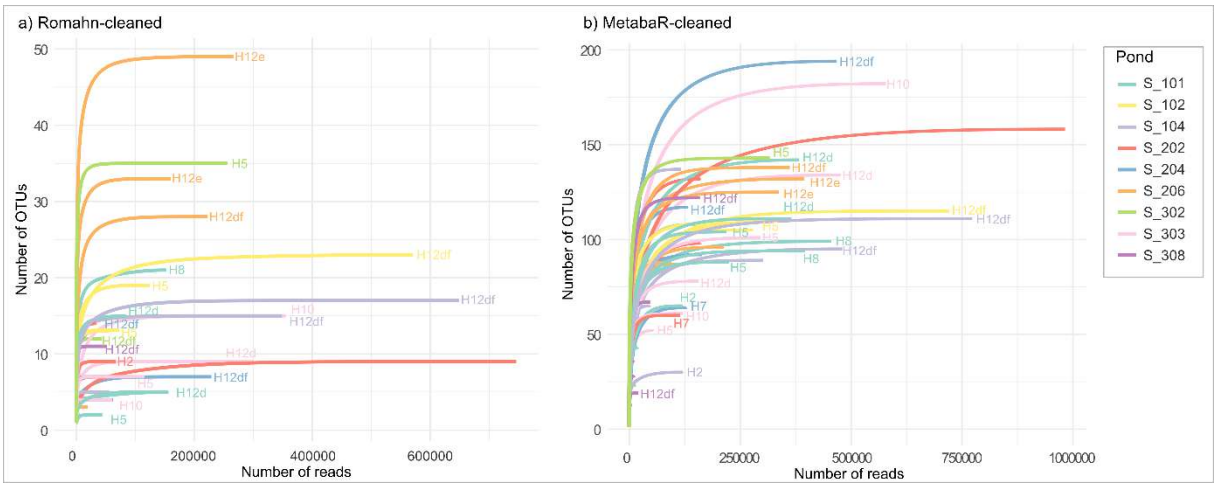


Fig. S.5.7. Rarefaction curves of the number of Operational Taxonomic Units (OTUs) per samples as function of the number of reads. Colours indicate pond identity (see legend) and text next to curves the mesohabitat from which the sample was taken, but for readability not all mesohabitat codes are given. a) Romahn- and b) MetabaR-cleaned datasets. Curves constructed with rarecurve() in vegan

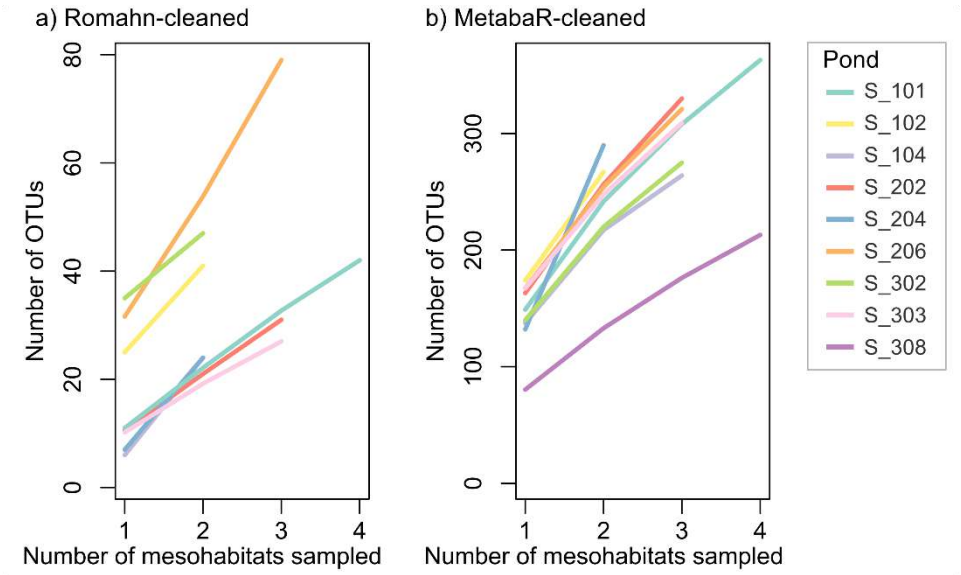


Fig. S.5.8. Operational Taxonomic Unit (OTU) accumulation curves per pond with colours indicating pond identity (see legend). Data from duplicate samples from the same mesohabitat were summed before construction of the curves. a) Romahn- and b) MetabaR-cleaned datasets. Curves constructed with specaccum(), method = random, in vegan.

Table S.5.7. Plant species detected and eDNA metabarcoding of with the ITS-3p62p1F1 & ITS-4unR1 (ITS2) primer pair in the samples of the nine studied silene ponds (Table 5.1). The proportion of the samples in which the species were detected for the Romahn-cleaned (Rom. Samp.) and MetabaR-cleaned (MbaR Samp.) datasets are given, as well as the median Ellenberg soil moisture indicator value (Ellen. F) from the Ecoflora database. The type is "exotic" for species without known occurrence in Latvia in GBIF (Global Biodiversity Information Facility, z.d.) and Plants Of the World Online (Royal Botanic Gardens, 2017), "aquatic" if Ellen. F ≥ 11, "amphibious" if 11 > Ellen. F ≥ 9, "terrestrial" if Ellen. Fs <9. For terrestrial species that are crops common names are given between brackets.

Species	Family	Rom. Samp.	MbaR Samp.	Ellen. F	Type
Acer campestre	*Sapindaceae*		27%	5	Terrestrial (tree)
Acer platanoides	*Sapindaceae*	6%	25%	5	Terrestrial (tree)
Acer pseudoplatanus	*Sapindaceae*		52%	6	Terrestrial (tree)
Aegilops speltoides	*Poaceae*		15%		Exotic
Aegopodium podagraria	*Apiaceae*		6%	6	Terrestrial
Alisma plantago-aquatica	*Alismataceae*	3%	2%	10	Amphibious
Amaranthus hybridus	*Amaranthaceae*		10%	4	Terrestrial (Amaranth)
Angelica sylvestris	*Apiaceae*		17%	8	Terrestrial
Anthriscus sylvestris	*Apiaceae*	6%	4%	5	Terrestrial
Astragalus depressus	*Fabaceae*		4%		Exotic
Bellis perennis	*Asteraceae*		15%	5	Terrestrial
Betula pendula	*Betulaceae*	11%	69%	5	Terrestrial (tree)
Bidens tripartita	*Asteraceae*		2%	8	Terrestrial
Cannabis sativa	*Cannabaceae*	3%	2%	6	Terrestrial (Hemp)
Carex stipata	*Cyperaceae*	3%	2%		Exotic
Carpinus betulus	*Betulaceae*		88%	5	Terrestrial (tree)
Carpinus fargesiana	*Betulaceae*		35%		Exotic
Centaurea scabiosa	*Asteraceae*	6%	8%	3	Terrestrial
Ceratophyllum demersum	*Ceratophyllaceae*	3%	13%	12	Aquatic
Cicer arietinum	*Fabaceae*		2%		Terrestrial (Chickpea)
Cicuta virosa	*Apiaceae*	3%	2%	9	Amphibious
Clematis vitalba	*Ranunculaceae*		10%	5	Terrestrial
Comarum palustre	*Rosaceae*	17%	73%	9	Amphibious
Corylus avellana	*Betulaceae*	3%	46%	5	Terrestrial
Daucus carota	*Apiaceae*	3%	8%	4	Terrestrial
Dioscorea polystachya	*Dioscoreaceae*		25%		Exotic
Echium vulgare	*Boraginaceae*		4%	4	Terrestrial
Epilobium palustre	*Onagraceae*	3%	33%	9	Amphibious
Eragrostis barrelieri	*Poaceae*		10%		Exotic
Erigeron canadensis	*Asteraceae*		8%	4	Terrestrial
Fagus sylvatica	*Fagaceae*		85%	5	Terrestrial
Filipendula ulmaria	*Rosaceae*	6%	33%	8	Terrestrial
Frangula alnus	*Rhamnaceae*		4%	8	Terrestrial
Glycine max	*Fabaceae*		4%		Terrestrial (Soybean)
Hedera helix	*Araliaceae*		56%	5	Terrestrial
Helianthus annuus	*Asteraceae*		4%		Terrestrial
Hippuris vulgaris	*Plantaginaceae*		44%	10	Amphibious
Holcus lanatus	*Poaceae*		17%	6	Terrestrial
Hordeum vulgare	*Poaceae*		2%		Terrestrial (Barley)
Humulus lupulus	*Cannabaceae*		23%	7	Terrestrial
Impatiens glandulifera	*Balsaminaceae*		29%	8	Terrestrial
Jacobaea vulgaris	*Asteraceae*	3%	2%	4	Terrestrial
Juglans hopeiensis	*Juglandaceae*		48%		Exotic
Juncus articulatus	*Juncaceae*	3%	2%	9	Amphibious
Lysimachia vulgaris	*Primulaceae*	8%	31%	8	Terrestrial

Species	Family	Rom. Samp.	MbaR Samp.	Ellen. F	Type
Lythrum salicaria	*Lythraceae*		25%	9	Amphibious
Medicago lupulina	*Fabaceae*		8%	4	Terrestrial
Myriophyllum spicatum	*Haloragaceae*		98%	12	Aquatic
Poa trivialis	*Poaceae*		31%	7	Terrestrial
Potamogeton natans	*Potamogetonaceae*		4%	11	Aquatic
Potamogeton nodosus	*Potamogetonaceae*		77%	12	Aquatic
Potentilla pensylvanica	*Rosaceae*		2%		Exotic
Prunus avium	*Rosaceae*		25%	5	Terrestrial
Prunus padus	*Rosaceae*	3%	65%	8	Terrestrial
Pteris vittata	*Pteridaceae*		38%		Exotic
Ranunculus bulbosus	*Ranunculaceae*	3%	12%	4	Terrestrial
Ranunculus flammula	*Ranunculaceae*	3%	42%	9	Amphibious
Robinia pseudoacacia	*Fabaceae*		48%	4	Terrestrial
Rorippa islandica	*Brassicaceae*		6%		Amphibious
Rosa canina	*Rosaceae*		52%	4	Terrestrial
Rubus idaeus	*Rosaceae*		17%	5	Terrestrial
Salvia moorcroftiana	*Lamiaceae*		8%		Exotic
Sambucus nigra	*Adoxaceae*		19%	5	Terrestrial
Securigera varia	*Fabaceae*	3%	67%		Terrestrial
Solidago gigantea	*Asteraceae*		29%	6	Terrestrial
Stellaria neglecta	*Caryophyllaceae*		8%	6	Terrestrial
Symphytum officinale	*Boraginaceae*		12%	7	Terrestrial
Syringa vulgaris	*Oleaceae*		2%	4	Terrestrial
Tripleurospermum inodorum	*Asteraceae*		4%	5	Terrestrial
Urtica dioica	*Urticaceae*	36%	77%	6	Terrestrial
Utricularia australis	*Lentibulariaceae*		65%	12	Aquatic
Utricularia minor	*Lentibulariaceae*	3%	2%	12	Aquatic
Viburnum prunifolium	*Adoxaceae*		4%		Exotic
Vicia faba	*Fabaceae*		4%	5	Terrestrial (Broadbean)
Vicia hirsuta	*Fabaceae*		4%	4	Terrestrial

Table.S.5.8. ITS2 OTUs assigned to genus level in the samples of Table 5.1 with the proportion of the samples in which the species were detected for the Romahn-cleaned (Rom. Samp.) and MetabaR-cleaned (MbaR Samp.) datasets.

Genus	Family	Rom. Samp.	MbaR Samp.
Acer	*Sapindaceae*	8%	17%
Agrostis	*Poaceae*		4%
Alnus	*Betulaceae*	25%	85%
Artemisia	*Asteraceae*	3%	12%
Betula	*Betulaceae*	33%	69%
Brassica	*Brassicaceae*		12%
Calamagrostis	*Poaceae*	3%	52%
Campeiostachys	*Poaceae*	3%	4%
Carex	*Cyperaceae*	3%	8%
Carpinus	*Betulaceae*		60%
Cirsium	*Asteraceae*	3%	8%
Comarum	*Rosaceae*	19%	29%
Drepanocladus	*Amblystegiaceae*	3%	2%
Epilobium	*Onagraceae*	3%	13%
Eragrostis	*Poaceae*		2%
Erythranthe	*Phrymaceae*	3%	23%
Fagus	*Fagaceae*		2%
Festuca	*Poaceae*		2%
Filipendula	*Rosaceae*		8%
Fraxinus	*Oleaceae*		46%
Geum	*Rosaceae*		8%
Impatiens	*Balsaminaceae*		19%
Jacobaea	*Asteraceae*		2%
Knautia	*Caprifoliaceae*		2%
Linaria	*Plantaginaceae*		6%
Lolium	*Poaceae*		29%
Lotus	*Fabaceae*	3%	27%
Lycopus	*Lamiaceae*	6%	8%
Melilotus	*Fabaceae*		8%
Myriophyllum	*Haloragaceae*		90%
Parietaria	*Urticaceae*		4%
Pinus	*Pinaceae*		8%
Piper	*Piperaceae*		6%
Populus	*Salicaceae*		6%
Potamogeton	*Potamogetonaceae*	22%	96%
Potentilla	*Rosaceae*		10%
Prunus	*Rosaceae*		31%
Quercus	*Fagaceae*		31%
Ranunculus	*Ranunculaceae*	6%	31%
Rosa	*Rosaceae*		13%
Rubus	*Rosaceae*	6%	71%
Salix	*Salicaceae*	11%	90%
Securigera	*Fabaceae*	3%	4%
Solidago	*Asteraceae*		6%
Sonchus	*Asteraceae*		2%
Stellaria	*Caryophyllaceae*		2%
Stuckenia	*Potamogetonaceae*		63%
Symphytum	*Boraginaceae*		4%
Tilia	*Malvaceae*	6%	10%
Trifolium	*Fabaceae*		21%
Tripleurospermum	*Asteraceae*		13%
Triticum	*Poaceae*		4%
Urtica	*Urticaceae*		2%
Veronica	*Plantaginaceae*		79%
Vicia	*Fabaceae*	3%	12%

Table S.5.9. ITS2 OTUs assigned to family level in the samples of Table 5.1, with the proportion of the samples in which the species were detected for the Romahn-cleaned (Juli. Samp.) and MetabaR-cleaned (MbaR Samp.) datasets.

Family	Rom. Samp.	MbaR Samp.
Apiaceae	8%	8%
Asparagaceae	3%	4%
Asteraceae	11%	50%
Betulaceae	36%	77%
Brassicaceae		2%
Bryaceae		2%
Ceratophyllaceae		2%
Characeae	14%	17%
Ericaceae	3%	10%
Fabaceae		42%
Fagaceae		33%
Haloragaceae		13%
Lamiaceae		2%
Lentibulariaceae	11%	37%
Malvaceae		2%
Nymphaeaceae		65%
Onagraceae		2%
Orobanchaceae		4%
Phrymaceae	6%	12%
Piperaceae		2%
Plantaginaceae	8%	19%
Poaceae	19%	62%
Potamogetonaceae		42%
Primulaceae		2%
Ranunculaceae	3%	4%
Rosaceae	28%	50%
Salicaceae	8%	12%
Sapindaceae	3%	4%
Urticaceae	6%	25%

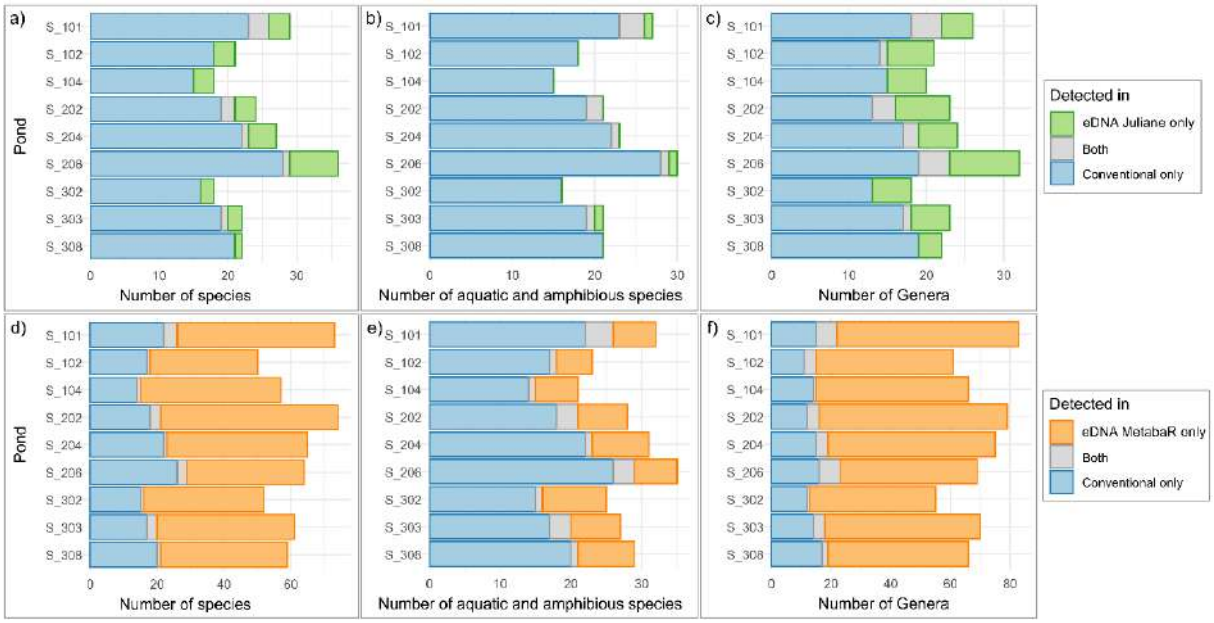


Fig. S.5.9. Number of taxa detected in each pond with conventional (blue), both conventional and eDNA (grey) and eDNA methods with a-c) Romahn (green "Juliane") and d-f) MetabaR (orange) data cleaning. a,d) species, d,e) aquatic and amphibious species, c,f) genera.

Table S.5.10. OTUs in the Romahn- and MetabaR-cleaned datasets assigned to crop species, species without known occurrence in Latvia and aquatic and amphibian species not detected with the conventional methods in the nine Silene ponds. For each OTU the NCBI GenBank accession number of the sequence it is most similar to (Best_match) and the similarity to this sequence (Best_id) is given as well as the proportion of reads in the dataset that belong to this OTU (Rel.Rds) and the percentage of samples in which the OTU is detected (%Smpl) are given. The Romahn-cleaned dataset consisted of 36 samples and 5.6 million reads and the MetabaR-cleaned dataset of 52 samples and 12.1 million reads.

				Romahn cleaned		MetabaR cleaned	
Species	OTU code	Best_match	Best_id	Rel.Rds	%Smpl	Rel.Rds	%Smpl
Crop species							
Cannabis sativa	OTU_010925	KC292629	100%	4E-4	3%	2E-4	2%
Amaranthus hybridus	OTU_007554	MH547548	100%			3E-6	10%
Cicer arietinum	OTU_009061	JN617194	100%			1E-6	2%
Glycine max	OTU_008632	EU288921	100%			1E-6	4%
Vicia faba	OTU_005068	FJ212318	100%			1E-6	4%
Hordeum vulgare	OTU_000390	FJ593180	97%			5E-7	2%
Species without known occurrence in Latvia							
Pteris vittata	OTU_006618	AM920401	97%			2E-7	2%
Pteris vittata	OTU_001241	HM559424	100%			7E-4	38%
Salvia moorcroftiana	OTU_011736	KP294358	97%			1E-6	8%
Eragrostis barrelieri	OTU_002480	KX282124	100%			8E-5	10%
Carpinus fargesiana	OTU_001074	MG923676	99%			1E-4	35%
Viburnum prunifolium	OTU_001828	KY860928	97%			1E-5	4%
Carex stipata	OTU_004875	MF669178	97%	7E-6	3%	3E-6	2%
Juglans hopeiensis	OTU_002143	KY652952	100%			1E-4	48%
Astragalus depressus	OTU_008989	KX954932	99%			1E-6	4%
Aegilops speltoides	OTU_008144	MF480406	99%			1E-4	15%
Potentilla pensylvanica	OTU_008756	MF543800	99%			6E-7	2%
Dioscorea polystachya	OTU_003132	FJ860072	100%			2E-3	25%
Aquatic and amphibian species not detected with conventional methods							
Rorippa islandica	OTU_010969	MN637875	98%			3E-6	6%
Utricularia australis	OTU_004085	MF543812	100%			4E-4	62%
Utricularia australis	OTU_011418	MF543812	96%			2E-7	2%
Utricularia australis	OTU_005782	MF543812	97%			5E-5	35%
Myriophyllum spicatum	OTU_007178	FJ426346	100%			2E-2	98%
Juncus articulatus	OTU_011128	MT796520	100%	4E-7	3%	7E-7	2%
Hippuris vulgaris	OTU_008095	OK523402	100%			2E-4	42%
Hippuris vulgaris	OTU_002989	OK523402	97%			1E-5	21%
Ceratophyllum demersum	OTU_005198	KM582604	100%	1E-3	3%	2E-3	13%
Ceratophyllum demersum	OTU_006938	KM582604	97%			1E-6	2%
Cicuta virosa	OTU_006240	MT784102	100%	2E-5	3%	1E-5	2%
Potamogeton nodosus	OTU_002379	LC374647	100%			2E-2	77%

Table S.5.11. Presence of OTUs of Table S.5.11 (for brevity only indicated by the assigned species name, but in the same order as in Table S.5.11) in data from sample, negative field, negative extraction, engative PCR, positive PCR and multiplex negative PCR control PCRs before cleaning with the Romahn and MetabaR cleaning methods. For each type of sample or control the proportion of reads that belong to this OTU (Rel.Rd) and the percentage of PCRs in which the OTU is detected (%PCR) are given.

	Sample		Negative field		Neg. Extract.		Neg. PCR		Positive PCR		Mulitplex neg	
Species	Rel.Rd	%PCR	Rel.Rd	%PCR	Rel.Rd	%PCR	Rel.Rd	%PCR	Rel.Rd	%PCR	Rel.Rd	%PCR
Crop species												
Cannabis sativa	1E-4	8%	5E-6	3%	5E-5	12%	1E-6	3%	2E-6	13%	3E-5	11%
Amaranthus hybridus	2E-5	20%	2E-4	23%	4E-5	19%	3E-6	6%	5E-5	25%	7E-5	18%
Cicer arietinum	2E-6	4%	2E-5	6%	3E-6	5%			2E-6	13%	5E-6	3%
Glycine max	3E-5	18%	3E-4	13%	1E-5	14%	4E-4	16%	8E-6	19%	2E-4	20%
Vicia faba	4E-6	10%			3E-5	12%			2E-5	19%	1E-5	9%
Hordeum vulgare	6E-7	2%			1E-6	2%					1E-6	2%
Species without known occurrence in Latvia												
Pteris vittata	6E-7	1%									9E-7	1%
Pteris vittata	5E-4	73%	3E-4	77%	3E-4	70%	4E-4	66%	6E-5	81%	3E-4	65%
Salvia moorcroftiana	7E-6	14%	2E-5	16%	2E-5	16%	3E-6	6%	3E-6	19%	2E-5	13%
Eragrostis barrelieri	1E-4	30%	1E-3	23%	3E-4	21%	1E-5	25%	3E-4	50%	6E-4	38%
Carpinus fargesiana	1E-4	53%	3E-4	35%	3E-4	56%	7E-5	41%	3E-5	44%	4E-4	45%
Viburnum prunifolium	6E-5	19%	1E-4	13%	6E-5	14%	2E-4	31%	4E-4	19%	3E-4	19%
Carex stipata	2E-6	1%										
Juglans hopeiensis	2E-4	72%	6E-4	74%	5E-4	70%	4E-4	75%	3E-4	75%	6E-4	73%
Astragalus depressus	8E-6	11%	8E-6	3%	7E-6	2%	8E-6	9%	3E-7	6%	2E-5	8%
Aegilops speltoides	7E-4	51%	9E-3	42%	5E-4	51%	3E-4	31%	3E-4	81%	8E-4	48%
Potentilla pensylvanica	1E-5	13%	9E-5	23%	2E-5	12%			5E-5	44%	5E-5	15%
Dioscorea polystachya	7E-3	82%	3E-2	68%	3E-3	86%	2E-3	94%	3E-3	100%	5E-3	85%
Aquatic and amphibian species not detected with conventional methods												
Rorippa islandica	6E-6	10%	2E-5	10%	4E-6	5%	3E-5	28%	6E-7	13%	1E-5	9%
Utricularia australis	7E-4	85%	2E-3	74%	4E-3	86%	3E-3	69%	3E-4	94%	3E-3	77%
Utricularia australis	3E-6	12%	1E-5	6%	9E-6	16%	1E-6	3%	3E-6	25%	1E-5	12%
Utricularia australis	2E-4	67%	8E-4	48%	6E-4	60%	3E-4	53%	7E-5	69%	7E-4	60%
Myriophyllum spicatum	2E-2	100%	8E-2	100%	3E-2	100%	7E-2	100%	1E-2	94%	8E-2	100%
Juncus articulatus	1E-6	4%					1E-6	3%			4E-6	4%
Hippuris vulgaris	3E-4	57%	1E-3	74%	9E-4	67%	1E-3	84%	2E-4	50%	1E-3	62%
Hippuris vulgaris	1E-5	23%	6E-5	26%	3E-5	26%	9E-6	9%	9E-7	19%	4E-5	20%
Ceratophyllum demersum	1E-3	71%	2E-4	61%	1E-3	65%	7E-4	59%	7E-5	69%	3E-4	66%
Ceratophyllum demersum	9E-7	3%										
Cicuta virosa	7E-6	3%	3E-6	3%	7E-7	2%			1E-6	6%		
Potamogeton nodosus	2E-2	99%	1E-1	100%	4E-2	100%	5E-2	100%	1E-2	100%	8E-2	100%

Appendix 6.A. Similarity environmental variables and macrophyte communities according to dispersal vector with geographic distance

The environmental dissimilarity between pond in terms of environmental conditions was calculated by performing a Principal Component Analysis (PCA) of the environmental variables described in **Chapter 3**, except for the distance to the nearest waterbody. For Neu-Woerr, the first PCA axis projected 38% of the inertia and the second axis 22%, for Silene the first and second axis projected 48% and 19% respectively. The environmental distance (Edis) was calculated as the Euclidean distance between ponds in the PCA's. The environmental similarity (1-Edis/max(Edis)) was plotted (*Fig. S.6.1*) The Mantel correlation between Edis and the geographic distance between ponds was calculated using the Spearman rank correlation coefficient and tested (α = 0.05) with 999 permutations using function mantel() in vegan. For Neu-Woerr the correlation between the environmental dissimilarity and distance between ponds was not significant, but for Silene there was a trend (*Table S.6.1*). For Silene, ponds closer than the halving distance (300 m, the distance at which the similarity between macrophyte community composition was half the original similarity, **Chapter 3**) were significantly more similar in environmental conditions than ponds further apart (Wilcoxon rank sum test, W = 291, p-value = 0.006).

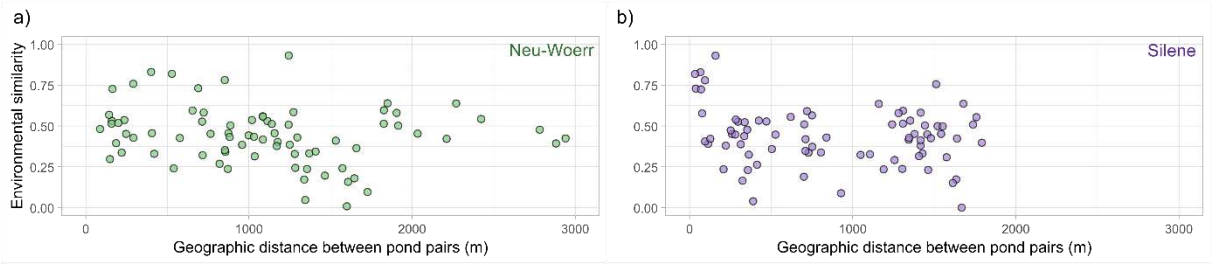


Fig. S.6.1. Environmental similarity as function of geographic distance between ponds for a) Neu-Woerr and b) Silene

Table S.6.1. Mantel tests of the correlation between environmental dissimilarity and geographic distance between ponds based on Spearman's rank correlation coefficient

	Mantel statistic	**p-value**
Neu-Woerr	0.19	0.16
Silene	0.18	0.052

The dispersal vectors of the propagules of the vascular macrophytes observed in Neu-Woerr and Silene were retrieved from Muller et al., 2021; Schou et al., 2023 and the CATMINAT database (Julve, 2025). Dispersal traits were available for all vascular plants except for *Eleocharis palustris*, *Eleocharis uniglumis* and *Stellaria palustris.* Presence or absence of the dispersal vectors wind, water and animals (including humans) was crisp coded (1 if present, 0 if not present in a species). Plants could have multiple dispersal vectors. The Bray-Curtis similarity (1 – dissimilarity) was calculated separately for macrophytes that could be dispersed by wind, water and animals. This was done on log(x+1) transformed percentage cover with the vegdist() function in vegan. The resulting similarities were plotted as function of the geographical distance between ponds (*Fig. S.6.2*). The Mantel spearman rank correlation between the Bray-Curtis dissimilarity and distance between ponds was calculated for macrophytes of each dispersal vector as above. For Neu-Woerr there was no significant (α = 0.05) correlation between the similarity and distance between ponds for any of the dispersal forms. For Silene the correlation was significant for the water and animal dispersed

macrophytes (*Table S.6.2*). A fuzzy Correspondence Analysis was performed on the dispersal vector and the life form for each site with the ade4 package. For both sites submerged plants were generally dispersed by water and animals while emergent plants were generally wind dispersed (*Fig S.6.3*).

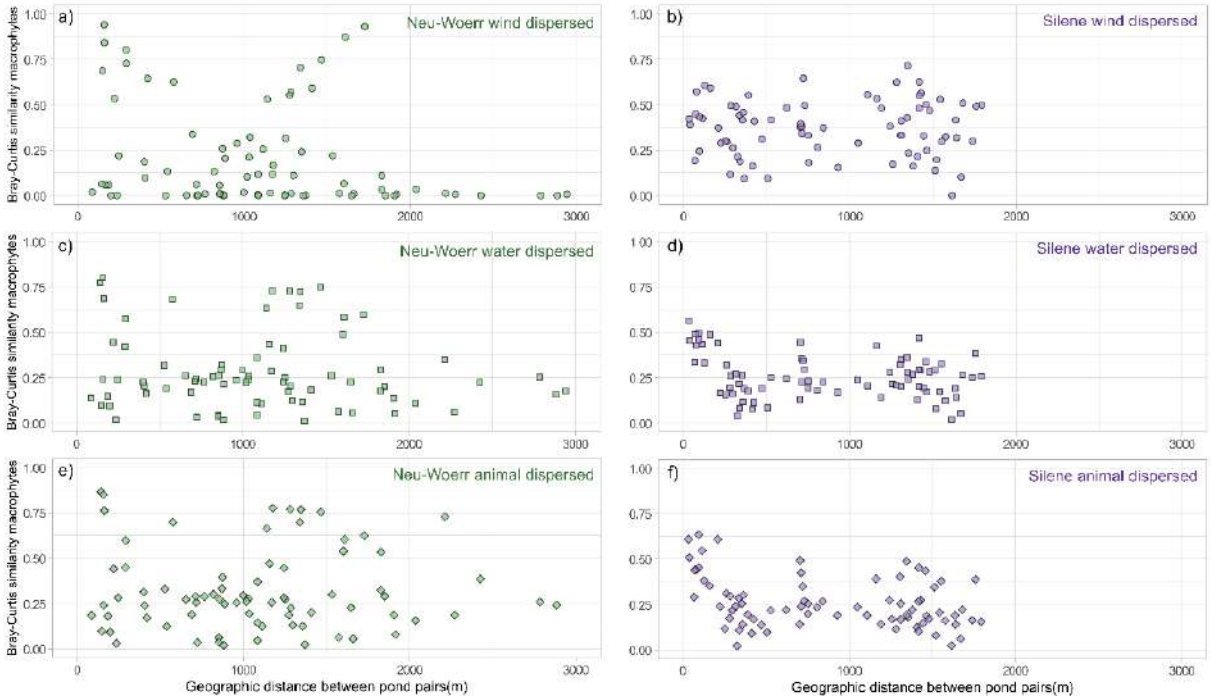


Fig. S.6.2. Bray-Curtis similarity as function of geographic distance between ponds for a,b) wind dispersed, c,d) water dispersed, e,f) animal dispersed macrophytes in a,c,e,) Neu-Woerr and b,d,f) Silene ponds networks.

Table S.6.2. Mantel tests of the correlation between macrophyte Bray-Curtis dissimilarity and geographic distance between ponds based on Spearman's rank correlation coefficient.

	Mantel statistic	**p-value**
Neu-Woerr wind dispersed	0.24	0.1
Neu-Woerr water dispersed	0.08	0.2
Neu-Woerr animal dispersed	-0.02	0.5
Silene wind dispersed	0.00	0.4
Silene water dispersed	0.23	0.03
Silene animal dispersed	0.36	0.005

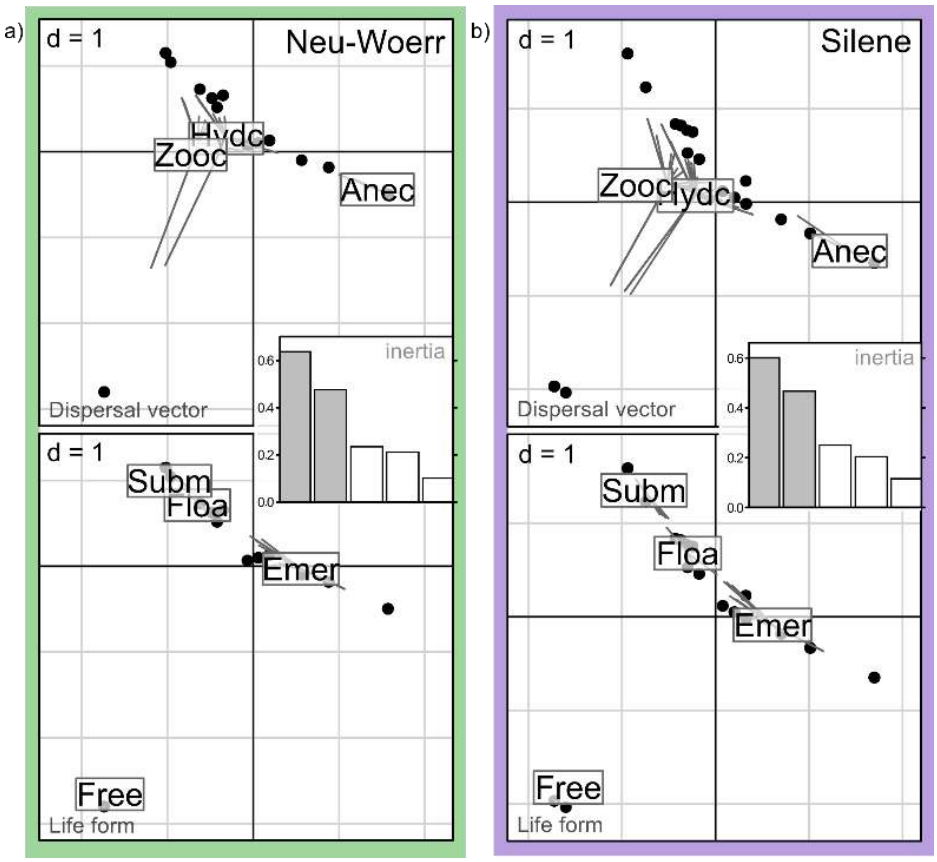


Fig. S.6.3. Fuzzy Correspondence Analysis axes 1 and 2 of dispersal vectors (upper panels, with Anec for wind, Hydc for water and Zooc for animal dispersed) and life forms (lower panels, with Subm for submerged, Floa for floating anchored, Emer for emergent and Free for free floating) of macrophytes in a) Neu-Woerr b) Silene. Inertia bar plots in insets.

Appendix 6.B. Time estimate of pond monitoring techniques

Table S.6.3 shows a very rough estimate of the time it took to perform the pond monitoring in the Silene site in 2022. For eDNA the estimates are for the seven primer pairs, targeting plants, invertebrates and vertebrates, together. It must be noticed that some eDNA steps were performed for all samples of *Table S.5.1* at once and that the time needed for some steps (e.g. reference database construction) does not depend on the number of samples. The estimate is for trained operators using established protocols (i.e. it does not include the time needed to optimize the eDNA extraction or PCR conditions). It does not include preparation (e.g. eDNA sampling material) or waiting time (e.g. for sequencing or bioinformatic processing). The monitored ponds were relatively small (surface area 70 to 1050 m^2) and easy to access, and no boat was used for the surveys and samplings. A pipetting robot was used to prepare the PCRs. In this case the macrophyte survey took the least amount of time per pond and the macroinvertebrate sampling and identification the most. This estimate is however not representative for pond monitoring in general, because many variables could influence the time needed. For example, the taxonomic richness of the pond can influence the time needed for identification and reference databases do not need to be constructed for each eDNA metabarcoding project.

*Table S.6.3. Time estimates for pond monitoring based on the Silene sampling campaign of 2022. Steps for which the time does not depend on the number of samples indicated with **

Task	Number of operators	Number of waterbodies	Total time (days)	Operator days per pond
Survey macrophytes	2	13	3.5	0.5
Identify macrophytes in the lab	1		1.5	0.1
Total macrophyte survey				0.7
Sample macroinvertebrates	2	10	2.5	0.5
Sort and identify macroinvertebrates	1		30	3.5
Total macroinvertebrate sampling and identification				4
Sample eDNA	2	9	2.5	0.6
Extract eDNA	1		5	0.6
PCR and library preparation	1	47	10	0.3
Quality control extraction and PCR	1		7	0.1
Bioinformatics * only for macrophytes	1		5	0.1
Reference database construction *	1		10	0.2
Data cleaning *	1	9	3	0.3
Total eDNA metabarcoding				2.1

Appendix 6.C. Minimal monitoring protocol for human-made ponds

This is a minimal monitoring protocol for pond networks created with the aim to increase freshwater biodiversity, and with the following targets:

- A quantified percentage of the total percentage of the pond network should be covered by macrophytes
- The pond network should have multiple life forms of macrophytes and their

This protocol can be carried out by operators without taxonomic training. It allows assessment against the targets as well as two variables, shade by surrounding trees and water transparency, that could impact the targets. The protocol should be performed in the macrophyte growing season (generally April-October, but dependent on the region), when the pond is not dry (> 50 % of the surface area covered with water) and when it does not rain. The pond surface area is delineated by the highest water level in normal years (without flood).

- Required equipment :
    - Notebook
    - Bucket
    - Pond Secchi tube
    - For large ponds : waders or a boat
    - For turbid ponds : rake or grapple

The operator walks around the pond, and when the pond is too large to observe from the shore, wade into the pond or use a boat. When waders or a boat are used, for safety reasons, at least two operators should do the assessment. The operators:

1) Estimate the percentage of the pond surface area that is covered by macrophytes
2) Estimate the percentage of the pond surface area that is covered by
    - Emergent macrophytes
    - Free floating macrophytes
    - Anchored floating leaved macrophytes
    - Submerged macrophytes
3) Estimate the percentage of the pond surface area that would be shaded by surrounding trees at midday
4) Collect surface water in a bucket and measure the transparency with a Secchi tube

If the pond is turbid and the bottom cannot be seen, a rake or grapple can be used to sample the pond. Starting from the point nearest to the shore towards the middle of the pond, every few metres the end of rake or grapple can be approached to the bottom to see if plants are growing on the bottom.

The visual aid in *Fig. S.6.4* can be used to estimate the cover percentages.

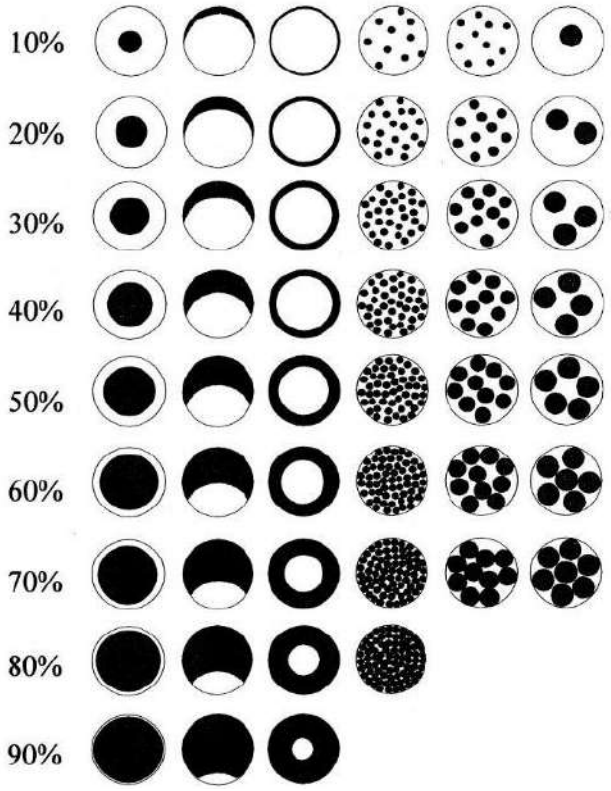


Fig. S.6.4. Visual aid for estimating percentage coverage. Figure retrieved from Ward & Wilkinson (2019).

Contributions

Chapter 3

The macrophyte surveys were performed by Isabelle Combroux, Jeanne Ricardo and Karina van der Zon, with the help of Corinne Grac. All fieldwork in Silene was assisted by Jana Paidere and Aija Brakovska, who also measured the environmental parameters. Fieldwork in Silene would not have been possible without the help of Mihails Pupiņš and Artūrs Škute. Fieldwork in Neu-Woerr was made possible by Uwe Meissner and Lydia Razafindralay. Chlorophyll-a concentrations were measured by Marie-Pierre Ottermatte. Crayfish data was collected by Marion Schaffner and Corinne Grac. Data curation was performed by Corinne Grac, Jana Paidere and Karina van der Zon.

Chapter 4

Invertebrate sampling was preformed by Corinne Grac and Karina van der Zon. All fieldwork in Silene was assisted by Jana Paidere and Aija Brakovska, who also measured the environmental parameters. Fieldwork in Silene would not have been possible without the help of Mihails Pupiņš and Artūrs Škute. Water chemistry analyses were performed by Marie-Pierre Ottermatte, Sergejs Osipovs and Aleksandrs Pučkins. Jeanne Ricardo, Frédéric Labat and Karina van der Zon identified the macroinvertebrates described in this thesis, with help from Jean-Michel Bichain, Etienne Chanez and Corinne Grac. Data curation was performed by Corinne Grac, Jana Paidere and Karina van der Zon.

Chapter 5

Environmental DNA sampling was performed Karina van der Zon, with the help of Corinne Grac. The macrophyte surveys were performed by Isabelle Combroux and Karina van der Zon. Fieldwork was made possible by Jana Paidere, Aija Brakovska, Mihails Pupiņš and Artūrs Škute. All eDNA work was performed in the laboratories of Miklós Bálint. Karina van der Zon optimized the eDNA extraction protocol, with the help of Leonie Schardt, Kathrin Theissinger and Corentin Fournier, and extracted the eDNA from the samples described in this thesis. Testing of eDNA primers and PCR conditions was performed by Melina Werner. Amplification of eDNA was done by Damian Baranski and Karina van der Zon with help of Leonie Schardt and Johannes Meka. Damian Baranski pooled the amplicons and sent them to the sequencing company. The reference database was created by Armando Espinosa Prieto, Juliane Romahn, Melina Werner and Karina van der Zon. Bioinformatic processing of the eDNA data was performed by Juliane Romahn. Juliane Romahn provided the data cleaning script.

Publications by the author

Roessink, I., **Van der Zon, K. A. E**., De Reus, S. R. M. M., & Peeters, E. T. H. M. (2022). Native European crayfish *Astacus astacus* competitive in staged confrontation with the invasive crayfish *Faxonius limosus* and *Procambarus acutus*. *PLoS One*, *17*(1), e0263133.

Čeirāns, A., Pupins, M., Skute, A., Nekrasova, O., Kirjusina, M., Combroux, I., Grac, C., Kvach, Y., **Van der Zon, K. A. E.**, Theissinger, K. & Georges, J. Y. (2024). Identification and use of suitable metrics for calling male count-based community assessments in amphibian monitoring in temperate Europe. *Ecological Indicators*, *168*, 112771.

Van der Zon, K. A. E., & Schallenberg, M. (2025). Patterns of nutrient limitation of algal productivity from headwaters to estuary. *Inland waters*, 1-14.

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Werner, M. J., Meka, J., **Van der Zon, K. A. E.**, Romahn, J., Espinosa Prieto, A., Bálint, M., Baranksi, D., Schardt, L., Georges, J.-Y., Theissinger, K., (Submitted). Passive eDNA sampling as a promising tool for standardizing wetland biodiversity assessments.

Oral presentations

Van der Zon, K. A. E., Grac, C., Georges, J.-Y., Meka, J., Pupins, M., Skute, A., Theissinger, K., Combroux, I. “Man-made pond networks as a restoration measure for wetland biodiversity”. Jahrestagung der Deutschen Gesellschaft für Limnologie, 19-23 September 2022, Konstanz, Germany

Van der Zon, K. A. E., Grac, C., Georges, J. Y., Pupins, M., Skute, A., Theissinger, K., Combroux, I. “Macrophyte habitat structure and biodiversity patterns in conservation pond networks”. Colloque international des mares ADREE-GHZH-SNPN-NaturAgora, 20-22 October 2022, Laon (Aisne), France

Van der Zon, K. A. E. “Environmental DNA”. River University, 31 July-4 August 2023, Kutzow See, Germany

Van der Zon, K. A. E. “Macrophyte communities in man-made pond networks”. PhD Congress ED413, 20 March 2024, Strasbourg, France

Van der Zon K. A. E., Georges, J.-Y., Grac, C., Pupins, M., Razafindralay, L., Skute, A., Theissinger, K., Combroux, I. “Wetland plants in pond networks: the same restorative action leading to different outcomes in two locations”. 14th European conference on ecological restoration, 26-30 August 2024, Tartu, Estonia

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Ponds for biodiversity and conservation: context, design and evaluation of restorative measures in Europe

Abstract

Creating pond networks could enhance freshwater biodiversity and support species conservation. Pond networks have been implemented in Europe and will be promoted by recent initiatives such as the EU Nature Restoration Regulation. However, guidance is lacking on how to effectively design, monitor, evaluate and manage these systems. To address these gaps, this PhD investigated the environmental and spatial factors structuring macrophyte and macroinvertebrate communities in human-made ponds, and suitable monitoring methods. Two pond networks were studied: Neu-Woerr (France/Germany) and Silene (Latvia). Silene showed high macrophyte diversity, while Neu-Woerr had low diversity, likely due to invasive crayfish. Macrophyte and macroinvertebrate community composition was linked to variables affecting light (shade from surrounding trees and water transparency), and for macroinvertebrates also to oxygen levels. Environmental DNA metabarcoding was tested for the detection of macrophytes, macroinvertebrates, amphibians and fish, but because of technical issues only the macrophyte data was analysed. This technique still needs improvement for ponds.

***Keywords**: wetland restoration, pondscape, man-made pond, eDNA, aquatic vegetation, calico crayfish, tagjump, beta diversity, environmental filtering*

Résumé

La création de réseaux de mares pourrait améliorer la biodiversité aquatique et favoriser la conservation des espèces. Des réseaux de mares ont été mis en place en Europe et seront promus par des initiatives récentes telles que le règlement européen sur la restauration de la nature. Cependant, il existe des lacunes concernant la conception, le suivi, l'évaluation et la gestion efficaces de ces systèmes. Afin de combler ces lacunes, cette thèse a étudié les facteurs environnementaux et spatiaux qui structurent les communautés de macrophytes et de macroinvertébrés dans les mares artificielles, ainsi que méthodes de suivi appropriées. Deux réseaux de mares ont été étudiés : Neu-Woerr (France/Allemagne) et Silene (Lettonie). Silene présentait une grande diversité de macrophytes, tandis que Neu-Woerr avait une faible diversité, probablement à cause d'écrevisses envahissantes. La composition des communautés de macrophytes et de macroinvertébrés était liée à des variables affectant la lumière (ombre des arbres environnants et transparence de l'eau) et, pour les macroinvertébrés, également aux niveaux d'oxygène. Le métabarcoding de l'ADN environnemental a été testé pour la détection des macrophytes, des macroinvertébrés, des amphibiens et des poissons, mais en raison de problèmes techniques, seules les données sur les macrophytes ont été analysées. Cette technique doit encore être améliorée pour les mares.

***Mots-clés** : restauration des zones humides, pondscape, étang, ADNe, végétation aquatique, écrevisse calicot, tagjump, diversité bêta, filtres environnementaux*