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Functional perspectives on macrophyte ecology: resource-use strategies and environmental drivers

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Thesis abstract

Macrophytes are key components of freshwater ecosystems because they fulfil multiple roles and promote the stability of colonized habitats. Compared to terrestrial species, they present peculiar adaptations to live in aquatic ecosystems, and they show a highly plastic response to environmental gradients. However, macrophytes are facing a worldwide decline due to anthropic impact: pollution, habitat degradation, climate change and the introduction of invasive species among major threats. Therefore, it is of crucial importance to better understand how these communities respond to environmental change in order to predict environmental consequences and favor good management policies. Functional traits – any morphological, physiological or phenological characteristic measurable at the individual level, that can influence a plant's fitness – represent a valuable tool to explore a number of ecological questions related to macrophytes at multiple scales. The aim of this PhD thesis is to collect information on the use of functional traits in the literature, and deepen our understanding of plant-environment interactions, with regard to the resource-use strategies of a group of rooted floating-leaved macrophytes. In Chapter I, I present a review on the use of functional traits in macrophytes studies, considering the last ten years of literature. Nearly 300 papers were analyzed, which covered around a third of the world macrophytes diversity (updated May 15th, 2020). The specific aim of the study was to analyze current research trends and highlight knowledge gaps, in order to provide a synthesis that could direct future research on the topic. I identified four main issues regarding the type of traits that have been used, the geographical knowledge gaps and the attention so far posed to interactions between macrophytes and other organisms. Most of the studies used morphological and productivity traits and were carried out in Europe and Asia. It emerges that roots and reproduction traits have been largely overlooked, hindering a full comprehension of macrophytes roles and functionality in the environment. Also, I evidenced a geographical gap, in which very few studies represent regions with the highest macrophytes diversity in the world, like South America. The remaining three chapters of this thesis focused on nymphaeids and their intraspecific variability in response to environmental conditions at different scales. First, I investigated the functional response of *Nuphar lutea* to environmental gradients (sediment and water features) at a very local scale (lake-scale). Then, I expanded the scale by including populations of the same species from four lakes systems located in norther and central Italy. A total of 28 *N. lutea* populations were functionally and environmentally characterized, measuring leaf structural and biochemical traits (including also genetic and spectral traits in Chapter III), and both water and sediment parameters. In both studies I found high traits plasticity, which was driven mainly by sediment quality and electrical conductivity, influencing the plant's resource-use strategies. Overall, a higher trophic level leads to an increase in *N. lutea* traits performance, but it also showed signs of a stressful effect. I also highlighted how petiole-related traits are important descriptors of performance for species like *N. lutea*, where petioles represent nearly half of the leaf biomass. Indeed, these studies demonstrated how different observation scales can lead to different results in terms of main drivers: water depth emerged as a key driver in local leaf traits plasticity (related to leaf size), however at a regional scale the effect of depth was only marginally observed. Finally, I widened the research focus further, by determining the functional and ecological niches of four nymphaeids species: *N. lutea*, *Nymphaea alba*, *Nymphoides peltata* and *Nelumbo nucifera*. Here, I aimed at comparing different species, including both native and invasive species, and species showing a declining trend, merging the information of both niches to explain observed population trends and better describe these species' resource-use strategies. I found that these species all share similar ecological conditions, tough invasive species *N. nucifera* tends to have a bigger ecological niche, but they are clearly distinguished by their functional expression, based on leaves efficiency. In fact, I identified three functional types: highly acquisitive leaves with high specific leaf area (SLA), low leaf dry matter content (LDMC) and high nutrients content (*N. peltata*), acquisitive leaves with large leaf size and high chlorophylls and nutrients content, though with conservative traits like high LDMC and low SLA (*N. nucifera*) and intermediate traits (*N. lutea* and *N. alba*). Besides, *N. alba* showed a limited plasticity coupled with low leaf nutrients content, which could explain its declining trend. Overall, I demonstrated that nymphaeids exhibit high traits plasticity in response to environmental conditions, and that this plasticity allows them to persist in a variety of conditions. Moreover, the implementation of traits derived from spectral measures and of genetic metrics increased the information obtained compared to traditional traits widening the spatial and temporal scale observed. Finally, I suggest that future studies should consider root traits as well as leaf traits, given the importance of sediments for rooted macrophtyes highlighted in this thesis.

Riassunto della tesi

Le macrofite sono componenti chiave degli ecosistemi di acqua dolce perché svolgono molteplici ruoli ecosistemici e promuovono la stabilità degli habitat colonizzati. Rispetto alle piante terrestri, esse presentano adattamenti particolari che permettono loro di vivere negli ambienti acquatici, e mostrano grande plasticità in risposta ai gradienti ambientali. Ciononostante, a causa dell'impatto antropico, le macrofite sono in declino a livello mondiale: inquinamento di acqua e sedimenti, perdita di habitat, cambiamenti climatici e l'introduzione di specie invasive sono tra le minacce più diffuse. È quindi di cruciale importanza approfondirne i meccanismi di risposta al cambiamento ambientale, per riuscire a predirne le ripercussioni a livello ecosistemico e favorire, al contempo, pratiche efficaci di gestione. I tratti funzionali – qualunque caratteristica morfologica, fisiologica o fenologica misurabile a livello di individuo, che influenzi la *fitness* della pianta – rappresentano un prezioso strumento in tal senso, per esplorare una gran quantità di questioni ecologiche legate alle macrofite. L'obiettivo di questa tesi di dottorato è quello di raccogliere informazioni sull'uso dei tratti funzionali in letteratura, e di approfondire le conoscenze sulle interazioni piante-ambiente con particolare riferimento alle strategie di utilizzo delle risorse di un gruppo di macrofite radicate a foglie flottanti, i ninfeidi. Nel Capitolo I presento una *review* sull'uso dei tratti funzionali applicati agli studi sulle macrofite, soffermandomi sugli ultimi dieci anni di letteratura (2010-2020, informazioni aggiornate al 15 maggio 2020). Sono stati analizzati quasi 300 articoli, che coprivano circa un terzo della specie conosciute di macrofite. L'obiettivo principale di questo studio è quello di analizzare le attuali linee di ricerca ed evidenziare eventuali lacune, per fornire una sintesi che possa dirigere la ricerca futura su questo argomento. Ho identificato alcune delle principali questioni riguardanti il tipo di tratti finora utilizzati, le lacune di tipo geografico e l'attenziona posta finora alle interazioni tra le macrofite e gli altri organismi. La maggior parte degli studi ha usato tratti morfologici o di produttività, e sono stati svolti in Europa e Asia. Ne emerge che i tratti radicali e riproduttivi sono stati largamente trascurati, impedendo una piena comprensione dei ruoli e della funzionalità delle macrofite. Inoltre, ho evidenziato l'esistenza di lacune geografiche in questo tipo di studi, per cui pochissimi dati sono disponibili per le regioni con la più alta diversità di macrofite, come il Sud America. I rimanenti tre capitoli della tesi riguardano i ninfeidi, e la loro variabilità intraspecifica in risposta alle condizioni ambientali a diverse scale spaziali. Inizialmente ho indagato la risposta di *Nuphar lutea* a gradienti ambientali (caratteristiche di acqua e sedimento) a scala locale (un singolo lago, Lago di Chiusi, SI). Dopodiché, ho allargato la scala di indagine includendo popolazioni della stessa specie presenti in quattro diversi sistemi lacustri. Complessivamente, 28 popolazioni di *N. lutea* sono state caratterizzate dal punto di vista funzionale e ambientale, misurando tratti fogliari strutturali e biochimici (e includendo anche tratti genetici e spettrali, si veda il Capitolo III), oltre a parametri di acqua e sedimento. In entrambi gli studi ho osservato una elevata plasticità nei tratti indagati, che risponde significativamente alla qualità del sedimento e dalla conducibilità elettrica, capaci di influenzare le strategie di utilizzo delle risorse. In genere, un livello trofico più alto porta a un aumento della *performance* nei tratti di *N. lutea*, ma innesca anche effetti di *stress*. Ho anche evidenziato come i tratti legati al picciolo sono descrittori importanti della *performance* di specie come *N. lutea*, dove il picciolo può rappresentare poco meno della metà della biomassa fogliare. Inoltre, questi studi dimostrano come al variare della scala di osservazione possono variare anche i determinanti ecologici: la profondità dell'acqua può essere un *driver* chiave

della plasticità dei tratti (legati alla dimensione della foglia) alla scala locale, ma tale effetto perde rilevanza a scala regionale. Infine, ho approfondito ulteriormente questi aspetti (vale a dire, le relazioni tra tratti funzionali e determinanti ambientali) quantificando le nicchie funzionali ed ecologiche di quattro specie di ninfeidi: *N. lutea*, *Nymphaea alba*, *Nymphoides peltata* e *Nelumbo nucifera*. Qui il mio scopo era quello di confrontare le diverse specie, includendo sia *taxa* nativi che invasivi e specie che mostrano significative tendenze di rarefazione. Da un punto di vista metodologico, ho comparato le informazioni relative alle due nicchie (funzionale ed ecologica) per spiegare i *trend* osservati nelle popolazioni e descrivere meglio le strategie di utilizzo delle risorse. Ho osservato che queste specie condividono le stesse condizioni ambientali, seppur la specie invasiva *N. nucifera* tenda ad avere una nicchia ecologica più ampia, ma sono ben distinte nella loro espressione funzionale, in termini di efficienza fogliare. Infatti, ho identificato tre tipi funzionali: i) foglie a strategia altamente acquisitiva, con alta area fogliare specifica (SLA), basso contenuto di sostanza secca (LDMC) e alto contenuto di nutrienti (*N. peltata*), ii) foglie a strategia acquisitiva con grandi dimensioni, alto contenuto di clorofille e nutrienti, ma con tratti conservativi come alto LDMC e basso SLA (*N. nucifera*) e iii) tratti intermedi (*N. lutea* e *N. alba*). Inoltre, *N. alba* presenta una plasticità limitata unita a un basso contenuto fogliare di nutrienti, che potrebbe spiegare la progressiva rarefazione di questa specie – e l'elevata sensibilità ai processi di eutrofizzazione di acque e sedimenti. Complessivamente, ho dimostrato come i ninfeidi mostrino una elevata plasticità nei tratti in risposta alle condizioni ambientali, e che questa plasticità permetta loro di sopravvivere in un'ampia varietà di condizioni. Inoltre, l'utilizzo di tratti derivati da misure spettrali o di metriche genetiche aumenta la quantità di informazioni ottenute rispetto ai tratti utilizzati tradizionalmente, ampliando la scala spaziale e temporale osservata. Infine, suggerisco di considerare anche i tratti radicali oltre a quelli fogliari nelle ricerche future, data l'importanza dei sedimenti per le macrofite radicate evidenziata da questa tesi.

1. Introduction

1.1 Rationale and relevance of the study

Freshwater ecosystems occupy only a small portion of the Earth's surface, less than 1%; however, they host an disproportionally high amount of biodiversity, around 10% of all known species (Søndergaard et al., 2005; Strayer and Dudgeon, 2010). They also provide a number of ecosystem services (Thomaz, 2021), spanning from freshwater provision (Li et al., 2017), to nutrient cycling (Vanni et al., 2002; Benelli and Bartoli, 2021) and climate regulation (Herbert et al., 2015), only to mention a few.

Macrophytes represent an important component of aquatic ecosystems, fulfilling several roles that favor the stability of these habitats and acting as ecosystem engineers that modify the environment (Bakker et al., 2013) (Fig. 1). Macrophytes exhibit specific morphological and physiological adaptations compared to terrestrial plants, that allow them to survive in a challenging environment (Bornette and Puijalon, 2011). In fact, rather than a taxonomical group of plants, they are better described by their functional adaptations.

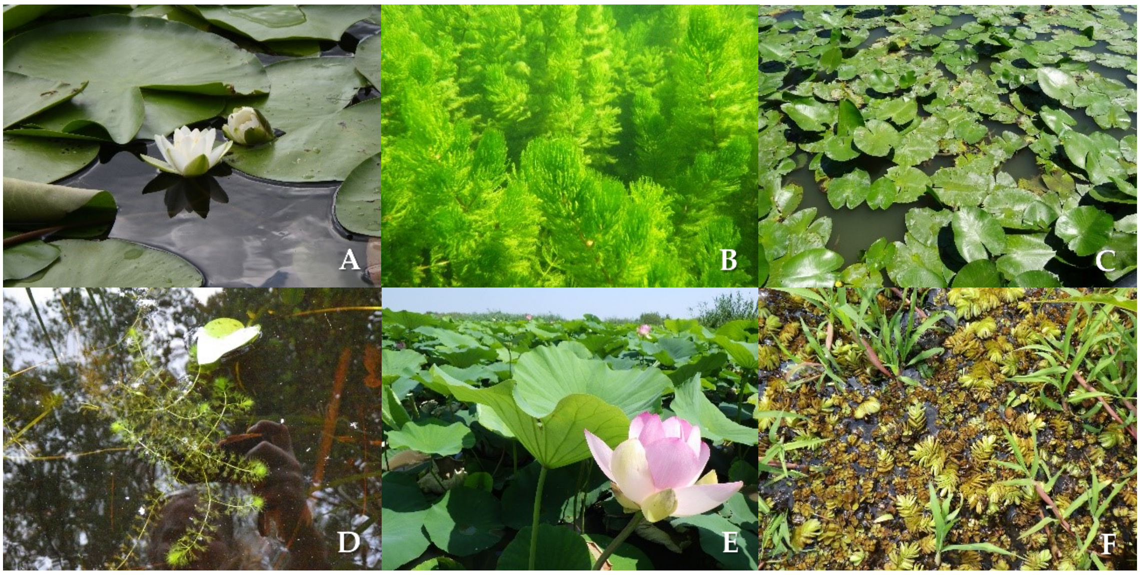

Fig. 1: *Examples of studied species. A -* Nymphaea alba *(Pusiano, CO, IT), B –* Ceratophyllum demersum *(Mantova, MN, IT), C –* Nuphar lutea *(Chiusi, SI, IT), D –* Aldrovanda vesiculosa *(Třeboň, CZ), E –* Nelumbo nucifera *(Mantova, MN, IT), F –* Salvinia *spp. community (Paranà, BR).*

Different macrophytes growth forms reflect the type of aquatic habitat they inhabit, and its ecological conditions (Fig. 2). We can distinguish submerged macrophytes, which may be rooted in the sediment or free floating, rooted macrophytes with floating or emergent leaves, free floating macrophytes that float on the water surface, or emergent macrophytes, with only part of their organs permanently or temporarily submerged (Wiegleb, 1991). All these growth forms are selected by some environmental filters, mainly related to light availability, temperature, nutrients availability, water velocity and hydrological regime (Bornette and Puijalon, 2011).

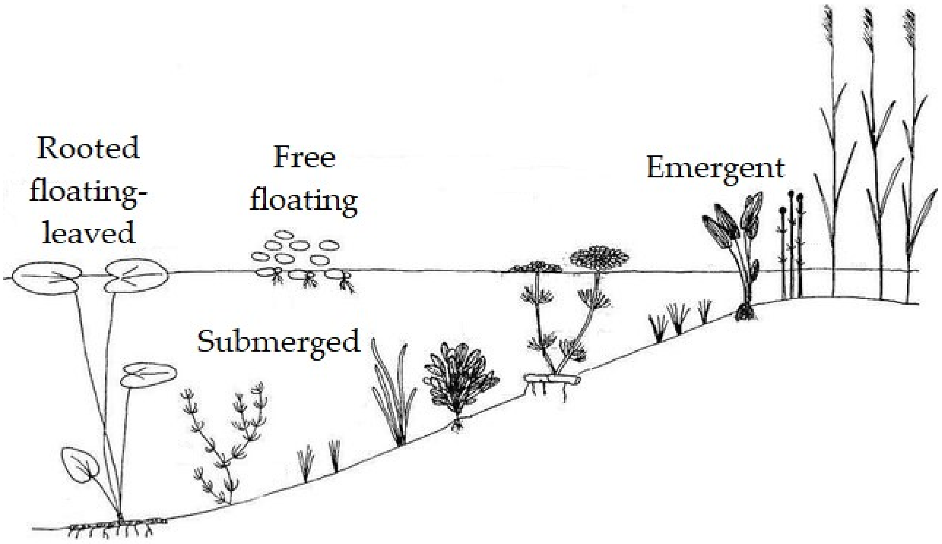

Fig. 2: *Different macrophytes growth forms with relation to the environment they occupy. Modified from Golovneva (2000).*

Freshwater habitats represent tough environments for macrophytes growth, for they show steep gradients in the above-mentioned drivers only within meters from the shallowest to de deepest waters (Bornette and Puijalon, 2011). Therefore, macrophytes are very plastic in their phenotypical response and this allows them to endure extreme or variable conditions (Pierce et al., 2012). In spite of that, they are undergoing an alarming worldwide decline (O'Hare et al., 2018; Bolpagni, 2021), due to threats direct posed by anthropic pressure or induced by climate change. On one side, the excessive input of nutrients in aquatic ecosystems and the structural modification of the environment are causing substantial habitat degradation (O'Hare et al., 2018), and on the other side the introduction of invasive species is threatening the persistence of native local populations (Bolpagni, 2021). The concern on macrophytes decline is not merely related to the loss of biodiversity, but also to the loss of important ecosystem services that these communities provide (Thomaz, 2021). Hence, it is crucial to better understand how macrophytes respond to stressors and changing environmental conditions, in order to be able to explain the current dynamics and potentially prevent further negative effects in the future. In this context, functional traits are a valuable tool to answer a variety of ecological questions. They are measures of any plant attributes at the individual level, so they mirror the response of communities to biotic and abiotic drivers (Violle et al., 2007). Besides, they allow to investigate different biological scales, from communities to individuals, reaching the intraspecific level, which would not be possible with a more traditional taxonomic approach.

This thesis aims to understand the use of functional traits in recent macrophyte studies, highlighting recent trends and gaps that might guide future research, and to investigate the effect of environmental drivers on macrophytes resource-use strategies, focusing on nymphaeids, a group of rooted macrophytes with floating or emergent leaves. The addressed topics will be presented in four chapters, the first being a review of recent literature, and the latter three addressing ecological questions at different scales, based on field data collected during the PhD, starting from the local scale (at single site level) and then moving on to a regional scale of investigation (Fig. 3).

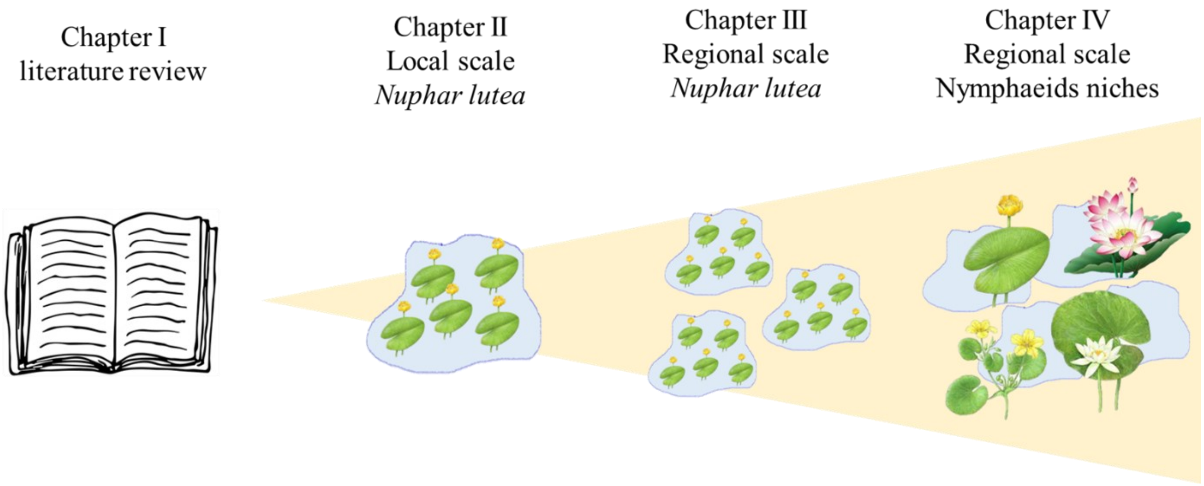

Fig. 3*: Outline of the present PhD thesis, showing the main topics of different chapters, starting from a preliminary literature review, and moving on to investigate different scales of functional traits. variation in nymphaeids. (Plants drawings modified from* www.e-ecodb.bas.bg *and* www.cleanpng.com*).*

1.2 Organization of the chapters

The first chapter includes a literature review of the last 10 years of research (2010-2020, updated May 15th, 2020) on functional traits in macrophytes studies (Dalla Vecchia et al., 2020). This represented a necessary step, because the topic has received increasing attention over the last years, reflected by growing literature; however, no one had so far attempted to summarize the available information. Rather than on the outcomes of the studies, our interest was concentrated on the research questions of the authors and how they used functional traits to answer them. One of the main assumptions in functional traits-based studies is that the trait variability among species is higher than that within species, and therefore species can be represented by their mean trait values in community studies (Dalle Fratte et al., 2019; García-Girón et al., 2019). This statement can be true at large spatial scales, but it may not hold at the local scale, where the effect of drivers acting locally is visible in intraspecific trait variability, so the use of mean species values only is likely to lead to an underestimation of processes, especially so in aquatic environments (Violle et al., 2012).

Chapters II and III of this thesis are thus dedicated to the variation of the functional response of the nymphaeid *Nuphar lutea* (L.) Sm. to environmental gradients, at two different spatial scales (Dalla Vecchia and Bolpagni, 2022; Dalla Vecchia et al., under review *a*). This species is common in many shallow lentic water bodies in Italy (as well as in northern temperate and some subtropical regions of Europe, northwest Africa, western Asia, North America, and Cuba) and is able to inhabit a range of environmental conditions, surviving in hyper-eutrophic waters, too (Longhi et al., 2008; Lastrucci et al., 2014; Pinardi et al., 2018). Chapter II deals with the effect of environmental drivers on leaf traits of *N. lutea* populations at the local scale (i.e., Lake Chiusi) and provides evidence of processes influencing this plant's resource-use strategies within a single lake system (Dalla Vecchia and Bolpagni, 2022). Then, in chapter III, a more complex approach is used, integrating different aspects of macrophytes diversity – functional, spectral and genetic – at a regional scale (Dalla Vecchia et al., under review *a*). Indeed, main drivers of traits variability may not be the same changing the observation scale, simply because the magnitude of the effect of certain variables may be masked by stronger differences among sites (Göthe et al., 2018). Besides, the implementation of leaf spectral responses in this study permitted to test the use of remote sensing-derived traits to investigate macrophytes responses, which could potentially widen both the scale and the precision of observations in space and time with a relatively lower sampling cost and effort. The integration of genetic diversity metrics eventually completes the picture, by explaining if the observed variability matches with high genetic diversity or if it is more likely due to phenotypic plasticity, and by providing information which is mediated in several generations, unlike traits that describe a snapshot of the populations' responses.

After focusing on one single species, in chapter IV I enlarged the topic including other species sharing similar morphological structure (Dalla Vecchia et al., under review *b*). In this chapter, on the other hand, the aim was to combine the information on ecological requirements (or tolerance) and functional expression of each species to understand the range of their niches and their current dynamics. I included both invasive (*Nelumbo nucifera* Gaertn.) and native [*N. lutea*, *Nymphaea alba* L., *Nymphoides peltata* (S.G.Gmel.) Kuntze] species, and the comparison between functional and ecological niches among species revealed important hints on the distribution, spread or regression of populations.

Overall, my PhD was carried out in the frame of the PRIN 2017 project "macroDIVERSITY" (https://twitter.com/macrodiv_proj), a national program funded by the Italian Ministry of University and Research [grant 2017CTH94H]. This project involved three research units, the Institute for Electromagnetic Sensing of the Environment of the National Research Council of Milan (coordinated by Dr. Paolo Villa), the University of Florence (coordinated by Prof. Andrea Coppi) and the University of Parma (coordinated by Dr. Rossano Bolpagni). The aim of the project was to integrate functional, spectral and genetic diversity of macrophytes to produce maps of traits variation and interactions for submerged, floating-leaved and emergent communities. This thesis focuses on the floating-leaved communities and most of the data presented here were collected during the macroDIVERSITY field activities. My PhD has, finally, benefited from the equipment and funds of the COMP-HUB Initiative, funded by the 'Departments of Excellence' program of the Italian Ministry of Education, University and Research (MIUR, 2018-2022).

1.3 Study period abroad

During the last period of my PhD, I spent 5 months (from September 2022 to January 2023) at the State University of Maringá (Paraná, Brazil), in the laboratory of Prof. Sidinei M. Thomaz. Here, I was actively involved in a project on the study of functional traits of tropical macrophytes. The activities included field samplings in the Paraná river floodplain, at the end of the dry season, exploring a variety of freshwater habitats (main river and tributaries, floodplain lakes, backwaters and reservoirs). The activities were carried out to achieve multiple aims. First, we worked on floating plant communities i) to test how different environmental conditions influence the community functional expression, therefore focusing on environmental filters; ii) to compare plant community taxonomical and functional indices, determining the extent of functional redundancy and diversity; iii) to explore the facilitation *vs* competition processes in highly diverse macrophyte communities, investigating the effect of diversity on community performance. Moreover, we particularly focused our attention on two groups of species (*Salvinia* spp. and *Eichhornia* spp.), to thoroughly characterize them functionally, in order to unravel the mechanisms of coexistence of very similar species in the same habitats, and to understand if they are positively or negatively affected by the presence of other species. This study will be of great interest not only for the local scientific community since most of the investigated species are native to Brazil but invasive elsewhere. Finally, we focused on two submerged species – *Egeria najas* Planch. (native) and *Hydrilla verticillata* (L.f.) Royle (invasive) – both occurring in similar habitats, often coexisting together, and presenting similar structural features. Both species are able to colonize lentic and lotic freshwater habitats, causing considerable nuisance to human activities and habitat modification, so we aimed to use a functional approach combined to environmental characterization to investigate the mechanisms influencing their performance. Overall, more than 80 floating and submerged communities were characterized, measuring water and sediment quality in each community, and more than 10 leaf traits and 3 root traits (for free floating species) for each species. The data collected are being analyzed now.

1.4 Side Research and Activities

My PhD included other studies and activities as well, which are not discussed in the main text of the thesis, so they will be briefly introduced in this section.

1. The first side activity was related to the record of habitats and species of conservation concern encountered during my field activities, even outside the study area of the project MacroDIVERSITY. In Gianguzzi et al. (2020), I evidenced the presence of the EU Habitat 3160 (Natural dystrophic lakes and ponds) in the province of Mantua (Italy), where we recorded the presence of the following phytosociological categories *Scorpidio-Utricularion minoris* Pietsch 1965, *Utricularietalia intermedio-minoris* Pietsch 1965 and *Utricularietea intermedio-minoris* Pietsch 1965 (Biondi and Blasi 2015). In Bartolucci et al. (2021), moreover, we reported the first record of *Ranunculus rionii* Lagger in the Emilia-Romagna region, and specifically in the province of Parma (Italy).

2. Besides, I was involved in studies regarding riparian and pioneer riverine vegetation, where my contribution was related to data statistical analysis. In Montanari et al. (2020), we tested the effect of artificial canals (HMWB = heavily modified water bodies) morphology on riparian plant community composition. This study supported the role of artificial canals in driving local community composition in lowland simplified landscapes. Then, with two additional studies we moved to pioneer riverine vegetation and the role of invasive species in three Apennine streams (Bolpagni and Dalla Vecchia, 2021) and three lowland temperate rivers in Lombardy (Bolpagni and Dalla Vecchia, 2022). Here, we examined the diversity patterns along a longitudinal and disturbance gradient. The main findings indicated that disturbance favors the entrance of alien species in the communities, determining a decrease in species diversity, though not necessarily coupled with a decrease in total vegetation cover. These activities were then implemented during the 2022 growing season (July to late August) through the enrichment of the database of the aquatic ecology laboratory of UNIPR relating to the functional traits of pioneer riverine plants. Overall, 101 species were characterized in the Parma and Baganza streams collecting more than 1500 original data referred to the leaf economic spectrum.

3. I was actively involved in the Cost Action ConservePlants (CA18201). I was selected to participate to an international training school on IUCN red lists risk assessment on plants, held in Montenegro in 2022 (29th March – 1st April), where I obtained the certificate of IUCN red lists assessor. Moreover, I applied for and won a Short-Term Scientific Mission (STSM) mobility grant funded by ConservePlants, and I spent a short period (8 days, from 5th to 12th July 2022) at the Botany Institute of Třeboň in Czech Republic – hosted by Dr. L. Adamec. Here, I developed a protocol on the measure of functional traits for aquatic carnivorous plants, characterizing 8 carnivorous species (*Aldrovanda vesiculosa* L., *Utricularia australis* R.Br., *U. bremii* Heer ex Koell., U. intermedia Dreves & Hayne, *U. minor* L., *U. ochroleuca* R.W. Hartm., *U. stygia* G.Thor., *U. vulgaris* L.) present in the wild or in the Botany Institute collection. The collected data – spanning from leaf to biochemical traits – are now being analyzed.

4. Starting from literature data concerning the spatial distribution of macrophytes in deep lakes (Azzella et al., 2017), with the help of Prof. Martin Wilkes (an ecologist of the University of Essex) and Dr. Alex Laini (a zoologist of the University of Turin), I carried out an innovative eco-spatial analysis based on the use of mMCA (multivariate multi-scale codependence analysis; cfr. Guénard and Legendre, 2018). From the analyses, a weak or absent macrophyte spatial structure emerged for the most impacted lakes (Vico and Nemi lakes), as well as for the most pristine lakes (Bracciano and Bolsena lakes). A well-defined structure was observed exclusively in Martignano Lake, an intermediate site both in terms of trophic status (37.7 µg TP L^{-1}) and size (2.02 km^2). Distinctive macrophyte patterns were found at the largest scales, reflecting a clear distinction between shallow (up to 3 m) and deep vegetated bands (> 3 m), dominated by vascular plants and large charophytes respectively. Conversely, no strong spatial structure was detected at the microscale. The low species diversity and the constant presence of only one dominant species can explain this result. Beyond light availability, sediment features (TP and organic matter content) also emerged as significant in determining vegetation structure. The work is now under review (Dalla Vecchia et al., under review *c*; Appendix V).

5. Finally, in collaboration with Dr. Mattia M. Azzella (botanist of the University La Sapienza in Rome), five different charophyte species [*Chara aspera* Willdenow 1809, *C. globularis* Thuiller 1799, *C. polyacantha* A.Braun, nom. inval. 1862, *C. tomentosa* Linnaeus 1753, *Nitellopsis obtusa*

(Desvaux) J.Groves 1919] have been analyzed in order to define an investigation protocol for their functional characterization. We characterized a total of 11 populations (three for *C. aspera*, *C. globularis* and *C. polyacantha*, and one for *C. tomentosa* and *N. obtusa*), collecting data on leaf traits (weight and area, elemental composition and pigments content). These data are now being analyzed.

1.5 Scientific approval

The results of this study were presented at the following conferences:

1. Conference of Young Botanists (CYBO) – Genova, 6-7th February 2020. Poster: "Strengths and weaknesses of the use of functional traits in recent studies on macrophytes".
2. 115° Congress of the *Società Botanica Italiana* onlus – online, 9-11th September 2020. Oral presentation: "Il valore dei tratti funzionali in recenti studi applicati alle macrofite".
3. 10° International Shallow Lakes conference – online, 1-5th March 2021. Oral presentation: "Local patterns and drivers of functional traits in *Nuphar lutea*".
4. *Incontro dei dottorandi e giovani ricercatori in Ecologia e Scienze dei Sistemi Acquatici* – online, 13-15th April 2021. Oral presentation: "Local plasticity of *Nuphar lutea* in a eutrophic shallow lake".
5. ASLO 2021 Aquatic Sciences Meeting – online 22-27th June 2021. Oral presentation: "Destructuring of submerged aquatic vegetation in deep volcanic lakes?".
6. 116° Congress of the *Società Botanica Italiana* onlus – online, 8-10th September 2021. Oral presentation: "*Structural gradients of submerged aquatic vegetation in deep lakes*".
7. XXX Congress of the Italian Ecology Society – online, 25-27th October 2021. Oral presentation: "Insights into submerged vegetation in pristine and impacted deep lakes".
8. Scientific meeting of the Società Botanica Italiana – online, 4th February 2022. Oral presentation: "*Contributo delle specie aliene alla vegetazione effimera fluviale in Appennino: influenza di disturbo idro-geomorfologico e qualità del sedimento*".
9. XXVI Conference of the *Associazione Italiana di Oceanologia e Limnologia* (AIOL) – 27th June / 1st July 2022. Oral presentation: "How hydrology and topography drive exotic plants in annual vegetation of mid-size lowland rivers".
10. 36th Congress of the International Society of Limnology – Berlin, 7-10th August 2022. Oral presentation: "Ecosystem drivers of leaf traits and resource-use strategies in nymphaeids".

Publications on the dissertation topic and side research (role covered in the publication indicated in square brackets; in bold if first author or corresponding author):

1. **Dalla Vecchia, A.**, Villa, P., & Bolpagni, R., 2020. Functional traits in macrophyte studies: current trends and future research agenda. Aquatic Botany, 103290. https://doi.org/10.1016/j.aquabot.2020.103290. [*data collection, analysis, writing*]
2. Montanari, I., Buldrini, F., Bolpagni, R., Laini, A., Dalla Vecchia, A., De Bernardini, N., ... & Chiarucci, A., 2020. Role of irrigation canal morphology in driving riparian flora in over-exploited

catchments. Community Ecology, 21(2), 121-132. https://link.springer.com/article/10.1007/s42974-020-00024-5. [*data analysis, text revision*]

3. Gianguzzi, L., Bagella, S., Bazan, G., Caria, M. C., Cerabolini, B. E. L., Dalla Vecchia, A., ... & Bolpagni, R., 2020. New national and regional Annex I Habitat records: from #13 to #15. Plant Sociology, 57, 65. https://doi.org/10.3897/pls2020571/07. [*data collection, text revision*]
4. Bolpagni, R, Dalla Vecchia, A., 2021. Pioneer annual vegetation of gravel-bed rivers: first insights on environmental drivers from three Apennine streams. Journal of Limnology 80(3): 2052. [data *analysis, text revision*]
5. Bartolucci F, Domina G, Argenti C, …& Lastrucci, L., 2021. Notulae to the Italian native vascular flora: 12. Italian Botanist 12: 85-103. https://doi.org/10.4081/jlimnol.2021.2052. [*data collection, text revision*]
6. **Dalla Vecchia, A.**, & Bolpagni, R., 2022. The importance of being petioled: leaf traits and resource-use strategies in *Nuphar lutea*. Hydrobiologia 849: 3801-3812. https://doi.org/10.1007/s10750-022-04803-1. [*data collection, analysis, writing*]
7. Bolpagni, R., & Dalla Vecchia, A., 2022. A longitudinal snapshot of pioneer plant patterns along lowland temperate rivers. River Research and Applications 38(6): 1129–1138. https://doi.org/10.1002/rra.3988. [*data analysis, text revision*]
8. Castellani, M. B., Lastrucci, L., Lazzaro, L., Bolpagni, R., Dalla Vecchia, A., & Coppi, A., 2022. The incidence of alien species on the taxonomic, phylogenetic, and functional diversity of lentic and lotic communities dominated by *Phragmites australis* (Cav.) Steud. Knowledge & Management of Aquatic Ecosystems 423: 5. https://doi.org/10.1051/kmae/2022001. [*data collection, text revision*]

Manuscripts on the dissertation topic and side research submitted [*under review*] (role covered in the publication indicated in square brackets; in bold if first author or corresponding author):

9. Castellani, M. B., **Dalla Vecchia, A**., Bolpagni, R., Natale, R., Piaser, E., Lastrucci, L., Coppi, A., & Villa, P. Role of genetic drift vs natural selection in affecting the evolution of two key macrophytes: *Phragmites australis* and *Nuphar lutea*. Freshwater Biology [*under review; data collection, text revision*]
10. Villa, P., Dalla Vecchia, A., Piaser, E., & Bolpagni, R. Assessing PROSPECT performance on aquatic plant leaves. Remote Sensing of Environment [*under review; data collection, text revision*]
11. **Dalla Vecchia, A**., Coppi, A., Castellani, M. B., Lastrucci, L., Piaser, E., Villa, P., & Bolpagni, R. Multidimensional trait variability in yellow water-lily, *Nuphar lutea*: functional, spectral and genetic drivers. OIKOS [*under review; data collection, analyses, writing*]
12. **Dalla Vecchia, A.**, Laini, A., Castellani, M. B., & Bolpagni, R. Eco-functional niches of nymphaeids: Trophic-mediated functional responses of macrophytes across spatial scales. Annals of Botany [*under review; conceptualization, data collection, analyses, writing*]
13. **Dalla Vecchia, A.**, Bolpagni, R., Laini, A., Nizzoli, D., Bresciani, M., Azzella, M. M., & Wilkes, M. M. An unexplored domain: eco-spatial rules of submerged macrophyte assemblages in deep lakes. Perspectives in Plant Ecology, Evolution and Systematics [*under review; data analyses, writing*]
14. Piaser, E., Berton, A., Bolpagni, R., Caccia, M., Castellani, M. B., Coppi, A., Dalla Vecchia, A., Gallivanone, F., Soana, G., & Villa, P. Impact of radiometric distortions on ultra-high resolution hyperspectral imagery over aquatic vegetation: preliminary results. Journal of Selected Topics in Applied Earth Observations and Remote Sensing [*under review; data collection, text revision*]

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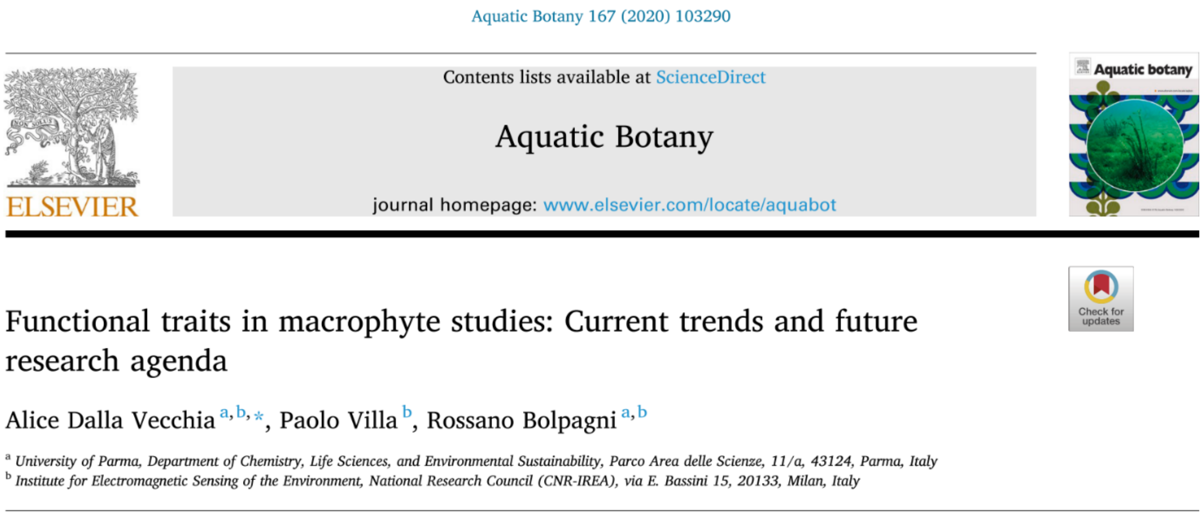
2. Chapter I

FUNCTIONAL TRAITS IN MACROPHYTE STUDIES: CURRENT TRENDS AND FUTURE RESEARCH AGENDA



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Functional traits in macrophyte studies: Current trends and future research agenda

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2.1 Introduction

The concept of functional traits is a relatively recent research approach that is rapidly establishing in ecology and is taking the place of purely taxonomic studies because of its high potential in exploring multi-scale environmental issues. Functional traits are defined as any morphological or phenological characteristic that is measurable at the individual level (Díaz et al., 1998; Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2013), and can mirror the relationships of a species to its habitat conditions, thus revealing the interactions of the plant with the environment (Fu et al., 2015). Moreover, a supplementary advantage of functional trait-based studies is that findings can be compared among different regions, since the different specific community composition does not represent a barrier anymore, thus allowing investigations at wider scales (Schoelynck and Struyf, 2016; Iversen et al., 2019).

The use of functional traits is of particular interest for aquatic ecosystems, which are environments of major concern when considering the threats posed by anthropic pollution, habitat degradation (land use change), and the introduction of non-native species, leading to a change in the community composition in terms of reduced biodiversity and functional homogenization (Bresciani et al., 2012; Phillips et al., 2016; Cantonati et al., 2020; Lindholm et al., 2020). The concern for biodiversity conservation is a critical concern for aquatic plants (O'Hare et al., 2018), which show a high diversity in sub-tropical to low tropical latitudes (Murphy et al., 2019), and in lowlands with higher water availability at the regional scale, coinciding with the strongest presence of anthropic activities (Bolpagni et al., 2018; Guareschi et al., 2020).

Aquatic plants are crucial in maintaining water transparency by absorbing nutrients from the water column and from the sediment, thus competing with phytoplankton for both nutrients and light (Scheffer, 1999), by releasing allelopathic substances that can inhibit the growth of phytoplankton (Hilt and Gross, 2008) and by favoring sediment stability and reducing resuspension (Van Donk and Van de Bund, 2002). Besides, macrophytes can influence hydrologic features of the water body, especially in lotic systems, by reducing water velocity and enhancing sedimentation of suspended particles (Rolland et al., 2015). They can also influence the chemical processes in the rhizosphere by releasing oxygen and other exudates from the roots (Soana and Bartoli, 2013). Moreover, their presence creates structure in the water column and offer habitat for zooplankton and fish (Schriver et al., 1995; Perrow et al., 1999) and finally, aquatic plants represent an important food source for a range of different organisms, as invertebrates, amphibians, fish, birds and mammals (Wood et al., 2017). Because of all these reasons, the presence of macrophytes promotes complex feedbacks that help maintaining the ecosystem stability (Bakker et al., 2013), but at the same time they can also trigger dystrophic events (Bolpagni et al., 2007) As the multiple pivotal roles of macrophytes in influencing the structure and the dynamics of the ecosystem have been widely recognized in the literature (e.g., Ozimek et al., 1990; Scheffer et al., 1993; Van Donk and Van de Bund, 2002), a deeper understanding in their functionality and interactions with the other components of aquatic systems should be a prerequisite for developing effective management actions.

The study of aquatic and terrestrial plants has long been based on a taxonomic approach in order to detect changes in the community species composition, using indexes like species richness or beta diversity (McGill et al., 2006; Lindholm et al., 2020). However, researchers have recently documented the use of functional traits for investigating important topics like the mechanisms explaining the structuring of the community (Van Gerven et al., 2015; Eckert et al., 2016; García-Girón et al., 2019a), the response of species and communities to environmental gradients (Zhang et al., 2019; Sebilian Wittyngham et al., 2019), the influence of anthropic activities and climate change (Huang et al., 2017; Yu et al., 2018), the design of effective restoration actions (Pereira et al., 2017; Pietrini et al., 2019), the spread of invasive species (Thiébaut et al., 2016; Villa et al., 2017), and the role of traits in determining biotic interactions (Grutters et al., 2016; Sun et al., 2018). The implementation of trait-based approaches has resulted in an increasingly abundant literature and in the institution of online databases containing plant functional traits values accessible to the scientific community (e.g., www.try-db.org, www.leda-traitbase.org, www.icestes.github.io). Nevertheless, a systematic and general synthesis on the use of functional traits in aquatic macrophytes studies is still missing. Given the high interest on these studies and the wide spectrum of application fields, we intend to answer the need of scrutiny for which functional traits, species and topics have been investigated so far in the context of aquatic macrophytes (Pan et al., 2019). We believe that this review has become necessary in order to evaluate what fields have been exhaustively researched and what other fields deserve further insight and to promote the standardization of procedures so that comparisons among studies are facilitated. For this reason, we aim to propose a research agenda highlighting the most critical aspects regarding trait-based approaches tackled so far and indicating what should be the next steps in this field.

2.2 Research strategy and analysis of articles

The systematic paper research was carried out on the Scopus database (www.scopus.com; last access 15th May 2020), addressing the words that identify aquatic plants and confining the research to functional traits. The string used was: TITLE-ABS-KEY (“aquatic plant*” OR macrophyte* OR hydrophyte* OR helophyte* OR pleustophyte* OR “water plant*”) AND TITLE-ABS-KEY (trait* OR “functional trait*”). We are aware that by using only the word “trait” we omitted a number of studies that investigated plant characteristics or attributes, though not explicitly referred to as “functional traits” (e.g., Fornoff and Gross, 2014; Marzocchi et al., 2019). However, we intended to delineate our research to studies that refer to a specific and homogeneous field of research (trait-oriented), adopting a consistent use of terminology. A total of 738 papers resulted from the research, published from 1969 onwards.

Only papers published between 2010 and 2020 were taken into consideration for this review, in order to focus on recent developments and current trends on the topic of functional traits applied to macrophytes, for a total of 520 papers (equal to 70 % of the selected papers). The papers were examined to check for relevance following the “matrix method” approach by Klopper et al. (2007). This method involves the creation of a matrix that summarizes the information found in the papers using a series of parameters of interest. The research was open to any macrophyte growth form and aquatic habitat, including estuarine and marine ecosystems.

Papers were considered relevant if they included the measurement of functional traits on one or more macrophyte species (primary studies) or the use of already measured traits from the literature (secondary studies) in order to address any ecological question. During the elaboration of results, we made no distinction between these two types of studies. Previous reviews on specific traits or topics related to macrophyte functional traits were also included, however none of these offered a wide-ranging overview as the present review. The TRY database list for functional traits (www.try-db.org) was consulted to check for consistency of the traits considered by the papers. The matrix of revision contained 10 parameters: *Geographic distribution*, *Habitat type*, *Study type*, *Macrophyte type*, *Name of the species*, *Species number*, *Functional trait category*, *Shoot/root functional traits*, *Environmental variables*, and *Main topic*, as listed in Table 1 (see also Appendix AI.1).

The first feature *Geographic distribution* is informative of the place where the study was conducted at the macro-spatial scale (e.g., continent), for both field investigations and/or laboratory experiments. *Habitat type* refers to where macrophytes were either measured in the field or collected for further analyses or experiments in the laboratory. Here we distinguished between i) lentic environments like lakes, ponds, and wetlands, including the small-standing water ecosystems *sensu* Bolpagni et al. (2019) that are characterized by a larger variability in the water regime as ephemeral systems, ii) lotic environments, including rivers, streams and canals, and iii) marine environments. The tag *Any* was assigned to studies not restricted to a single habitat type and can include more than one habitat where the target macrophyte species were present and investigated. The parameter *Macrophyte type* includes the three main growth forms, i.e. submerged, free-floating, and emergent (Fu et al., 2019a; García-Girón et al., 2019b);

rooted emergent (e.g., *Nelumbo nucifera*) and rooted floating-leaved (e.g., *Nuphar lutea, Nymphaea alba*) were grouped together because often there was no clear distinction in some of the papers examined.

Table 1. *Number of papers focusing on each trait category ("Functional traits") to address certain ecological questions ("Application") for the first ten most studied species. For this table only articles studying one to three species were considered, to concentrate on more specific studies. Targeted species: Myr_spi* = Myriophyllum spicatum*; Cer_dem* = Ceratophyllum demersum*; Phr_aus* = Phragmites australis*; Pot_cri* = Potamogeton crispus*; Pot_pec* = Potamogeton pectinatus*; Elo_can* = Elodea canadensis*; Pot_per* = Potamogeton perfoliatum*; Lem_min* = Lemna minor*; Hyd_ver* = Hydrilla verticillata*; Pol_amp* = Polygonum amphibium. *In "Application", the category "Invasive species" includes studies on their performance and comparisons and interactions with native species; "Environmental conditions" concern water and climate parameters and landscape characteristics; "Competition" is referred to plant species competition and it also includes studies on community structure. "Interactions oth. organisms" includes interactions between plants and bacteria, algae or animals. References can be found in Appendix I (AI.3). The quoted papers not included in the references are listed in the AI.2.*

	Functional traits								**Application**								
Targeted species	Morphology	Elemental composition, DMC	Productivity	Physiology	Biomecanics	Reproduction	Root anatomy	Contaminants accumulation	Herbivory, palatability	Invasive species	Environmental conditions	Sediment characteristics	Eutrophication and pollution	Mechanical stress	Competition	Interactions oth. organisms	Intraspecific variability
Myr_spic	5	4	8	2				1	2	2	2	5	1	5	7	2	
Cer_dem	7	7	2	5	1				2	7	5		8	1			5
Phra_aus	2	1	4	3			2	1	1		3	2	3			2	
Pot_cris	2		1	1	2	3						3	3			4	2
Pot_pect	4	1	1		3	2			3		3		3	3			3
Elo_can	6	1	9	5	2				1	7	7	2			4	2	5
Pot_perf			1	1							1				1		
Lem_min	1		5	3		3					4		8		7		
Hydr_vert	5	2	4		1				1	4	4	5					
Poly_amph	1		1												1		

The tag *Any* was given to papers analyzing the whole community including more than one macrophyte growth form present in the study area. Under *Study type, field/lab* refers to whether traits were measured from samples of plants grown under natural conditions (field) or grown in manipulated conditions (laboratory). Reviews were listed separately (e.g., Colmer et al., 2011; Heino et al., 2015). For *Species number* we chose three categories defined based on preliminary check of the selected papers, in order to distinguish those papers addressing specific questions to single or very few species (tag 1–3), papers considering a limited number of species (4–6) and lastly papers studying more than 6 species (tag >6), which may be representative of the whole community. The *Functional traits* considered by the papers where classified into 10 categories: *Growth form*, when this was considered as a variable relevant for the issue investigated; *Morphology*, including measures of the size and plant structure (e.g., height, stem diameter, root length); *Productivity*, related to fresh and dry weight and biomass allocation, together with growth rates measured on a biomass basis; *Physiology* includes traits related to physiological processes like photosynthesis, respiration and enzyme activity (pigment content is also included in this group); *Biochemistry* refers to the elemental composition of tissues, namely content of C, N, P or other elements; the traits included in *Reproduction* concern any feature related to vegetative or sexual reproduction (e.g., number of flowers, seed size, number of vegetative propagules); *Ecological preferences* take into consideration indexes like the Ellenberg indicator values applied to identify the plant niche along environmental gradients (Ellenberg et al., 2003); the category *Biomechanical traits* includes plant features linked to the resistance to mechanical stress, like wind, waves or water flow velocity. Typical measured traits are flexural rigidity and flexural strain (Łoboda et al., 2018, 2019). *Biotic interactions* identify traits related to the nutrient uptake strategy facilitated by other organisms, which may be mycorrhizal fungi or bacteria (see Cornelissen et al., 2003). In this category we did not include traits that can determine other types of interactions, such as the elemental composition of tissues or the dry matter content, which are already mentioned in previous groups. The last category (*Other*) includes all other traits.

Shoot or root FTs points out whether the papers dealt with only aboveground or belowground traits or with both types: belowground traits were those measured specifically on roots or rhizomes, while aboveground traits were those measured on stems, leaves and reproductive organs. As for pleustophytes, when biomass was provided, it was considered a shoot trait unless a distinction between shoot and root biomass was made. Papers were also scanned for *Main environmental variables* that were measured and related to the functional traits. They were in turn classified into: *Water*, including physical and chemical parameters of the water column like temperature, pH or nutrient concentration; *Sediment* characteristics such as granulometry or organic matter content; *Climate*, concerning meteorological variables together with changes in the atmospheric composition (e.g., increased CO2); *Anthropic* refers to the influence of anthropic activities, for example land use and pollution; the tag *Depth/light* addresses specifically the effect of a reduction of available radiation both because of shading or increased water depth, while *Hydrology/topography* includes the information on the hydrologic regime or physical habitat characteristics. Papers were finally assigned to one or more of the seven *Main topic* categories: *Environmental gradients* groups papers addressing how community or species traits vary with relation to one or more environmental variable; *Community structure* studies include questions on the mechanisms that rule the interactions among plant species and how different species occupy space within the community; *Anthropic pressure* refers to the studies that investigate the effect of pollution, habitat degradation and climate change on plant traits; the topic *Biotic interactions* explores the effect of plant traits on other organisms both above and belowground, including phytoplankton, bacteria and fungi, as well as interactions with herbivores; *Invasiveness* clearly refers to studies investigating relationships between traits and potential invasiveness and management implications; *Species characteristics* is a broad category that was assigned to studies investigating relationships among functional traits of single or few species, without the aim of finding any relation with other variables. The last topic (*Other*) includes all other questions.

2.3 General findings

The first functional trait-based studies on macrophytes were published in the late 1960s and the trend is so far considerably increasing, with the majority of the papers being published in the last ten years (520 out of 738, equal to 70 % of total publications; Fig. 1).

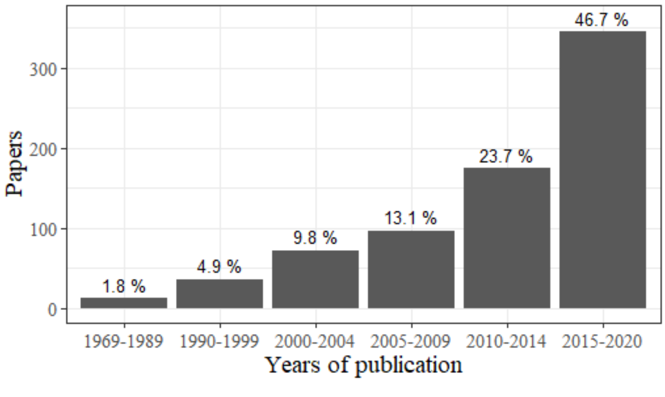

Fig. 1. *Publication trend of the 738 macrophyte trait-based studies.*

In this review, the papers published between 2010 and 2020 were screened for relevance. Of these, 296 papers were considered relevant and included in this study (40 % of initial set of papers; AI.2). Most of the studies were carried out in Europe (41.4 % of the total amount of papers considered) and Asia (31.5 %), followed by North and South America, and only very little attention was given to this topic in Oceania and Africa (Fig. 2a). As for the habitat type, lakes are the most investigated (30 %), but also lotic environments and wetlands received considerable attention (21.1 % and 20 %, respectively) (Fig. 2b).

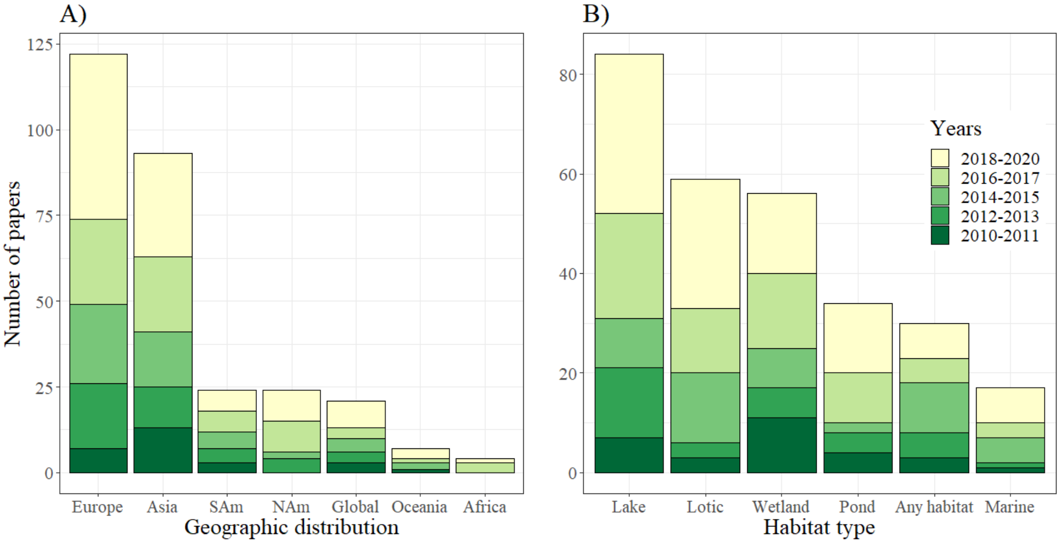

***Fig. 2**. The number of papers conducted in different continents (Geographic distribution, A), South America (SAm), North America (NAm), and Global Studies (Global), and Habitat type (B). Stacked bars show the repartition in years of publication.*

Authors dedicated most of their attention specifically to submerged (39.0 %) and emergent macrophytes (29.9 %) rather than free-floating ones (8.8 %). However, there is a noticeable number of papers (71 papers, 22.3 %), which dealt with all three growth forms (Fig. 3a). Studies were equally divided into field and lab studies (44 % and 42.3 %, respectively) and 16 studies used a combined approach of controlled and field experiments (Fig. 3b).

Within the period considered in this study, 24 review papers concerning some delineated aspects of functional traits were published (highlighted in bold in AI.2). However, the aim of these papers was not to provide a general framework as in this review. Besides, considering the number of species studied in each paper, the vast majority of the studies focused on 1–3 species (57.3 %), and about a third (32.5 %) on more than 6 species.

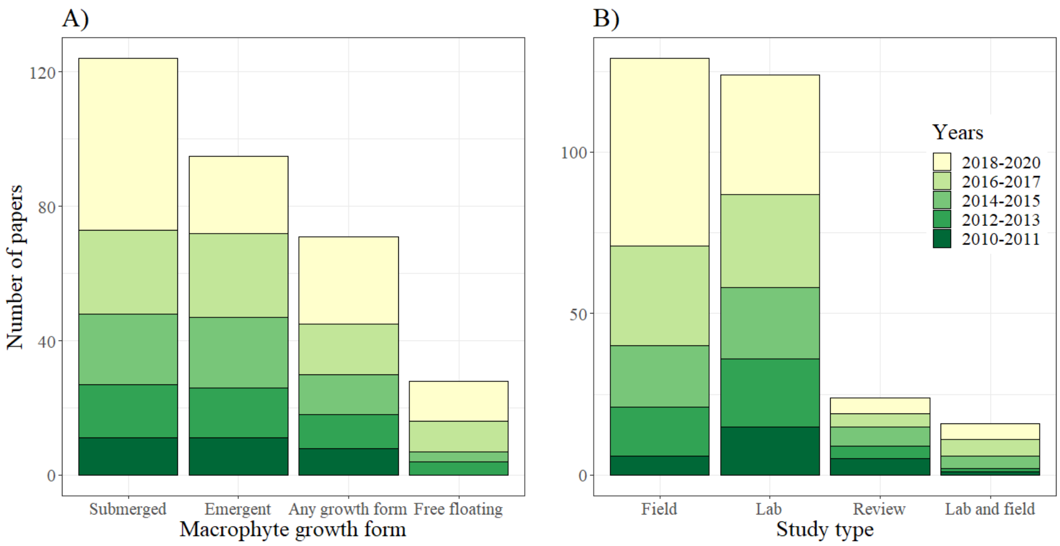

***Fig. 3**. The number of papers focusing on different macrophyte Growth forms (A) and Study type (B). Study types include field studies (field), controlled-conditions experiments (lab), combined approaches (lab and field) and reviews.*

The most investigated functional traits categories are *Morphology* (27.7 % of the papers), *Productivity* (22.6 %), *Reproduction* (13.7 %) and *Physiology* (12.2 %; Fig. 4a).

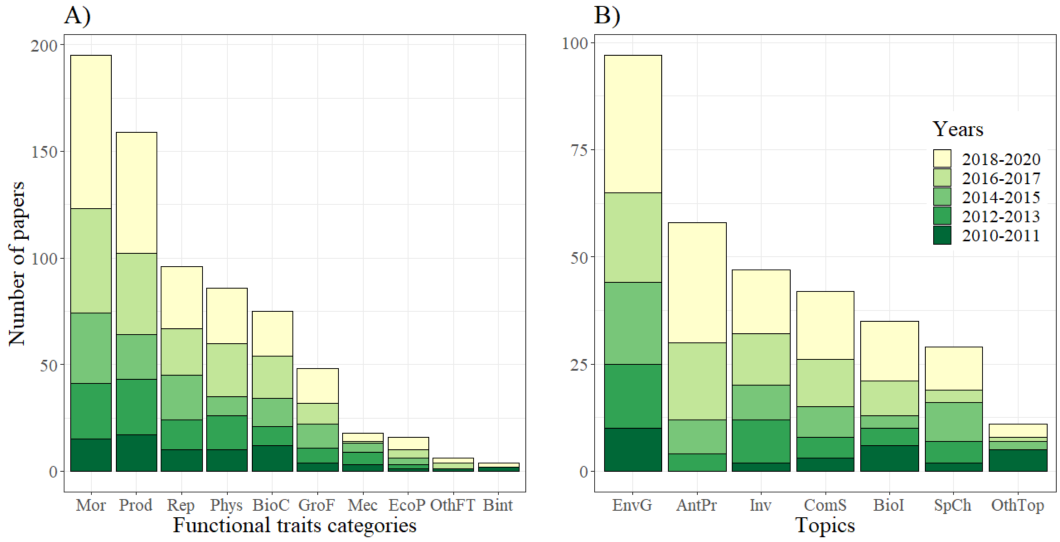

***Fig. 4**. Categories of functional traits that have been measured by the authors or acquired from the literature and used to reach the aim of the study (A) and main topics investigated (B). The categories of functional traits are: Morphology (Mor), Productivity (Pro), Reproduction (Rep), Physiology (Phys), Biochemistry (BioC), Growth form (GroF), Ecological preferences (EcoP), Biomechanical traits (Mec), Other (OthFT) and Biotic interactions (Bint). Main topics are: Environmental gradients (EnvG), Anthropic pressure (AntPr), Invasiveness (Inv), Community structure (ComS), Biotic interactions (BioI), Specific characteristics (SpCh) and Other topics (OthTop).*

Traits are measured in most cases on the aboveground portion of the plant (57.4 %) and often also on belowground organs (38.4 %). Only 12 studies focused exclusively on *Root traits*.

Among the environmental variables related to macrophyte traits, *Water* parameters are the most frequently studied (34.2 %), followed by *Depth* and *Light* (17.7 %), *Hydrology* and *Topography* parameters (15.2 %) and *Sediment* characteristics (14.1 %) (Fig. 4b). As for the main topics, *Environmental gradients* have received by far the greatest attention (30.4 %); other importantly explored topics are related to *Anthropic activities*, namely anthropic pressure (18.2 %) and *Invasiveness* (14.7 %).

2.4 Analysis of current research trends

Geographic distribution

Europe is the continent showing the greatest number of studies on macrophyte functional traits (Fig. 2a). Research groups are well spread around the countries, and we can list examples from all Europe (e.g., Mermillod-Blondin and Lemoine (2010) in France; Anjum et al. (2013) in Portugal; Villa et al. (2017) in Italy; Lindholm et al. (2020) in Finland). The same cannot be said for Asia, the second continent for number of studies, where China accounts for most of the publications and very few studies have been carried out outside China (e.g., Kato and Kadono (2011) and Amano et al. (2012) in Japan; Bashir Shah et al. (2014) in India). The other continents lay far below in the list, but we noted emerging studies in the Brazilian wetlands present along the Amazon basin (e.g., Delatorre et al., 2019; Catian et al., 2018). Studies conducted in Oceania mainly concern the topic of invasive species, for example the research on effective management actions (Eller et al., 2015; Ellawala Kankanamge et al., 2019) or the impact of disturbance due to anthropic activities on native and invasive species (Mouton et al., 2019). Similar topics related to invasiveness can be found also in African studies (Venter et al., 2017), together with studies investigating community assembly rules in South African wetlands (Sieben and Le Roux, 2017).

Habitat type

Lakes result as the most studied habitat, which in part mirrors the wide number of studies conducted in Chinese lakes (e.g., Xing et al., 2016; Wang et al., 2017; Fu et al., 2018; Su et al., 2019; Fig. 2b). Here, shallow lakes have been chosen to investigate the effect of water depth on macrophyte population stability and traits intraspecific variability (Fu et al., 2018; Zhou et al., 2019) and wind disturbance combined with eutrophication effects on traits (Zhu et al., 2018a), or the drivers influencing functional diversity in different macrophyte communities (Fu et al., 2019a, Fu et al., 2019b). After lakes, lotic environments and wetlands are roughly equally studied. In both environments, aspects related to the hydrologic regime are particularly investigated, namely the effects of water level changes and water flow disturbance on biomechanical or life history traits (e.g., Colmer et al., 2011; Miler et al., 2014) or the relationship between sediment properties and plant performance (Sutton-Grier and Megonigal, 2011). Papers belonging to the category *Any habitat* include some reviews (e.g., the review by Schultz and Dibble, 2012), focusing on how invasive macrophytes may influence fish and macroinvertebrates communities, the paper by Eckert et al. (2016) on the consequences of clonal and sexual reproduction for aquatic plants, or the review by Schoelynck and Struyf (2016) exploring the role of silicon as a trait for aquatic vegetation, and many studies on single species, in which samples are collected for trait measurements in several environments where the species of interest was found (e.g., Efremov et al., 2015; Kwong et al., 2017).

Macrophyte type

All macrophyte growth forms (e.g., Korol and Ahn, 2016; Dong et al., 2017; Huang et al., 2018) have been well represented in the trait-based studies we analyzed, except for a lower number of studies regarding free floating species, a result that could be expected due to the relatively lower number of species included in this group (Chambers et al., 2008; Fig. 3a). These species have mainly been used to investigate responses to water contamination and possible uses of these plants in phytoremediation (Mesa et al., 2017; Pietrini et al., 2019) or aspects related to the dispersal and proliferation of highly invasive species like *Eichhornia crassipes* or *Pistia stratiotes* (Gao et al., 2012; Fan et al., 2013; Venter et al., 2017). On the other hand, submerged macrophytes represent the most studied growth form. They have been investigated for a variety of purposes, and in particular they were selected to investigate the responses to and effects on sediment properties (Lemoine et al., 2012; Zhu et al., 2012), or to explore the use of different forms of inorganic carbon to support underwater photosynthesis (Hussner and Jahns, 2014; Eller et al., 2015). Emergent macrophytes have also been widely explored, especially with a focus on trait plasticity in relation to water parameters and water level fluctuation (Demetrio and Barbosa, 2014; Standen et al., 2018) and responses to disturbance by wind or water flow (Cao et al., 2016; Wang et al., 2010).

Study type

Studies carried out under natural conditions or under controlled conditions (*field* and *laboratory* studies) are equally abundant in this research, however laboratory studies include almost exclusively papers considering only few species (Fig. 3b), and often try to explain the adaptation (i.e., intraspecific trait variability) of a species trait to changes in a certain environmental condition determined by biotic or abiotic factors (Nuttens et al., 2016; Silveira and Thiébaut, 2017; Thouvenot et al., 2017). Field studies tend to bypass intraspecific variability, and more often aim at detecting changes in the community trait composition, thus determined by a different species composition and relative abundance rather than due to variability at the species level (Fu et al., 2014; Lindholm et al., 2020). 16 studies have used a dual approach to compare results obtained in the two experimental conditions or combine information from different kinds of experiments. For example, Kordyum et al. (2017) compared the aerenchyma formation and enzyme biosynthesis in two emergent species (*Sium latifolium* and *S. sisaroideum*), under natural and experimental conditions, and Paz et al. (2019) analyzed palatability traits to herbivores for three macrophyte species (*Egeria densa*, *Gymnocoronis spilanthoides*, *Ludwigia peploides*) in the laboratory, and later transplanted them in the field to assess actual consumption under natural conditions. Among the 24 reviews scrutinized, the topic of invasive species is very common: traits were used to explain the effects of invasive species on the ecosystem and on interactions among the components (Strayer, 2010) or for the redaction of risk assessments based on functional traits (Gordon et al., 2012; Azan et al., 2015). Other topics debated in these reviews are linked to specific questions such as the response of aquatic vegetation to abiotic factors (Bornette and Puijalon, 2011), the role of silica in aquatic plants (Schoelynck and Struyf, 2016) or effects of water level fluctuations (Carmignani and Roy, 2017). Root functional traits were taken into consideration in 11 out of 24 review papers: Fusconi and Mucciarelli (2018) explored arbuscular mycorrhiza, while the most extensive review we found on root functional traits is by Ali et al. (2019), focusing on nutrients and heavy metal abatement.

Trait category

We observed that *Morphology* and *Productivity* traits are the most investigated among the analyzed papers and show an increasing trend in the last four years (Fig. 4a). Many of these traits are considered "soft traits", relatively cheap and easy to measure in the field, such as leaf area or plant height (Cornelissen et al., 2003), which make them a good choice for field studies at the community level and are also available for many species in online databases. They are often used to compute indices that synthetize functional characteristics within a community, such as the "functional trait diversity" index (FD_Q) and functional beta diversity, the "community weighted means" index (CWM), the SES_{MPD}, namely the standardized effect size of abundance-weighted mean pairwise distances between species for each trait (Fu et al., 2014, 2019b; Lukács et al., 2019). These metrics all take into consideration both trait values and species abundance within the community. In this sense, researchers are not interested in catching the trait variability at the species level, rather they use traits as an indication of the mean species characteristics, thus revealing the function of the species at the community scale: at this scale intraspecific variability is believed to have a negligible influence (e.g., Fu et al., 2014; García-Girón et al., 2019b). *Morphology* and *Productivity* traits often appear together in studies because they include traits describing the leaf and plant economic spectrum, along with elemental composition (e.g., Specific Leaf Area, Leaf Area, Leaf Dry Matter Content, Leaf Nitrogen Content, Specific Root Length, Leaf Area Index) (e.g., Pierce et al., 2012; Li et al., 2019a). The economic spectrum is considered explicative of existing trade-offs between, for example, growth and tissue construction; its strength may vary along an environmental gradient and in turn it influences the ecosystem functions (Díaz et al., 2004, 2016). These trait categories have been applied to the most disparate research purposes other than the insight into community assembly rules and community responses to environmental conditions, such as in the response to anthropic activities like the introduction of invasive species and pollution. For instance, Chmura and Molenda (2012) evaluated the phenology and growth response of three emergent species (*Phragmites australis*, *Scirpus sylvaticus*, and *Typha latifolia*) to thermally polluted water, and Thiébaut et al. (2017) used these morphology and productivity traits, along with tissues elemental composition, to assess palatability to gammarid herbivores in two invasive species, *Elodea canadensis* and *E. nuttallii*. Such studies addressing more specific ecological questions often take into consideration also the trait plasticity, as mentioned above, in order to understand what are the factors that determine the variability at the species level (e.g., Xie and Yu, 2011b; Glover et al., 2015). *Reproduction* traits have been widely used to investigate dispersal abilities, how they are affected by environmental conditions and how they influence the community structure (Qian et al., 2014). In this context, Chmara et al. (2015) found a strong relationship between traits (including *Reproduction* and *Morphology* traits) and the acidity gradient, demonstrating the importance of carbon availability in determining aquatic plants performance. *Reproduction* and growth-related traits have also been used to detect differences in growth strategies and resource allocation between sexes in the dioecious species *Vallisneria spinulosa* (Li et al., 2019b). *Physiology* traits are very often measured in what we defined laboratory studies, because of logistic constraints (i.e., linked to physical, time, and economic resources) connected to collecting direct measures of such traits in the aquatic systems (e.g., Saha et al., 2016; Tang et al., 2018). Besides, physiology-related measurements are very sensitive to changes in environmental conditions, which can be difficult to control when in the field and bias the response of plants to defined treatments, e.g., photosynthesis efficiency under different levels of CO_2 (Hyldgaard and Brix, 2012). Again, a widespread purpose for the use of these traits was the assessment of effects of pollution and climate change: photosynthetic and enzymatic responses to specific pollutants like cadmium (Huang et al., 2017; Liu et al., 2017), copper (Roubeau Dumont et al., 2019), herbicides (Nuttens et al., 2016) and perfluoroalkyl substances (Pietrini et al., 2019) were investigated. *Physiology* traits and especially photosynthesis-related traits and allelopathic activity have been used to understand the advantages of invasive species that lead to their successful competition against natives, in the context of increasing temperatures and CO_2 availability (Thouvenot et al., 2015; Gillard et al., 2017). To this regard, the recent development of more portable and less expensive instruments for measuring chlorophyll fluorescence (Kuhlgert et al., 2016; Chen et al., 2019; Gomez-Sanchez et al., 2019), currently targeted at agronomic applications, should enable the collection of larger amount of data on some synthetic metric of physiological performance (e.g., photosynthetic yield), thus allowing the increment of cover and extent of physiology traits studies.

Interactions with herbivores were often studied using a combination of traits that describe the palatability of a species: usually these traits include the elemental composition of tissues, the phenolic content, and the Plant Dry Matter Content or Leaf Dry Matter Content, in order to detect differences in the response to herbivores between native and invasive species and outline a possible management solution against invasive species, and understand the reasons for their successful competition (Grutters et al., 2016; Thiébaut et al., 2017), or to determine the effects of the introduction of invasive herbivores, so that the choice of poorly palatable species in restoration action can prevent the spread of herbivores (Yam et al., 2016). Similarly, relations with herbivores and palatability traits are used to identify the most suitable (e.g., less palatable) species to introduce in constructed wetlands and other restoration actions (Paz et al., 2019). On the other hand, the least investigated traits directly describing *Biotic interactions* in terms of relations with bacteria and fungi as an uptake strategy. We found only four papers focusing on this subject, of which three are reviews that discuss the role and importance of traits describing the interactions with bacteria (Bornette and Puijalon, 2011) or mycorrhiza (Fusconi and Mucciarelli, 2018; Ali et al., 2019). The only study we found that experimentally measured bacterial associations is by Rejmánková et al. (2011), who attempted to explore plant strategies for phosphorus uptake and related phosphatase activity to bacteria associated to roots. In general, as we mentioned above, root traits have been quite understudied. Within our research there are several papers (n = 111) that deal with combined shoot and root traits, however, most of the time they principally concern root biomass, to calculate the root-shoot ratio (e.g., Fu et al., 2013; Hussner and Jahns, 2014; Dong et al., 2017).

Multi-scale trait patterns

Exploring plant functional variability at different scales in both spatial (from community to ecosystem, up to landscape) and temporal (from daily to seasonal dynamics, up to long-term changes) dimensions requires an approach that is at the same time effective and feasible (Abelleira Martinez et al., 2016; Anderson, 2018). Remote sensing provides high-throughput data and techniques that can be translated into quantitative metrics related to vegetation features and overcome logistic and economic constraints of directly measuring most of the plant species inhabiting all biomes (Jetz et al., 2016; Gamon et al., 2019). Remote sensing applications to trait-based vegetation studies have shown an increasing trend during the last couple of decades (Homolová et al., 2013; Wang and Gamon, 2019), with a particular focus on terrestrial plant communities, especially in forest and grassland ecosystems (e.g., Asner et al., 2015; Schneider et al., 2017; Schweiger et al., 2018), but some studies on aquatic plants have recently emerged, implementing and extending in situ measurements (Villa et al., 2014, 2017).

In our research, we found 9 papers applying remote sensing techniques to macrophyte studies, focusing in particular on floating and emergent growth forms. Interactions between light and plant canopy elements, in particular reflectance and transmittance due to leaves, shape vegetation spectral response; these interactions result in a strong link between anatomical and biochemical properties (Leaf Pigments Content, Specific Leaf Area, Leaf Tissue Density) and optical properties (Klančnik et al., 2014; Klančnik and Gaberščik, 2016), which in turn can be exploited to model the performance and productivity of macrophytes stands (Liu et al., 2011). For example, Wang et al. (2012) used indices obtained from multispectral remote sensing data (Normalized Difference Vegetation Index and Vegetation-Water Index) to classify vegetation functional types in relation to water level dynamics. An approach based on remote sensing has found application also in the determination of traits favoring invasion success: Santos et al. (2012) used airborne imaging data to compare pigments and light use efficiency of native and non-native submerged species, and Tóth et al. (2019) characterized morphological and physiological traits with leaf reflectance for autochthonous and allochthonous emergent species. The contribution of remote sensing data in this context allows for a larger scale sampling and a prompter evaluation of seasonal variability of the macrophytes stands (Tóth et al., 2019). Reflectance and transmittance spectra of floating-leaved species were also measured as specific traits that influence light availability in the water column and then alter the environmental conditions underneath the water surface, and these properties can be explained by species exhibiting different morphological and biochemical leaf traits (Klančnik et al., 2018).

Species covered

The papers included in our review have applied functional traits to a total amount of 1124 aquatic *taxa*, which were in most cases identified to the species level, but for few studies the identification reached only the genus level (e.g., Molnár et al., 2015; Cao et al., 2016; Cornacchia et al., 2019). Some papers included also terrestrial species (Zhang et al., 2017; Dalle Fratte et al., 2019), but they were not considered in the evaluation of the diversity of species studied in this review. The world macrophyte species diversity has been estimated to count on 3457 species (Murphy et al., 2019), so our study revealed that in the last ten years about one third of the total macrophyte diversity has been explored in terms of functional traits.

However, if we consider only the two categories of papers that focused on up to six species, the taxa investigated are only 213. This suggests that specific ecological questions have been asked only on a very limited portion of the total macrophyte diversity, while most of the diversity is explored in the context of vast community studies (e.g., Monção et al., 2012; Török et al., 2013), in which mainly "soft traits" are used (e.g., morphology traits), even if it is "hard traits" (e.g., physiology traits) that could be more explicative of plant functionality (sensu Hodgson et al., 1999; Cornelissen et al., 2003), although more difficult and expensive to measure. According to our results, the ten most studied species are: *Myriophyllum spicatum* (63 papers), *Ceratophyllum demersum* (52 papers), *Potamogeton crispus* (41 papers), *Stuckenia pectinata* (40 papers), *P. australis* (39 papers), *E. canadensis* (35 papers), *Potamogeton perfoliatus* (31 papers), *Lemna minor* (30 papers), *Hydrilla verticillata* (28 papers) and *Persicaria amphibia* (28 papers). Common applications of traits for these species include the research of features determining plant palatability, physiological adaptations in response to eutrophication and the presence of contaminants, and plant adaptations to hydrological stress (Table 1, AI.3). Most of these species were well represented both in community studies and in specialized experimental studies: for *M. spicatum* see Thouvenot et al. (2019) and Fu et al. (2020); for *C. demersum* see Fu et al. (2017) and Sun et al. (2018); for *P. australis* see Yam et al. (2016) and Sikorska et al. (2017). However, species belonging to the genus *Potamogeton* (including *S. pectinata*), although widely spread across aquatic plant communities, were very poorly represented in the latter category of studies (3, 2, and 1 papers, respectively; Amano et al., 2012; Gillard et al., 2017; Riis et al., 2018; Zhu et al., 2018a; Zhang et al., 2019; Pätzig et al., 2020), indicating a need for further examination of their functionality.

Connections among topics

If we consider how papers are connected with each other in terms of the examined categories and topics, it is quite difficult to observe distinct clusters of narrative trends: most subjects are quite evenly linked with each other (Fig. 5). However, it is still possible to detect at least one strong narrative trend, which, to some extent, had already emerged in the above discussed paragraphs: studies that investigate the topic of environmental gradients mainly use morphology and productivity traits measured in field, with a notable portion of laboratory studies on submerged species (and less frequently on emergent ones), and relate these traits to water parameters especially in lakes, covering sites located in Asia and Europe. This pattern is consistent with the most studied features observed for each category (Fig. 2, Fig. 3, Fig. 4). The works of a group of scientists of the Chinese Academy of Sciences from Wuhan and Beijing are emblematic of this trend (e.g., Fu et al., 2013; Zhu et al., 2018a, b; Su et al., 2019). For example, Su et al. (2019) investigated how plant size and biomass of submerged species could establish feedbacks determining water transparency in subtropical shallow lakes. In this case, they found that small, bottom dwelling macrophytes were more effective in maintaining water transparency because they impeded more efficiently sediment resuspension, and released more oxygen to the water column, thus probably contributing to phosphorus immobilization. Overall, the pattern of connections among features shows that the topic of *Environmental gradients* has been exhaustively explored and linked to nearly all the subjects we considered in this review (Fig. 5). Other topics do not show the same amount of coverage: for instance, *Anthropic pressure* and *Invasiveness* are strongly linked only to water parameters, among all environmental variables. Nevertheless, *sediment* or *hydrology* characteristics have been demonstrated to be fundamental in determining the variability of root (Ali et al., 2019) and shoot traits (Zhu et al., 2018a), and therefore plant function in the ecosystem, especially in the context of invasive species (Venter et al., 2017). On the other hand, we mentioned root traits received far less attention than shoot traits, and we therefore suggest implementing the integration between root traits and sediment characteristics in future studies. At the same time, the topic of *Invasiveness* has been studied mainly from the point of view of *morphology* and *productivity* traits, setting aside *reproduction* traits. Although vegetative propagation seems to be the main mechanism of spreading of aquatic invasive species (Bashir Shah et al., 2014; Urban and Dwyer, 2016), sexual reproduction may also be important in spreading dynamics. This could either lead to loss of genetic diversity, due to hybridization with native species, or higher vigor to hybrids in case of hybridization with non-natives, as observed for *Ludwigia* spp. in Brazil (Thouvenot et al., 2013). Moreover, Kwong et al. (2017) found that fruit weight and fruit number in *Sagittaria platyphylla* was higher in introduced ranges than in native habitats, due to the absence of specialist herbivores. This work suggests the importance of evaluating the effects of biotic interactions on various traits categories and not only on biochemistry and productivity, as in most papers analyzed here (e.g., Grutters et al., 2016; Jiménez-Ramos et al., 2018). *Reproduction* traits resulted the third most studied trait category, however it does not keep the same position as for number of links, being related mainly only to the topics of environmental gradients and anthropic pressure (Fig. 5). Finally, we observed that the two continents that count the highest number of papers are not equally connected to all the subjects considered in this review: on one side Asian studies mostly stick to the most common pattern of lake studies on plant responses to water parameters, and on the other European studies basically encompass all the other subjects; the rest of continents are extremely underrepresented.

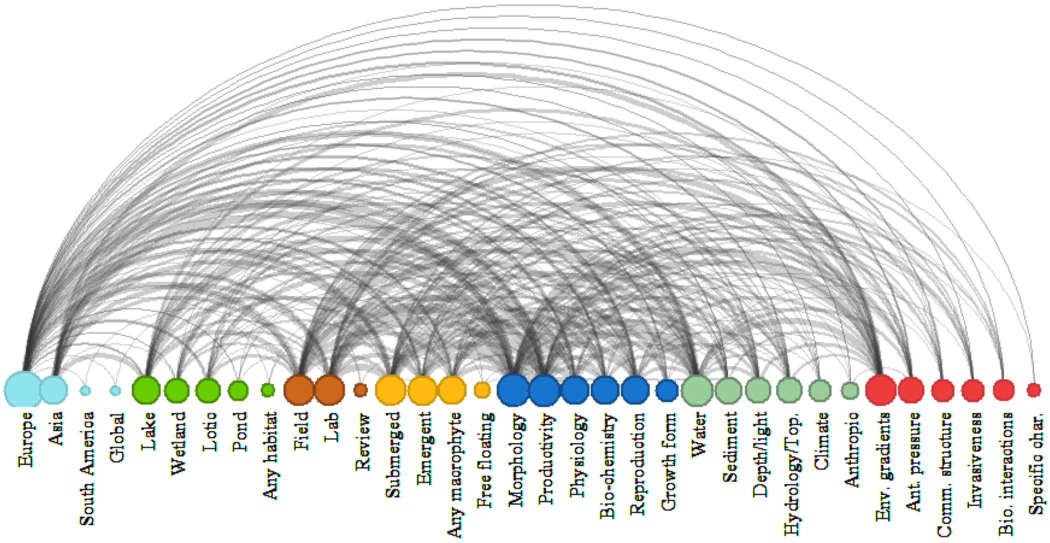

__Fig. 5__. Diagram illustrating the major links among the features considered in this review. Arc line width is representative of the strength of the link between two nodes, i.e., the number of papers including both nodes in the study, and circle size is proportional to how many connections the node installs with other nodes. For clarity links weaker than 15 (less than 15 studies showing that connection) are omitted, just as nodes not showing links of this strength.

2.5 A research agenda

What emerges from this systematic review is that the use of functional traits in aquatic botany studies enormously increased in recent years (almost doubling in the last 5 years compared to the period 1969–2014). Indeed, researchers have long been dealing with macrophytes functional characteristics: see for example the works on the macrophyte productivity by Hogeland and Killingbeck (1985) or plant strategies by Murphy et al. (1990). However, only recently this research field has benefited from a standardization of measurements and a sharing of the information collected in online databases. Although so far studies have been very heterogeneous in their purposes and methods, highlighting the vast range of the research fields that can be investigated using a functional trait approach, here we tried to offer a unified perspective. This allows researchers to identify a few aspects that can be represent a starting point for future developments in studying traits applied to macrophytes:

i. In the papers examined in this review, sediment characteristics have been associated to traits almost as often as other parameters like hydrology or water depth and light availability, confirming the importance of substrate type influencing plant traits (Xie and Yu, 2011a; Anjum et al., 2012) and performance (Bolpagni and Pino, 2017). Roots of aquatic plants colonize the sediment and so they represent the plant interface between the water column and the rhizosphere, and although aquatic plants are able to absorb nutrients from shoots as well, roots are not only passive organs in charge of ensuring anchorage to the substrate, but they have an active role in determining plant performance (Huang et al., 2018; Moe et al., 2019). However, we noticed a consistent lack of interest towards root traits, except for root biomass and number (e.g., Glover et al., 2015; Silveira and Thiébaut, 2017), whereas much less attention has been given to anatomy and physiology traits such as root lacunal volume and different tissues proportions, elemental composition, exudates and uptake strategies, which could reveal crucial implications for a deeper understanding of macrophytes functions (Kordyum et al., 2017; Ali et al., 2019). Again, we believe that traits related to root biotic interactions (we refer to bacterial and mycorrhizal associations) should receive further attention, because of their potential in influencing plant functioning (Rejmánková et al., 2011; Fusconi and Mucciarelli, 2018). It has been demonstrated that structural and physiological root traits play an important role in influencing other levels of biotic interactions, so their collection should be implemented: for example, root density was related to plant ability to regrow after herbivores damage (Wood et al., 2018). Therefore, we would like to stress the need of further collection and processing of macrophytes root traits and the study of the relationships with sediment characteristics, in view of a change of perspectives, which will see plant roots as major actors of life dynamics and not only as shoot subordinates.
ii. A main goal for future studies in this field will be to effectively capture the complexity that is intrinsic in natural systems dynamics, especially in aquatic ecosystems. The environmental heterogeneity characterizing macrophytes habitat, connected with their high phenotypic plasticity (Vivian-Smith, 1997), results in fine-scale patchiness of aquatic

plant communities, and disentangling trait variability among and within species in more than few ecosystems would require an amount of data impossible to collect in the field using traditional data collection techniques. Integrating remote sensing into the functional measurements and monitoring pipeline can enable the effective upscaling of some relevant community traits (Anderson, 2018), especially for emergent or floating-leaved species, thus helping to study the spatial variability of functional traits across systems, and its links with ecological processes (Funk et al., 2017). Optical passive data can be integrated with acoustic systems (i.e., side-scan sonar, echo sounders, and multibeam sonar) for tri-dimensional characterization of submerged macrophytes, especially canopy structural parameters (Bučas et al., 2016; Mizuno et al., 2018). The multiple roles of macrophytes are well known and they state that macrophytes, as primary producers, do not live in isolation but they constantly interact with the other biotic and abiotic components (O'Hare et al., 2018). It will be essential to deepen our understanding of these interactions by applying traits-oriented frameworks, e.g., the Biodiversity-Ecosystem Functioning approach (Tilman and Downing, 1994), in order to have a more complete view of ecosystems functioning, avoiding separating different compartments during the assessment.

iii. Invasive species represent one of the main threats to biodiversity and ecosystem functioning that aquatic habitats are facing in recent years (Strayer, 2010; Bolpagni et al., 2015; Rumlerová et al., 2016). Biological invasions correspond indeed to one of the most investigated topics among the papers we analyzed; however, we noticed some research gaps in this field, related to the type of traits studied and the environmental parameters associated. We promote the extension of these studies to other functional traits besides morphology, productivity and elemental composition, since there is evidence that also physiological and reproduction traits play an important role in non-native species establishment and colonization success (Kwong et al., 2017; Tóth et al., 2019). Moreover, the role of traits in invasive species has seldom been associated to environmental variables other than water chemical and physical parameters, although other parameters have been demonstrated relevant effects, such as light availability, in driving competition with native species, especially in the first phases of establishment (Ellawala Kankanamge et al., 2019), and hydrology parameters, in determining important consequences in propagule dispersion and plant resistance to variable water regimes (Urban and Dwyer, 2016; Zhang et al., 2016). Remotely sensed data, allowing quantitative, standardized measures of specific traits (Tóth et al., 2019), can make the allochthonous vs. autochthonous species comparison feasible across scales and sites, thus facilitating the assessment of environmental drivers for invasiveness (Rocchini et al., 2015; Niphadkar and Nagendra, 2016), at least for floating and emergent plants. We also encourage the investigation of invasive species and their biotic interactions, focusing in detail on the effects of specialist herbivores rather than generalists and on their foraging strategy (e.g., foraging on meristems and flowering organs rather than on mature leaves), which could be more effective in the control of invasive alien aquatic plants (Grutters et al., 2016).

iv. One of the main purposes of trait-based studies should be to allow for comparisons at multiple scales, as wide as possible. However, our review highlights how most of the recent research in the context of aquatic macrophytes has been carried out in Europe and China, while entire continents like Africa and Oceania have been almost neglected. Besides, very little attention has been given to some important hotspots of macrophyte biodiversity, like Brazil, which alone hosts more than one fifth of the global macrophyte species pool (Murphy et al., 2019). The same study from Murphy et al. (2019) divided the globe into squares of 10 × 10° latitude × longitude in order to evaluate global macrophyte diversity, and it states the urgency of not neglecting any part of the world, since all the squares contained at least 55 different species. It is then clear how global research on macrophyte functional traits is omitting some of the regions hosting the highest diversity. In this context, collaboration within the scientific community is essential in order to share the expertise and reach a faster advance in macrophyte functional traits research. The pledge of favoring a wider and immediate collaboration has already been launched in the context of carbon emissions from inland aquatic habitats (Marcé et al., 2019), and we believe that this concept is particularly fitting our field as well. Moreover, remote sensing-based trait maps can increase the resolution of current knowledge we have on plant diversity, by improving the spatial scale of analysis where trait data available are already abundant, and providing a mean to fill gaps where species or traits data are scarce (Jetz et al., 2016).

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3. Chapter II

THE IMPORTANCE OF BEING PETIOLED: LEAF TRAITS AND RESOURCE-USE STRATEGIES IN *Nuphar lutea*

Hydrobiologia
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ECOLOGY OF SHALLOW LAKES

The importance of being petioled: leaf traits and resource-use strategies in *Nuphar lutea*

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3.1 Introduction

Macrophytes are rapidly declining worldwide, mainly due to increased stress induced by water exploitation, habitat degradation, eutrophication, and the accumulation of alien and invasive species (O'Hare et al., 2018; Miguel-Ruano & Sanchez-Carrillo, 2020; Bolpagni, 2021). However, a deep and sound understanding of macrophytes functioning and dynamics along environmental gradients (e.g., trophic and depth) is still needed to prevent their decline (Azzella et al., 2017; Ghirardi et al., 2019; Angove et al., 2020).

Functional traits have been used to address a variety of ecological questions and have become more and more popular in the study of aquatic macrophytes over the last few years (Dalla Vecchia et al., 2020). The potential of functional traits lies in the opportunity to investigate in detail the response of single species or entire communities to a range of biotic and abiotic factors influencing their life cycle, functioning, and environment (Fu et al., 2015; Villa et al., 2017; Dalla Vecchia et al., 2020).

A common trade-off has been observed in the resource-use strategy in aquatic as well as in terrestrial plants, namely between "fast and acquisitive" and "slow and conservative" strategies, which is particularly evident in leaf traits that imply a trade-off between fast growth on the one hand and tissue construction and protection costs on the other hand (Díaz et al., 2016; Zervas et al., 2019). To investigate the resource-use strategies of plants, a focus on traits belonging to the plant and "the leaf economics spectrum" (LES) has been proposed (Wright et al., 2004). The LES includes a complex of traits that addresses the resource allocation and investment in the photosynthetic organs, like, among others, the specific leaf area (SLA), the leaf dry matter content (LDMC), the leaf chlorophyll content, and the leaf nitrogen and phosphorus content (LNC and LPC, respectively) (Cornelissen et al., 2003; Dalle Fratte et al., 2019).

Trait variability is usually higher among species rather than within species (Siefert et al., 2015). However, accounting for intraspecific trait variability (ITV) is awakening a renewed interest among researchers in recent years (Benavides et al., 2020), because it could provide valuable information on the interactions among different plant species and between species and the environment (Violle et al., 2007). The importance of intraspecific compared to interspecific trait variability can be relatively high: Siefert et al. (2015) reported that, on average, ITV accounted for 25% of the total within community trait variance in terrestrial plant species. Neglecting to account for it could lead to only partial interpretation of the results and could mask important processes happening at the local and larger scales (Wong & Carmona, 2021).

In this context, macrophytes have been described to show a high plasticity in traits that help them deal with the natural variability of the aquatic environment (Wells & Pigliucci, 2000). Macrophytes are considered as highly acquisitive species, in agreement with their usually high values for SLA and high relative growth rates (Poorter et al., 2009; Grime et al., 2014). However, SLA is highly variable in macrophytes as well, and Pierce et al. (2012) show that its variability is even greater than in terrestrial plants. Moreover, among different macrophytes growth forms, they also found that nymphaeids presented the widest variability in leaf size and mass traits. Therefore, it will be particularly interesting to investigate the mechanisms lying behind ITV for macrophytes and nymphaeids, in particular.

The decision whether to include petioles in leaf trait measurements clearly depends on the specific research questions of the study (Pérez-Harguindeguy et al., 2013), but in some cases including or omitting the petiole will determine significant differences in the trait values, especially for traits like SLA and LDMC (Wang et al., 2017; Wu et al., 2019). Among macrophytes growth forms, nymphaeids present a considerable plant height variability which will depend on water level and may grow in water up to few meters deep (Sculthorpe, 1967). To bring the leaves to the water surface, these plants need to invest in building a long petiole, which will represent a substantial proportion of the leaf mass and area (Richards et al., 2011, 2012). Besides reaching the water surface, the petioles of nymphaeids also work as ventilating system: oxygen flows in young leaves through the petioles and pushes out carbon dioxide and methane from older leaves, promoting colonization in deeper water (Richards et al., 2012). The scientific literature offers examples of studies that explain the relationship between water depth and macrophytes traits (i.e., Demetrio et al., 2014; Fu et al., 2014b; Li et al., 2018); however, to our knowledge, a clear evaluation of how nymphaeids' functional traits respond to both water and sediment qualities is still missing (but see Henriot et al., 2019).

With this study we aim at investigating the ITV of leaf traits of a representative nymphaeid species, the yellow water lily (*Nuphar lutea* (L.) Sm.) at a very local scale. Specifically, we mean to unravel the resource-use strategies of *N. lutea* in a hyper-eutrophic shallow lake, in relation to the resource allocation in leaf petioles and blades along local gradients of water and sediment conditions. We hypothesize that water depth has a strong influence on both petiole and blade structural traits of *N. lutea*, inducing the plant to optimize the resource allocation in blades and petioles in deeper water, and that sediment features, specifically those related to trophic level, would also determine changes in the resource-use strategy of this species, suggesting more stressed conditions for the plant under high trophic level.

3.2 Materials and methods

Study site

Lake Chiusi (43° 03′ 22.11″ N, 11° 57′ 55.79″ E) is a shallow hyper-eutrophic lake located in Tuscany, central Italy (Fig. 1). The lake lies at 251 m a.s.l. and has a surface of 3.9 km2 and an extended drainage basin of 105 km2. Fischer et al. (2016) reported a mean water depth of 3.5 m and a maximum depth of 5.8 m. However, the same authors report that the lake is subjected to rapid siltation and sedimentation, so the mean depth is likely to be decreasing over the years (Lastrucci et al., 2014). The main tributary is the stream Tresa and there is one emissary, the Fosso della Ripa, which brings waters to the nearby Lake Montepulciano. Bigi & Rustici

(1984) reported a mean annual air temperature of 13.8°C and a mean annual rainfall of 736 mm.

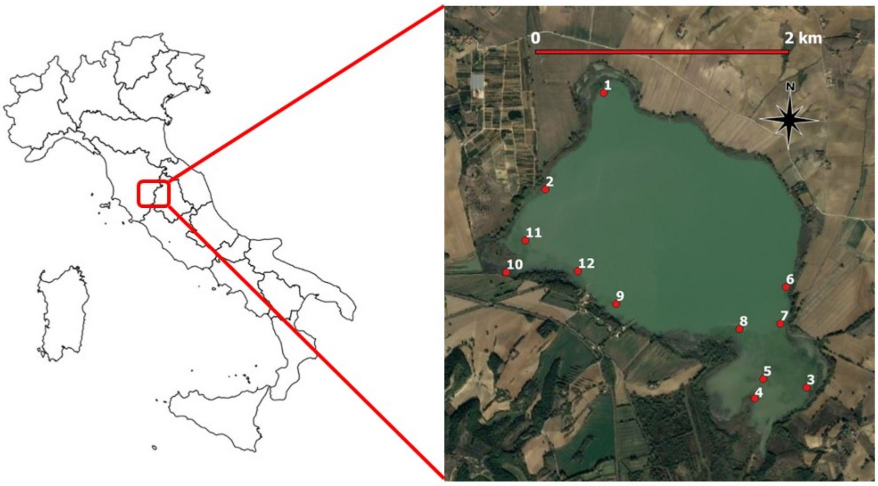

Fig. 1: *Map of Lake Chiusi showing the location of sampling plots*

Evidence for the high trophic state of the lake is documented since the 1980s (Arrigoni & Ricceri, 1981; Balsamo, 1990). The most recent available data from the Italian monitoring program report a mean annual concentration of Chlorophyll-*a* of 49 μg l^{-1}, classifying the lake in a bad state of conservation (Cavalieri et al., 2018). High nutrients inputs come from the surrounding intensive agricultural activities; however, human pressure is homogeneously diffuse on the lake without clear patterns (SIRA ARPAT Toscana, 2005). The lake itself is exploited for recreational and professional fishery, but these activities are not intensively affecting the study area nor are they directly exploiting the macrophytes stands. Nevertheless, to avoid any possible direct human disturbance on the studied *N. lutea* plots, leaves and environmental samples were collected only from the internal sectors of stands (at least at 3 m from the edges of the target stands). The site is also a protected area named "Lago di Chiusi" and in the context of the European 92/43/EEC 'Habitats' Directive it represents a site of community importance (SCI). The lake vegetation is constituted by extended monospecific populations of *N. lutea*, with the sporadic presence of *Nymphaea alba* L. Helophytes communities are represented by *Phragmites australis* (Cav.) Trin. ex Steud., *Carex elata* All., and *Carex riparia* Curtis, while in the more riparian zone we can find examples of hygrophilous forest, like *Salix alba* L., *Salix cinerea* L., and *Populus nigra* L. (Lastrucci et al., 2014).

Field sampling and environmental characterization

The field sampling was carried out at the end of July 2020 and was part of the field campaigns programmed for the macroDIVERSITY project. 12 spatially independent plots of 16 m^2 (monospecific *N. lutea* stands; see Fig. 1) have been chosen and characterized by sampling water, sediment, and leaves. Water temperature, dissolved oxygen (DO), conductivity (Cond), and pH were measured directly in the field using a multiparameter probe (YSI 556 MPS). Secchi disc and photosynthetically active radiation on the water surface and 1 m below the surface were also measured. Water samples were filtered in the field with GF/C glass fiber filters (Whatman), kept refrigerated in cool boxes, and frozen at − 20°C as soon as possible for further nutrients analyses. A sediment core was collected and the top 5 cm homogenized and refrigerated for further analyses. In the laboratory, soluble reactive phosphorus (SRP) and dissolved inorganic carbon (DIC) were determined within 72 h. SRP was determined spectrophotometrically following Valderrama (1977), and DIC was determined using titration with HCl 0.1 N (Anderson et al., 1986). Sediment features (density, water content, porosity, organic matter content) were determined gravimetrically (Buchanan et al., 1984). Sediment total phosphorus (TP) was determined spectrophotometrically after acid extraction on a subsample of sediment ashes muffled at 450° for 4 h, following Aspila et al. (1976). At each plot, 8 undamaged, full-expanded leaves (including petioles) were collected, which presumably belonged to different individuals and represented similar life stage. Leaf samples were kept moist in wet paper towels and sealed in plastic bags and then refrigerated and processed within 72 h.

Functional traits measures

For this study we measured a set of structural traits (Table 1) – leaf area (LA), leaf fresh weight (LFW) and dry weight (LDW), specific leaf area (SLA), and leaf dry matter content (LDMC) – for the whole leaf, including petiole, for the leaf blade only, and for the petiole only. Biochemical traits – phosphorus content (leafTP), chlorophyll-*a* (Chl-a), chlorophyll-*b* (Chl-b), carotenoids (Car), and the ratio between chl-*a* and chl-*b* (Ca/Cb) and between chl-*a* plus chl-*b* and Car (Chl/Car) – were measured in leaf blades only and reported on dry weight basis for TP and fresh weight basis for pigments ($\mu g\ g^{-1}$). The SLA is the ratio between the LA (expressed in mm2) and the LDW (mg), and a high SLA is considered an acquisitive trait, because it implies a lower investment in structural tissues for the photosynthetic organs, therefore a higher photosynthetic capacity per unit investment (Dalle Fratte et al., 2019). The LDMC is the ratio between LDW and LFW (g); a high LDMC, on the other hand, indicates a higher investment in structural tissues, revealing a more conservative strategy (Díaz et al., 2016). LeafTP and chlorophyll content reflect the plant photosynthetic capacity but also the tissues nutritional quality; however, they can vary quite consistently with the environmental availability of N and P (Wright et al., 2004; Pérez-Harguindeguy et al., 2013). To ensure traits comparability among leaves and plots, only full-expanded leaves without signs of herbivory have been collected as a proxy of similar age class. Indeed, the outermost *N. lutea* leaves (compared to the central portions of the stands) showed visible signs of herbivory that can be linked to a more prolonged emerging phase.

***Table 1**: list of measured leaf traits with corresponding units of measurement and identification tags used in the text.*

Trait	Unit of measurement	Trait tag
Leaf area	mm^2	LA
Blade area	mm^2	bladeA
Petiole area	mm^2	petA
Leaf fresh weight	g	LFW
Blade fresh weight	g	bladeFW
Petiole fresh weight	g	petFW
Leaf dry weight	g	LDW
Blade dry weight	g	bladeDW
Petiole dry weight	g	petDW
Specific leaf area	$mm^2\ mg^{-1}$	SLA
Specific blade area	$mm^2\ mg^{-1}$	bladeSLA
Specific petiole area	$mm^2\ mg^{-1}$	petSLA
Leaf dry matter content	$mg\ g^{-1}$	LDMC
Blade dry matter content	$mg\ g^{-1}$	bladeDMC
Petiole dry matter content	$mg\ g^{-1}$	petDMC
Total phosphorus content	$mg\ g^{-1}$	leafTP
Chlorophyll-*a* content	$mg\ g^{-1}$	Chl-a
Chlorophyll-*b* content	$mg\ g^{-1}$	Chl-b
Carotenoids content	$mg\ g^{-1}$	Car
Chlorophyll-*a* to Chlorophyll-*b* ratio		Ca/Cb
Chlorophylls to carotenoids ratio		Chl/Car

Water-saturated leaves were delicately washed free of debris and epiphytes, scanned for LA, and immediately weighted for LFW. They were subsequently dried at 50°C until constant weight and then LDW was determined. LA was determined analyzing scanned images with the software ImageJ (Rasband, 1997-2018). LeafTP was determined using the same method described for sediment TP. Pigment content was determined after 24-h extraction in 80% acetone (Wellburn, 1994). Structural traits were measured on five leaves per plot, while biochemical traits were determined on three leaves per plot, for a total of 96 leaves processed.

Statistical analyses

To inspect for general patterns of *N. lutea* trait responses to environmental variables, a redundancy analysis (RDA) was performed, which fits a multivariate multiple regression of response over constraining matrices (in this case trait values over environmental variables) and performs a principal component analysis on the resulting matrix of fitted values (Legendre & Legendre, 2012). Traits mean values for each plot were used to build the response matrix. We first included water depth, pH, conductivity, DIC, SRP, sediment porosity (por.sed), sediment organic matter content (OMC.sed), and sediment total phosphorus (TP.sed) as environmental variables, but they were then checked for collinearity and those with a Pearson's correlation coefficient higher than 0.7 were omitted. We omitted water temperature and DO a priori because, in a shallow hyper-eutrophic lake, like Lake Chiusi, they were highly variable throughout the day and plots were not sampled at the same time each day. All trait values and environmental variables included in the analysis were centered to their mean, to avoid bias related to the different scales or units of measurement of variables. A permutation test was performed after running the RDA to check for significance of the canonical axes.

To complement the RDA, we fitted linear mixed models to check for more direct relations between environmental variables and each trait and give more consistency to the analysis. This time, all the trait values from the 96 leaves measured were implemented. We use the plot number of samples as a random effect in the model. All the available environmental variables mentioned above for the RDA (omitting water temperature and DO) were employed for modeling and were standardized. We built one model for each trait, and specifically for the whole leaf trait, the blade and petiole-only traits. We proceeded with the selection of the best fitting model using the function {dredge} and selecting the model with the lowest Akaike Information Criterion (Greenacre & Primicerio, 2014). Trait values were log transformed when patterns in the distribution of residuals were encountered.

In order to assess the contribution of the petiole and the corresponding allocation of resources, the proportion of area and fresh and dry weight of petioles over the whole leaf were calculated. Linear mixed models were then fitted to petiole proportion to investigate if the most important variable leading the variation of leaves structure could also influence the resource allocation between petioles and blades. Again, the plot number was included as random effect.

All statistical analyses were carried out with the software R (R Core Team, 2020), using the packages *vegan* (Oksanen et al., 2020), *corrplot* (Wei & Simko, 2017), *mgcv* (Wood, 2011), *lmerTest* (Kuznetsova et al., 2017), *usdm* (Naimi et al., 2014), and *MuMIn* (Barton, 2020) and finally *ggplot2* (Wickham, 2016), *dplyr* (Wickham, 2021), *forcats* (Wickham et al., 2021), and *gridExtra* (Auguie, 2017) for graphical outputs.

3.3 Results

Observations of the environmental variables and the *N. lutea* traits are available in Appendix II (AII.1 and AII.2). Despite the relatively small size of the studied lake (3.9 km^2), both environmental and functional data showed a wide variability between stands. For instance, colonized water depth, conductivity, and sediment TP ranged from 0.3 to 1.9 m, 533 to 608 µS cm^{-1}, and from 277 to 833 µg P g^{-1}, respectively (AII.2). At the same time, LA and LDW (as representative of functional traits) ranged from 250 to 1250 cm^2 and 2 to 16 g, respectively (AII.1).

Redundancy analysis

Several of the environmental variables were highly correlated ($r > 0.7$), so to run the RDA only water depth, DIC, SRP, and TP.sed were included. These variables contributed to explain 48.1% of the total variance in the data (constrained variance). Regarding the constrained components, the first two canonical axes cumulatively explained 86.0% of the variability, with RDA1 explaining 59.8% and RDA2 25.7% of the total constrained variance. The first RDA axis mainly described the water depth gradient, with depth decreasing with increasing values of RDA1 (RDA1 score = -0.99), but TP.sed and DIC also marginally contributed to the opposite direction of water depth (RDA1 scores = 0.49 and 0.45, respectively). The second RDA axis, on the other hand, was mainly related to sediment characteristics, with TP.sed having the highest RDA2 score (-0.62).

The analysis showed a clear differentiation between structural and bio-chemical traits, with structural traits (LA, LFW, and LDW) increasing their values with decreasing RDA1, thus with increasing water depth, and bio-chemical traits (pigments content) increasing their values with increasing RDA2, in relation with sediment characteristics (Fig. 2). The traits that are most related to RDA1 are petA (RDA1 score = -0.71), LA (RDA1 score = -0.70), bladeA (RDA1 score = -0.68), petSLA (RDA1 score = -0.68), and bladeFW (RDA1 score = -0.67), while those most related to RDA2 are Chl-a (RDA2 score = 0.54), Car (RDA2 score = 0.51), Chl-b (RDA2 score = 0.46), and SLA (RDA2 score = 0.45). However, after the permutation analysis of the model, only RDA1 resulted marginally significant (P-value < 0.1), while the second canonical axis did not show significance.

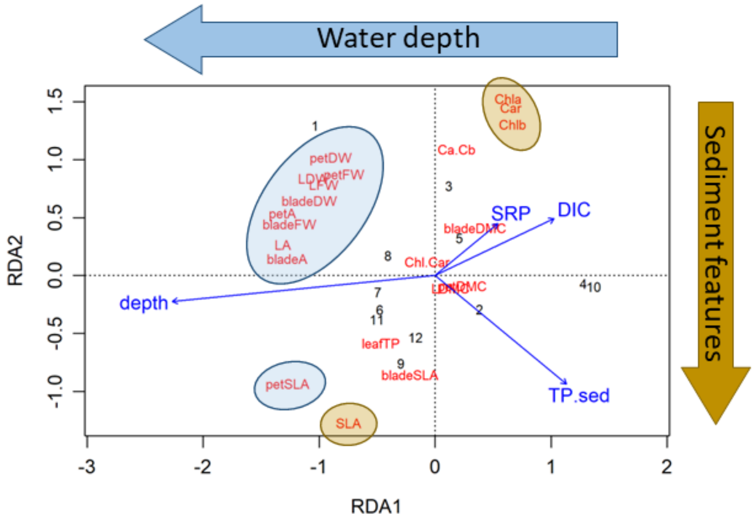

Fig. 2*: Results of the RDA representing the first two canonical axes. Environmental variables are shown in blue, traits in red, and plot numbers in black. The first canonical axis indicates a water depth gradient, and traits varying along this axis are highlighted in blue. The second canonical axis is related to sediment features, and traits more related to this axis are highlighted in brown.*

Linear mixed models

The linear mixed models partly confirmed the results of the RDA, identifying significant correlations between environmental variables and traits. Indeed, only water depth, conductivity, and sediment TP were significantly correlated with *N. lutea* traits (Table 2, graphical outputs available in AII.1). Water depth was significantly positively correlated with most of the structural traits, namely LA, bladeA, petA, bladeFW, LDW, bladeDW, and petDW.

Water conductivity was significantly positively correlated with pigments content (Chl-a, Chl-b, and Car) and negatively with petSLA. Sediment TP was positively correlated with bladeSLA and negatively with Chl-a. Some traits showed nearly significant relationships with environmental parameters, as was the case for LFW with depth (positive trend), or for SLA with conductivity, and bladeSLA with sediment OMC (negative trend) (P-values reported in Table 2). Besides these traits, no significant relationship with environmental parameters was found also for petFW, DMC of whole leaves, blades nor petioles, leaf TP, and ratios between pigments content (Chl-a/Chl-b and Chl/Car).

Table 2*. Results of the linear mixed models. Only traits and environmental variables (Depth = water column dept; Cond = conductivity at 20 °C; OMC.sed = sediment organic matter content; TP.sed = sediment total phosphorous) that showed significant relationships are reported. Asterisks indicate the level of significance resulting from the models (* p<0.001; ** p<0.01; * p<0.05). Orange colors reveal positive relations, blue colors negative ones; their intensity is related to the level of significance. P-values for nearly significant relationships are also reported (for the traits' tag see Table 1).*

Trait	Depth	Cond	OMC.sed	TP.sed
LA	**			
bladeA	**			
petA	***			
LFW	p=0.0519			
bladeFW	**			
LDW	*			
bladeDW	*			
petDW	*			
SLA		p=0.0639		
bladeSLA			p=0.0579	*
petSLA		***		
chla		***		**
chlb		**		
car		**		

Petiole contribution

Considering all samples measured for structural traits, the petiole area was on average 20.97% (± 3.98 sd) of the total leaf area, 67.29% (± 5.20) of the total leaf fresh weight, and 43.72% (± 6.21) of the total leaf dry weight (Fig. 3). Area and fresh and dry weight proportions were tested against water depth, and only petiole area proportion increased significantly with increasing water depth (P-value = 0.0154), while fresh and dry weight proportions did not show significant correlations with depth (graphical outputs available in AII.1).

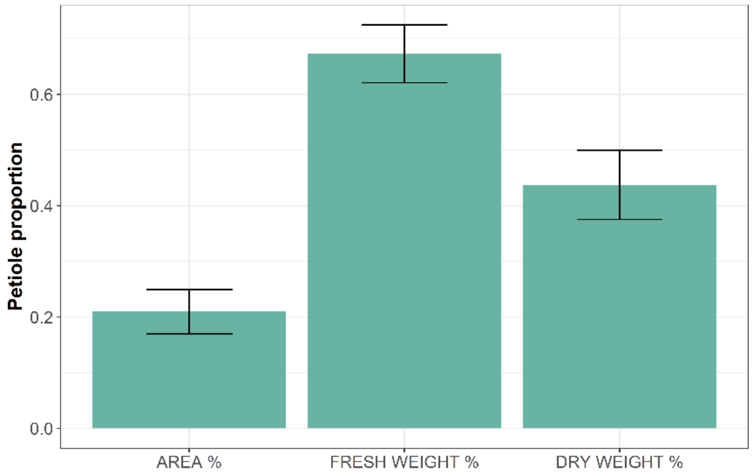

***Fig.* 3**: *Mean proportions of petiole area and fresh and dry weight on the leaf total measure (n = 60). Error bars represent standard deviation.*

3.4 Discussion

Our findings clearly highlight how intraspecific leaf trait variability in *N. lutea* is modulated by the environmental conditions at the very local scale (few hundred meters). Other studies confirmed the influence of the environment on *N. lutea* performance (Henriot et al., 2019); however, to our knowledge, this is the first attempt to unravel processes happening within a single ecosystem. Indeed, Lake Chiusi was an appropriate site to test the intraspecific variability of *N. lutea* at the local scale because it is a nearly shallow stagnant system, with no stratification and negligible water mixing by the tributary and emissary streams. In this system, the influence of the sediment on the water column defines clear environmental gradients especially in the littoral zone, even in such a small water body (3.9 km^2). The importance of water depth and substrate for the occurrence and survival of *N. lutea* was already evidenced in the literature (Jakubas et al., 2014).

Surprisingly, nearly half of the leaf biomass was overall allocated in the petiole (in the range 55-77% and 29-59% for fresh and dry weight, respectively). This information confirms the non-negligible role of petioles in contributing to the leaf organ functionality, highlighting the impossibility to omit petioles from leaf traits of *N. lutea* and nymphAeids, more in general (Li et al., 2011). Therefore, studies including only blade traits would eventually underestimate ecological processes and adaptive responses associated to this key macrophytes group for freshwaters.

Further, the petiole area increases proportionally more than blade area along the depth gradient, although the fresh and dry weight proportions remained constant. This indicates that *N. lutea* is allocating a constant relative amount of resources to petioles, even in deeper water: a new interesting evidence about the structural and eco-physiological behavior of *N. lutea* due to the pivotal importance of petiole aerenchyma or photosynthetic extra surfaces for nympheids to fit the environment. This opens the door for additional and deeper data: widening the environments and the ecological conditions to investigate is the next needed step to strengthen our understanding of the roles of petioles in macrophyte ecology.

Environmental drivers of **N. lutea** *traits variation*

As postulated, among the environmental parameters evaluated, we found that water depth and conductivity were the main drivers of the observed traits variability. Water depth is crucial for *N. lutea* and nymphAeids in general because it defines how much investment the plant needs to undertake to reach the surface with its leaves (Jin et al., 2017). Water conductivity, on the other hand, is a proxy for the trophic level of the surroundings of the sampling site (Yuwono et al., 2015; Smith et al., 2011) and in shallow waters it mainly reflects the influence of the sediment processes on the water column (Aftabuddin et al., 2017). In fact, water depth revealed to be highly negatively correlated with water conductivity (Pearson's correlation coefficient = 0.84), suggesting that processes happening at the sediment–water interface clearly influence the whole water column at shallower water depths.

However, patterns resulting from the RDA have to be interpreted with caution, because of the relatively small number of *N. lutea* stands (12) included in the analysis. This is especially true for the second RDA axis that was related to pigments content of leaves. Further efforts will be needed to widen the water parameters (as N forms) to be included among the explanatory variables. In the present study, we focused our attention on P forms (SRP in water and TP in sediments) observing a driving role only for the sedimentary pool, whereas SRP does not seem to be directly related to the observed ITV due to strict control locally exerted by phytoplankton (with recurrent summer blooms and peaks up to ~ 50 $\mu g\ l^{-1}$, Cavalieri et al., 2018).

Nevertheless, RDA outputs were confirmed by the linear mixed models. LA increased with increasing water depth, but not only due to the increased petiole length. Indeed, we also observed an increase in the blade area. This result is informative of the investment the plant needs to face in building petioles: the construction costs need to be rewarded by a bigger photosynthetic surface. This finding is in accordance with Richards et al. (2011), who additionally observed that a single individual built fewer leaves when living in deeper water, a trend that was found also under other potential stressors for the plant, like low nutrients, pH, and alkalinity (Klok & van der Velde, 2017). Another evidence for the costs related to the construction of petioles lies in the significant increase of their dry weight, but not fresh weight, along the depth gradient, indicating the need for supporting tissue. This reinforces the importance of open spaces (e.g., aerenchyma) to boost the transport of gas to root systems for guaranteeing their functions in a hostile medium (e.g., reduced sediments). In this respect, new data are urgently necessary to explore the adaptive structural responses of petioles along environmental gradients (depth and tropic level).

In our study site, the LDMC was not correlated with any variable investigated, suggesting that the variations in fresh and dry weight were able to modulate the variability of this trait. The SLA, instead, did show to be related with some environmental drivers, in this case those related to the trophic level of the system. This is in accordance with the hypothesis of Fu et al. (2014a), who did not find SLA to vary with water depth and proposed that nutrients could be more determining for aquatic plants. Considering the SLA an acquisitive trait, we can expect that a healthy plant can afford to have a higher SLA and keep a higher growth rate (Poorter et al., 2009). The negative relationship observed between SLA and variables related to the trophic level of the system (conductivity and sediment OMC) suggests that the plant is probably facing a stressful condition and is switching to a more conservative strategy. This relationship was only a trend for whole leaf and blade SLA, but it was clear for the petiole SLA. A high trophic level acts as fertilizer and promotes plant growth (Lehmann et al., 1997; Bornette & Puijalon, 2011), which in our case was confirmed by the increase in the blade SLA with increasing sediment TP or in the higher pigments content at higher conductivity values. Henriot et al. (2019) also found positive relationships between sediment phosphorus and growth and reproduction traits in *N. lutea*. But this situation can also trigger processes with negative impacts for plant growth: a high productivity in the system induces high respiration rates from the bacterial community, resulting in anoxia and consequent possible accumulation of toxic compounds (Muri & Wakeham, 2006; Wu et al., 2013; Henriot et al., 2019).

3.5 Conclusions

A very limited number of trait studies have focused their attention on petioles so far, especially for macrophytes, and international protocols for traits collection do not pay enough attention to aquatic plant's morphological peculiarities in defining, for example, whether petioles should be included or omitted in the trait analyses. Our results report that omitting information on petioles in nymphaeids would lead to a very partial understanding of plant responses to local environmental conditions. Petioles not only have a support and transport function but represent "functional-morphological hot-spots" capable of contributing significantly to the performance of individuals. The present results suggest the need to deepen the functional roles of petioles opening new research lines to better capture the biotic–abiotic interactions and dynamics in freshwaters. At the same time, rather than to find general laws of traits variation in the environmental space, with this study we also aimed to highlight that there are processes happening even within the lake scale (few hundred meters) that can influence the plant performance in multiple ways. Our dataset was limited in the number of samples, so a future integration with a higher number of observations in different sites will allow to deepen the trends observed in traits variation in this study for *N. lutea*.

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4. Chapter III

MULTIDIMENSIONAL TRAIT VARIABILITY IN YELLOW WATER-LILY, *Nuphar lutea*: FUNCTIONAL, SPECTRAL AND GENETIC DRIVERS[1]

4.1 Introduction

Macrophytes can colonize various aquatic environments and are present in different growth forms. Among them, rooted floating-leaved macrophytes represent a link between all components of aquatic environments: sediments, water and atmosphere. These species usually occur in lentic shallow waters: in fact, they fear deep water where leaves would struggle to reach the surface and avoid strong water current which could uproot them (Bornette and Puijalon 2009). In these environments they are top competitor species, for they have access to both water and sediment nutrients, and they shade submerged species with their – usually big – floating leaves (Pierce et al., 2012; Temmink et al., 2021; Dalla Vecchia and Bolpagni, 2022), and therefore can influence the composition of the local macrophyte communities.

Several anthropic pressures are posing serious threats to macrophytes stability and persistence worldwide: climate change, land-use change and habitat degradation, water pollution and the introduction of alien and invasive species are responsible for a general decline of macrophytes populations (Carpenter, 2005; Van der Wal et al., 2013; Lind et al., 2022). The first macrophyte group expected to disappear is submerged macrophytes, due to increased turbidity in the water. Specifically, rooted floating-leaved species are predicted to increase in abundance over submerged species in the transition from clear oligotrophic to turbid eutrophic water (Baattrup-Pedersen et al., 2016; Lind et al., 2022), however Zervas et al. (2019) also observed a shift towards emergent life forms because of eutrophication. In a context of rapidly changing habitats, it is of uttermost importance to understand how rooted

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[1] submitted to OIKOS.

floating-leaved species respond to variability of environmental conditions (Bolpagni et al., 2016).

To investigate the response of plants to environmental gradients, functional traits have often been implemented in ecological studies (Dalla Vecchia et al., 2020 and references therein). Traits can capture the influence of biotic and abiotic factors on morphological, physiological, phenological or productivity characteristics of a community or of a species (Violle et al., 2007). Indeed, traits are a valuable tool that allows to explore the intraspecific level of variability, enabling to truly understand the effect of the environment on plants performance. As stated by Siefert et al. (2015), intraspecific trait variability in terrestrial plants can account for 25 % of the total within-community trait variability and omitting to account for this variability could lead to a misinterpretation of processes. The use of traits including intraspecific variability is being encouraged in ecology (Liu et al., 2021), over the use of mean trait values only, to avoid a strong underestimation or overestimation of a species niche and performance (Violle et al., 2012). In addition to typically measured leaf traits, *in vivo* reflectance spectroscopy has been increasingly employed over the last few years as a tool to quantify foliar spectral properties, which can be related to structural, biochemical (e.g., pigments) and physiological parameters in macrophytes (Klančnik et al., 2018; Villa et al., 2021). The application of advancements in reflectance spectroscopy of plant traits to remote sensing data (airborne and spaceborne) could enable the acquisition of a great quantity of information at different ecological levels covering spatial and environmental gradients, which are valuable towards advancing functional plant ecology and biodiversity studies (Abelleira Martínez et al., 2016, Cavender-Bares et al., 2022).

Besides, plants phenotype is given by the interaction between plasticity and genetics (Violle et al., 2012), and if the measure of functional traits and spectral properties can provide a snapshot of the plant's features, genetic information can integrate the life history of a population. In fact, the degree of genetic differentiation within and among populations can explain part of the observed trait variation and suggest what could be the adaptation potential to changing environmental conditions (Agrawal et al., 2008; Parsons et al., 2011). In this regard, the degree of genetic differentiation in a population could also be, in part, a response to environmental pressures that select certain characteristics, and thus represent a view on the past driving forces (Pavoine et al., 2011).

Present environmental conditions also drive plants traits variability. Many studies have so far investigated the effect of environmental gradients on traits of different macrophytes growth forms (e.g., Fu et al., 2019; Iversen et al., 2019; Sharba et al., 2020; Castellani et al., 2022). Among the main drivers, temperature, water level and its fluctuation, light and nutrients availability (and eutrophication processes) have been associated to rooted floating-leaved species performance (Khanday et al., 2015; Henriot et al., 2019; Klok and van der Velde, 2022). Temperature determines the metabolism activation. Limitation of growth rates and leaves development exist at low or high temperatures (Amano et al., 2012), for example Klok and van der Velde (2022) observed an optimal interval between 10 and 30 °C for the growth of *Nymphoides peltata*. Leaf thickness and pigments content have been observed to change according to the position of the leaf, submerged in water or floating on the water surface in *Nuphar lutea* (Kordyum and Klimenko, 2013). Water depth also has an influence on plants economics, since floating leaves of rooted species must reach the water surface to be fully developed and functional, investing considerable resources in plant elongation (Richards et al., 2011; Dalla Vecchia and Bolpagni, 2022). Water depth, along with turbidity, also influences light availability. Higher light availability increases the photosynthetic activity and an increase in carbon stock, together with changes in the root/shoot ratio, have been observed in the rooted floating-leaved species *Nymphaea advena* (Cronin and Lodge, 2003). Light availability in the water column has a lower impact on emergent than on submerged species (Pan et al., 2017), however it is a determinant factor for submerged leaves development and seedling survival (Smits et al., 1990). Nonetheless, increased turbidity can indeed affect submerged leaves of emergent species. Eutrophication processes in aquatic environments are better described by the increase in water nitrate, phosphate and electrical conductivity (Baattrup-Pedersen et al., 2015). Increased nutrients were shown to increase growth and reproduction traits in *N. lutea*, but also caused toxicity-induced responses in both *N. lutea* and *N. advena* (Cronin and Lodge, 2003; Henriot et al., 2019).

The effect of all these drivers on plant traits, however, may vary according to the spatial or temporal scale observed, just as they influence species distribution (Alahuhta et al., 2018). The use of traits to highlight scale-dependent responses to environmental gradients is more appropriate than using species presence only, because traits show a closer relation to environmental variation than does species turnover, especially at local scales (Göthe et al., 2017). Li et al. (2022) found a difference in the magnitude of tree species traits at different spatial scale, rather than in the direction of relationships. However, Da et al. (2022) also found that main drivers of leaf traits variation in terrestrial species were not the same at different spatial scales, with climatic factors being more important than soil characteristics at larger scales. Approaches relying on remote sensing, based on high-throughput spectral data and quantitatively connected to ecologically meaningful information drawn from limited but high quality *in situ* measures, are a feasible and efficient option for thoroughly investigating plant functional variability across multiple scales (e.g. intra- to inter-specific, population to ecosystem, within- and among-sites, local to regional, up to global) and dimensions (structure, biochemistry and physiology) (Abelleira Martinez et al., 2016; Gamon et al., 2019; Dalla Vecchia et al., 2020).

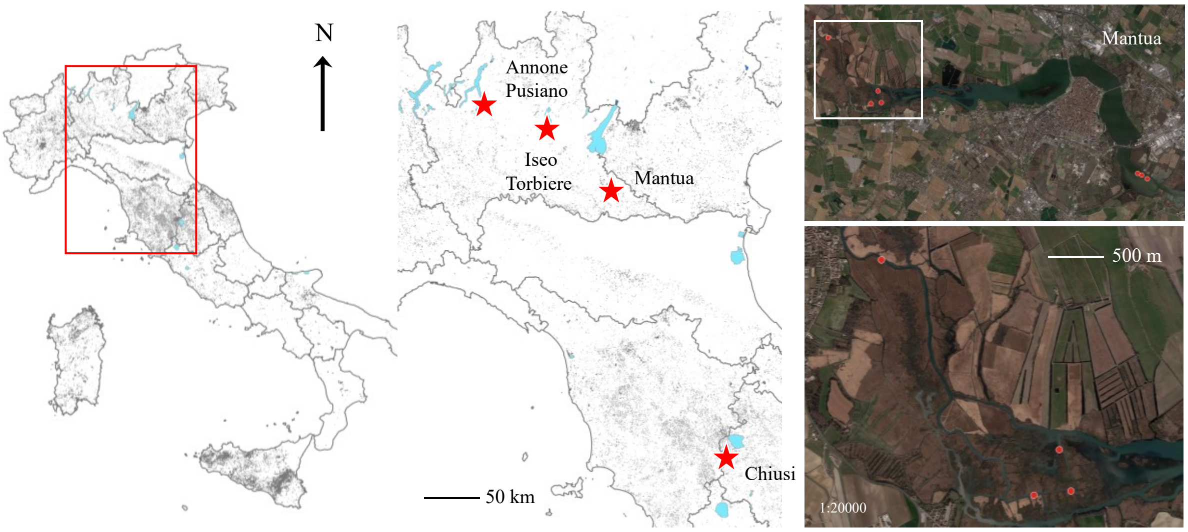
In this study we combine functional, spectral and genetic variability of a rooted floating-leaved macrophyte species to investigate its drivers along an environmental gradient. Previously, it has been demonstrated that *N. lutea* leaf traits vary with water depth and conductivity at the local scale (Dalla Vecchia and Bolpagni, 2022). Here we aim at testing the effect of environmental filtering on leaf traits and genetic diversity of 28 populations of *N. lutea* in four lake systems in Italy. We hypothesize that nutrients, rather than depth, represent primary drivers of multidimensional trait variability at the regional scale, because the effect of local depth differences would be overcome by the gradient of trophic levels among sites. Moreover, we also hypothesize that a directional variation of trait values may be promoted by selective pressure on DNA loci and the reduction of genetic diversity at the population level.

4.2 Material and methods

Study sites

This study was carried out in four sites located in central and northern Italy: lakes Pusiano and Annone (9.3° E, 45.8° N), Lake Iseo and Torbiere del Sebino (10.0° E, 45.7° N), Mantua lakes system (10.8° E, 45.1° N) and Lake Chiusi (12.0° E, 43.1° N), ordered by location from North to South (Fig. 1).

***Fig. 1**: Map of the study sites located in North-Central Italy, and a focus on the Mantua lakes sampling area, showing the spatial arrangement of sampling plots.*



Both Pusiano and Annone are sub-alpine lakes located south of Lake Como, between its two branches. Lake Pusiano has a surface area of around 5 km^2 and a maximum depth of 24 m, and is now considered to be phosphorus-limited, though it reached hypereutrophic conditions between the 1970s and 1980s (Legnani et al., 2005). Lake Annone covers a similar area of around 5 km^2, and has a maximum depth of 11 m, but it is divided into an eastern and a western basin connected by an isthmus, and it is classified as eutrophic (Rusconi et al., 2022). Lake Iseo is a deep sub-alpine lake, with a surface area of 61 km^2 and a maximum depth of 256 m (Pilotti et al., 2018). It is regulated by a dam built in the first reach of its outflow, the Oglio River. It is a meso-eutrophic lake and has shifted to meromictic the 1980s (Scibona et al., 2022). Immediately south of Lake Iseo shores lies the Torbiere del Sebino wetland, a protected area hosting well developed macrophytes stands. Mantua lakes system is composed of three smaller lakes (Superior, Middle and Inferior) and two connected wetlands: the Valli del Mincio and Vallazza, respectively located upstream and downstream of the lakes. The lakes system is located along the city of Mantua and fed by the Mincio river, emissary of Lake Garda, and has been regulated since 1190 (Bresciani et al., 2009). Together, the three lakes have a surface area of 6.2 km^2 and a maximum depth of 17.5 m (Pinardi et al., 2021). The system has eutrophic waters, and hosts different submerged, floating, and emergent macrophyte communities (Pinardi et al., 2021). Lake Chiusi is the southernmost sampling site, located in

Tuscany, Central Italy. It has a surface of 4 km^2, and a maximum water depth of 5.8 m, decreasing over the years due to high rates of sedimentation (Lastrucci et al., 2014). It is categorized as hyper-eutrophic, with mean concentrations of chlorophyll-*a* up to 49 μg l^{-1} (Cavalieri et al., 2018).

Sampling design

28 populations of *Nuphar lutea* were sampled in the four investigated lake systems: 12 in Chiusi, 7 in Mantua, 5 in Iseo-Torbiere and 4 in Pusiano-Annone (Fig. 1). Populations were chosen among *N. lutea* stands in the study site, starting from floating macrophyte maps derived from very-high-resolution satellite images (2 m pixel) and drawing plots randomly within 3 m of the waterfront of stands that were larger than 100 m^2 and logistically reachable with a light boat, stratifying selection on three levels of canopy density (modelled via spectral proxies), to ensure representativeness in within-site variability. In each population, a 4 m^2 plot was delineated, in which species presence and % cover were obtained, and water, sediment and plant samples were collected for environmental characterization and trait analyses. Samplings were carried out in July 2020 and 2021, during the peak season of *N. lutea* development.

Environmental characterization

In each plot water depth (hereafter depth, m), specific conductivity (SPC, μS cm^{-1}), nitrate concentration (NO3, μg l^{-1}), sediment organic matter content (sed.OM, %) and sediment total phosphorus content (sed.TP, μg g^{-1}) were measured. SPC was determined *in situ* using a multiparameter probe (YSI 556 MPS), while site water was filtered with glass fiber filters (Whatman) of 0.7 μm pore size and kept refrigerated. Within two days from collection, samples were filtered a second time with 0.2 μm pore size nylon filters, and NO3 concentration was determined by means of ion chromatography (883 Basic IC plus Metrohm, Herisau, Switzerland). A sample of 50 ml of sediment was collected from the upper 5 cm sediment layer in each plot, stored in falcon tubes and frozen as soon as possible after collection. In the laboratory, sediment samples were defrosted, homogenized and a subsample was dried at 60°C for 24 hours. After grounding the dried sediment to fine powder, an aliquot of ca. 0.3 g was weighed and incinerated at 450°C for 4 hours. Sed.OM was determined gravimetrically from the weight loss of the ashes compared to the dry sample (Buchanan, 1984). Sed.TP was analyzed spectrophotometrically from the ashes, after extraction in HCl (Aspila et al., 1976).

Leaf traits

In each plot, 8 leaves by as many randomly selected branches of *N. lutea* were collected, excluding leaves belonging to the same branching and trying to collect them from different individuals. Indeed, random collection of leaves from the stands can lead to unexpected trait variability which could largely be due to differences in the life stages of the leaves. Therefore, to standardize the collection, only young, i.e. fully developed leaves without mechanical damages or herbivory signs were selected from different rosettes and gently washed from dirt and epiphytic algae. Spectra of fresh leaves were collected within seconds of leaves detachment with a portable spectroradiometer (SR-3500, Spectral Evolution, Lawrence, USA; range covered: 350-2500 nm). Radiance reflected from the adaxial side of each leaf laid on dark background (black neoprene with absolute reflectance factor < 3%) was measured and converted to reflectance via readings taken over white standard panel (Spectralon®, with absolute reflectance factor > 95%).

After spectra readings, leaves were stored in sealed plastic bags and refrigerated until processing, which occurred within few hours. Five leaves per plot were used to determine the following structural traits: leaf area (LA, mm^2), specific leaf area (SLA, mm^2 mg^{-1}), leaf dry matter content (LDMC, mg g^{-1}) and proportion of leaf dry weight allocated to petioles (pet.propDW, %). Fully hydrated leaves, including petioles, were weighed and scanned with a portable scanner at 300 dpi, and successively dried at 60°C for 48 hours. LA was calculated using the software imageJ (Rasband, 1997-2018), SLA is the ratio between LA and leaf dry weight, LDMC is the ratio between leaf dry and fresh weight (Pérez-Harguindeguy et al, 2013), and pet.propDW is the ratio between petiole and whole leaf dry weight, expressed in percentage. Leaf chlorophylls content (chlab, μg cm^{-2}), expressed as sum of chlorophyll-*a* and chlorophyll-*b* on an area basis, were analyzed spectrophotometrically on the remaining three fresh leaves after extraction in 80% acetone for 24h (Wellburn, 1994). These traits were chosen as broadly representative of the trade-offs underlying the leaf economic spectrum (Wright et al., 2004). Indeed, SLA and LDMC explain the resource investment in leaves and the plant's resource-use strategies, with species with higher SLA and lower LDMC values are considered to have a fast and acquisitive strategy, while species with lower SLA and higher LDMC have a slow and conservative strategy. In addition, Pierce et al. (2012) have highlighted the role of LA in delineating size-resource orthogonal axes in hydrophytes, and Dalla Vecchia and Bolpagni (2022), demonstrated that petioles strongly influence *N. lutea* leaves strategies, with Pet.propDW accounting for more than 40% of leaf dry weight on average, suggesting an important investment in leaves construction costs. Chlab, calculated on an area basis for consistency with spectral information, is related to the photosynthetic activity of leaves and N content and is informative of light use efficiency and productivity (Evans and Poorter, 2001).

Derived from leaf reflectance spectra measured, we selected four specific reflectance features connected to pigments content and leaf structure, to be included as foliar traits in further analysis, namely: i) r440, i.e. the mean reflectance within 430 and 450 nm, linked to leaf surface roughness and composition, e.g. presence of trichomes (Sims and Gamon, 2002); ii) r515, i.e. the mean reflectance within 510 and 520 nm, linked to absorbance band for carotenoids and anthocyanins (Féret et al., 2017; Peters and Noble, 2020); iii) r625, i.e. the mean reflectance within 610 and 640 nm, linked to chlorophyll-*a* and -*b* content and balance (Villa et al., 2021); and iv) r800, i.e. the mean reflectance within 780 and 820 nm, linked to mesophyll structure complexity (Féret et al., 2017).

Genetic analyses

The DNA extraction and AFLP protocol were carried out starting from a total of 206 dried leaf samples divided per four sites. DNA extraction was performed using the 2x cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990), and the quality and quantity control of the extraction product was assessed by a spectrometric survey using a Bio-Photometer (Eppendorf). AFLP analysis followed the standard procedure (Vos et al., 1995), modified at the final amplification step (Coppi et al., 2014 and references therein). Two combinations of primers were selected for the final PCR amplification: hex_EcoRI-ACG/MseI-TTA and fam_EcoRI-CTA/MseI-CTC. AFLP profiles obtained by capillary electrophoresis were analyzed using GeneMarker v1.5 (SoftGenetics LLC, State College, PA, United States). The analyses of genetic variation at sampling plot and site level were performed as average genetic diversity within a sampling plot (AGD) using Arlequin v2.000 software (Schneider et al., 2000). The percentage of outlier loci (OUTLIER) was detected following Yang et al. (2016) using BayeScan v2.01. Following Foll (2012), outliers were determined as loci that fall over a threshold value set on the logarithm of posterior odds values (LogPO). The number of pilot runs was kept at 20, with a length of 10 000 iterations each one (Coppi et al., 2021).

Statistical analyses

To investigate the interlinks between ecological drivers and variation in functional traits (including reflectance features) and genetic metrics, GAM models were used, because species responses to environmental variables were often not linear. GAM models were built in R environment (R Core Team, 2022). All traits were tested in separate models (11 models in total) including all five environmental variables (Depth, SPC, NO3, sed.OM and sedTP), using the function {gam} of the package *mgcv* (Wood, 2011). For traits with multiple measures per plot (i.e., all except genetic diversity metrics) the plot number was added to the model as a random factor. The function {gam.check} was used to check the model assumptions, and response variables where log-transformed when necessary. Model selection was performed with aid of the function {dredge} of the package *MuMIn* (Barton, 2020), and the model with the lowest AIC criterion was selected. In case the trend of the model was linear (few cases), the additive model was used nonetheless to keep consistency with all other traits' models. GAM models were graphically visualized with the implementation of the packages *ggplot2* (Wickham, 2016), *gratia* (Simpson, 2022) and *gridExtra* (Aguie, 2017). To investigate if the variability of environmental conditions is correlated to a site effect, and thus if sites themselves are related to trait variation, the differences among sites on traits and on environmental variables were tested. Towards this, a preliminary PCA (Principal Component Analysis) was performed with environmental data, to visualize if sites were distinguished in the environmental space. The package *ggbiplot* (Vu, 2011) was used to visualize the first two axes of the PCA and the distribution of sites. Then, one-way ANOVA tests were performed using site as a categorical explanatory variable and traits and environmental variables as continuous response variable, after visually checking if model assumptions were acceptable. The ANOVA was then followed by Tukey post-hoc comparisons to identify differences among pairs of sites. Moreover, the partition of genetic variation was evaluated by an analysis of variance framework using analysis of molecular variance (AMOVA). The AMOVA was performed using Arlequin software ver. 3.5.2.2 (Excoffier et al., 2010) at three different hierarchical levels, i) within populations (plots), ii) among populations, and iii) among hypothetical groups of populations (study sites).

4.3 Results

Environmental gradients

The plots investigated showed a wide variability in terms of environmental conditions: depth ranged between 0.1 m in Mantua and Iseo-Torbiere and 1.9 m in Chiusi and Iseo-Torbiere; SPC ranged between 216 μS cm^{-1} in Pusiano-Annone and 608 μS cm^{-1} in Chiusi; N-NO_3^- had values <0.01 μg l^{-1} in 13 plots from Chiusi, Iseo-Torbiere and Pusiano-Annone, and peaked in Mantua with 9.49 μg l^{-1}; sed.OM ranged between 1.2% in Iseo-Torbiere to 38.1% in Chiusi, and sed.TP ranged between 277.5 μg g^{-1} in Chiusi and 1788.5 μg g^{-1} in Mantua. The first two axes of the PCA on environmental variables together explain 62.1% of the variation and showed that Chiusi is environmentally distinct from the other sites, mainly due to higher SPC and lower NO3 (AIII.1). The other three sites cluster together; however, Mantua system occupies a bigger portion of the environmental space, having plots with higher water and sediment nutrients content (AIII.1). The ANOVA tests showed no difference among sites for depth and sed.OM, whereas significant differences were detected for SPC, NO3 and sed.TP (see AIII.2 for complete sites comparisons). SPC was significantly higher in Chiusi, intermediate in Mantua and lower in Iseo-Torbiere and Pusiano-Annone. NO3 was significantly higher in Mantua, which also showed the highest variance, than in Chiusi and Pusiano-Annone, while Iseo-Torbiere was not statistically different from any other site, although this is due to one single plot (IS24) with particularly high values of NO3 (5.63 μg l^{-1}). Sed.TP was again statistically higher in Mantua.

Genetic Diversity

The AFLP protocol was performed on 203 samples and produced a total of 190 polymorphic loci. 94 loci were detected for the hex_EcoRI-ACG/MseI-TTA primer combination, whereas the amount of 96 was shown for the fam_EcoRI-CTA/MseI-CTC pair of primers combination.

The range of bp varied from 50 to 442 and 50 to 347 for the hex_EcoRI-ACG/MseI-TTA and fam_EcoRI-CTA/MseI-CTC, respectively.

Table 1*: a) Partition of genetic variance among populations. AMOVA was performed at two hierarchical levels testing the differentiation among and within populations. The table shows: degrees of freedom (d.f), Sum of squared deviations, Variance component estimates, percentages of total variance contributed by each component; b) Partition of genetic variance among groups of populations performed on a hypothetical subdivision in two groups of populations. Data show the degrees of freedom (d.f), the Sum of squared deviations, the Variance component estimates, the Percentage of total variance contributed by the among-group level.*

a)

Source of Variation	**d.f**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among populations	27	3845.676	16.97461 Va	46.55
Within populations	175	3411.496	19.49427 Vb	53.45
Total	202	7257.172	36.46888	

b)

Source of Variation	**d.f**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among groups	1	2554.961	24.80706 Va	51.18
Among populations within groups	26	1290.715	4.16863 Vb	8.6
Within populations	175	3411.496	19.49427 Vc	40.22
Total	202	7257.172	48.46996	

The mean value of AGD and the mean OUTLIER were 0.207 and 34, respectively. The site with lower levels of AGD and the frequency of OUTLIER was Chiusi (0.169 and 27), whereas the other sites showed comparable higher levels of AGD (AIII.2). A higher percentage of outliers was registered for Lake Iseo (45), indicating a possible higher impact of selective pressure on the gene pool of the five populations analysed The AMOVA analysis (Table 1a) showed that genetic variation among populations (plots) is high (FST = 0.465). The higher portion of the total genetic differentiation (53.5 %) was due to intra-populations differences rather than among-populations differences (46.5 %). The relatively high level of differentiation among populations indicates a possible genetic separation among geographically separated sites. Among all hypothetical groupings of populations examined, the one formed by the Chiusi populations, separated from the Mantua, Iseo and Pusiano-Annone populations, accounted for the highest percentage of among groups percentage of variation (51.2 %; P < 0.0001; Table 1b).

Traits drivers

The GAM models indicated that most traits were significantly correlated with one or more of the environmental variables included in this study (Table 2, AIII.3).

Table 2: *Summary of the results of GAM models and traits difference among sites. Significant variables in GAM models are marked with "x". In the comparisons among sites, "=" indicates no difference, "+" indicates first site has higher values than second site, the opposite for "-". Depth=water depth (m), SPC=specific conductivity (µS cm^{-1}), NO3=water nitrate concentration (µg l^{-1}), sed.OM=sediment organic matter content (%), sed.TP=sediment phosphorus content (µg g^{-1}), Dev. explained=percentage of deviance explained, R-sq (adj.) = adjusted R square, CH=Chiusi, IS=Iseo-Torbiere, MN=Mantova, PA=Pusiano-Annone. LA=leaf area, SLA=specific leaf area, LDMC=leaf dry matter content, pet.propDW=proportion of leaf dry weight allocated to petioles, chlab=leaf chlorophylls content, AGD=average genetic diversity, OUTLIER=percentage of outlier loci, r440 to r800 refer to the mean leaf reflectance at the given wavelength.*

TRAIT	Depth	SPC	NO3	sed.OM	sed.TP	Dev.Ex (%)	R-sq. (adj.)	IS-CH	MN-CH	PA-CH	MN-IS	PA-IS	PA-MN
LA				x	x	83.0	0.792	=	+	+	+	+	=
SLA		x			x	71.5	0.661	=	−	−	−	−	=
LDMC		x				74.2	0.687	=	+	+	+	=	=
pet.propDW		x				37.3	0.285	+	=	=	=	=	=
chlab	x				x	59.8	0.495	+	=	=	=	=	=
AGD		x				45.8	0.414	+	+	=	=	=	=
OUTLIER		x				31.8	0.292	=	=	=	=	=	=
r440		x				65.6	0.614	+	+	−	=	−	−
r515						//	//	+	+	=	=	−	−
r625		x	x		x	75.7	0.727	+	+	−	=	−	−
r800		x				72.6	0.692	+	+	=	−	−	−

LA was influenced by sed.OM and sed.TP (p<0.05). Leaf area tended to increase with sed.OM when this was lower than 10% or higher than 25%, while in the range from 10% to 25% the trend was inverted. Phosphorous content in sediments had a tendentially negative effect on LA until concentration was lower than 1000 µg g^{-1}, over which the trend became positive (Fig. 2a-b). LA was statistically higher in Mantua and Pusiano-Annone than in Iseo-Torbiere (both p<0.001) and Chiusi (p<0.001 and p=0.028 respectively). SLA showed linear correlations with SPC (positive, p<0.001) and sed.TP (negative, p<0.005) (Fig. 2c-d). SLA was significantly higher in Chiusi and Iseo-Torbiere than in Mantua (p<0.001) and Pusiano-Annone (p<0.001). LDMC was significantly correlated only to SPC (p=0.0015), showing a linear negative relation, opposite to that observed for SLA (Fig. 2e).

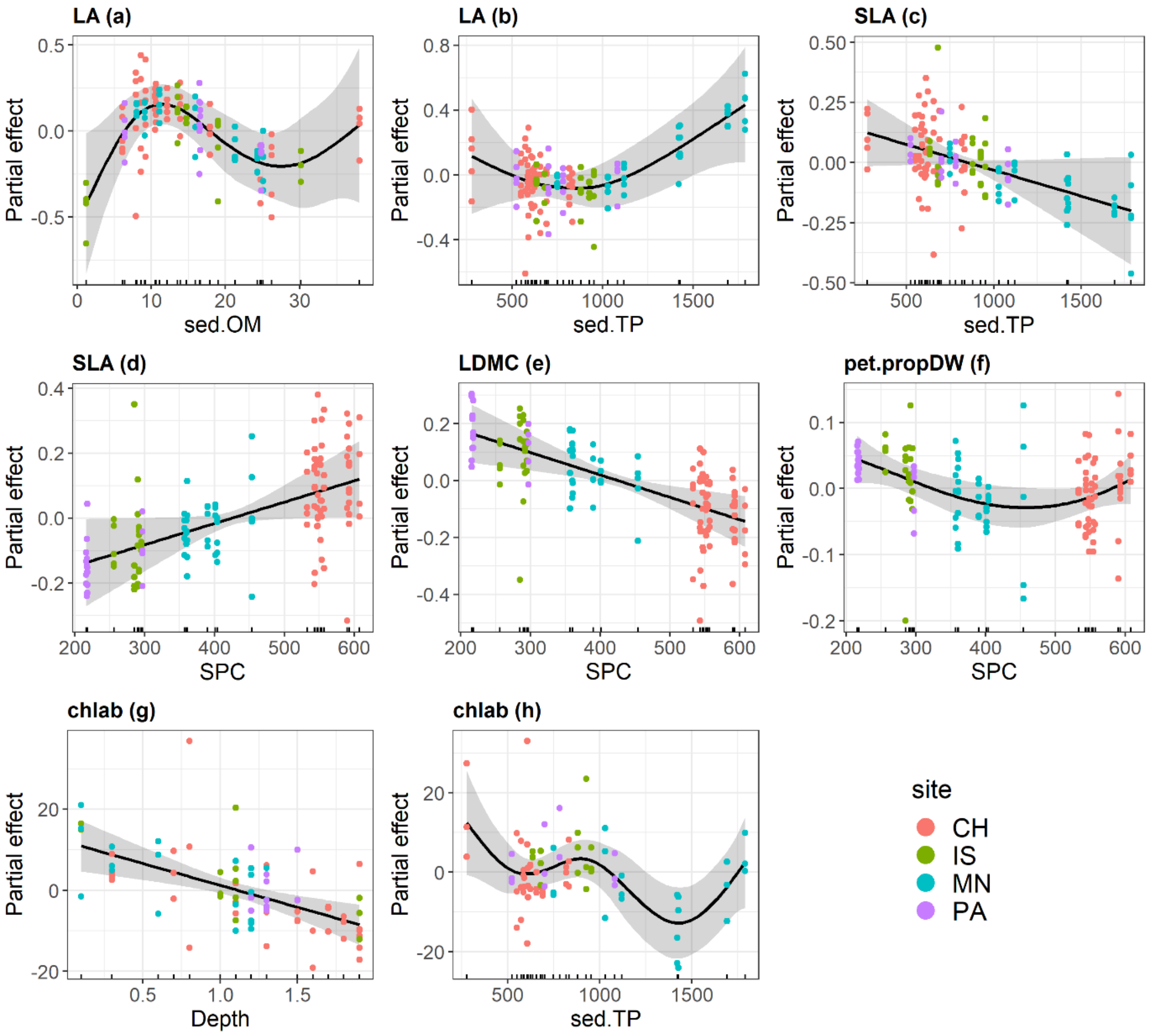
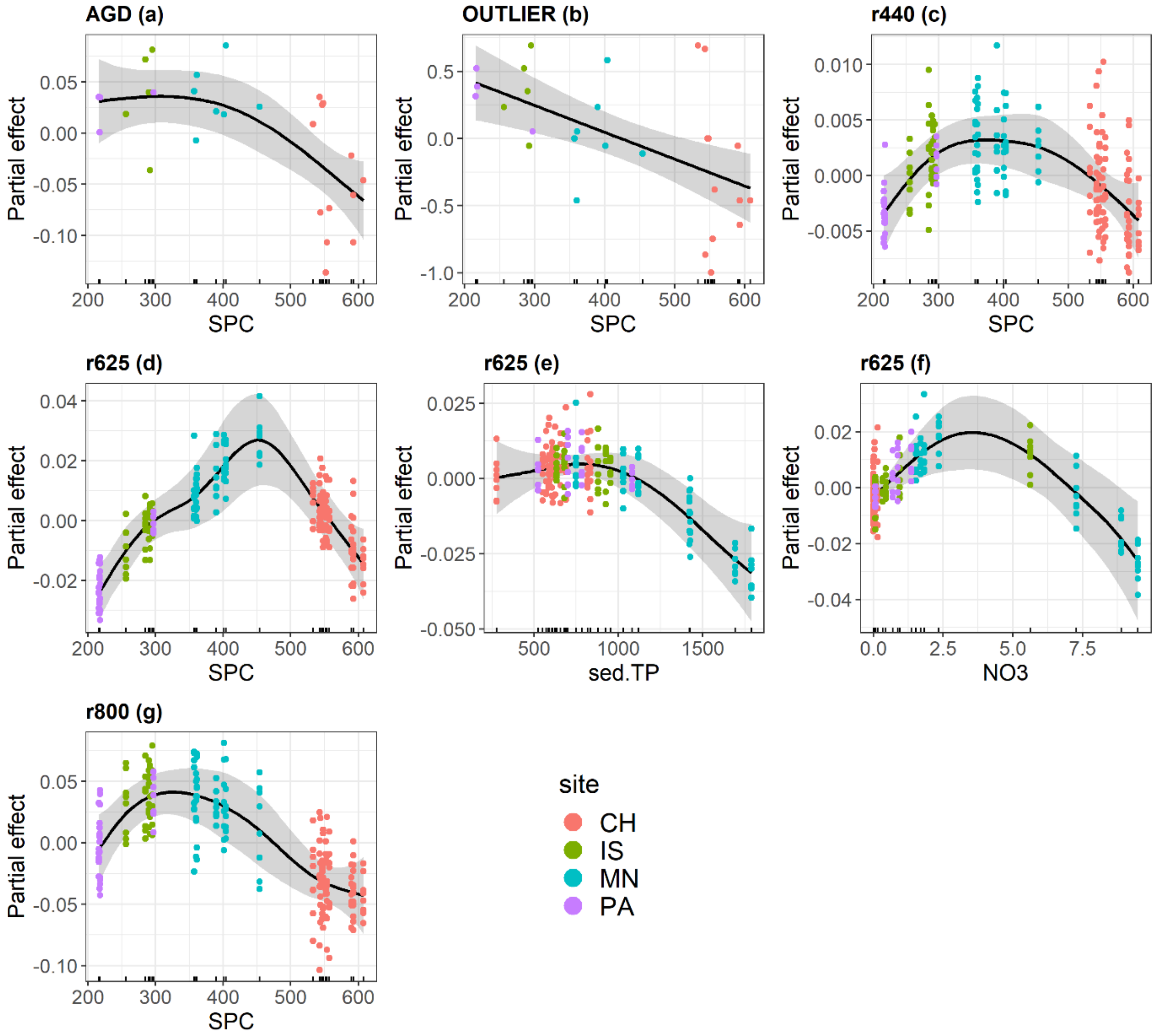


***Fig. 2**: Results of the GAM models for measured traits and genetic metrics. Residuals are plotted against the given environmental variables; different points colours refer to different sites. Gray shade shows confidence interval, and only significant relationships are shown. LA=leaf area, SLA=specific leaf area, LDMC=leaf dry matter content, pet.propDW=proportion of leaf dry weight allocated to petioles, chlab=leaf chlorophylls content, Depth=water depth (m), SPC=specific conductivity (μS cm^{-1}), sed.OM=sediment organic matter content (%), sed.TP=sediment phosphorus content (μg g^{-1}).*

Differences among sites in LDMC were quite mixed, with Pusiano-Annone and Mantua tending towards higher scores, and Iseo-Torbiere and Chiusi occupying the lower bound of the range. Pet.propDW showed limited differences among sites (but high within-site variability) – with only significant differences between Iseo-Torbiere and Chiusi (p=0.014) – which would suggest limited plasticity to environmental conditions. Indeed, Pet.propDW was significantly correlated with SPC (p=0.035), tending to decrease for plots with SPC < 450 μS cm^{-1}, with signs of inversion in the trend crossing this threshold, although the model only explained < 40% of variance (Fig. 2f). Chlab was influenced by Depth (p<0.001) and sed.TP (p=0.027). While Depth had a linear negative effect, the sed.TP – chlab trend was more waggling: generally negative for P content < 1500 µg g^{-1} and inverting its shape for higher concentrations (Fig. 2g-h). Among sites, plots in Iseo-Torbiere had higher chlab than in Chiusi (p=0.017), while all other pairs did not significantly differ.

Fig. 3*: Results of the GAM models for spectral traits. Residuals are plotted against the given environmental variables; different points colours refer to different sites. Gray shade shows confidence interval, and only significant relationships are shown. Plots titles (c to g) refer to the mean leaf reflectance at the given wavelength, AGD=average genetic diversity, OUTLIER=percentage of outlier loci, Depth=water depth (m), SPC=specific conductivity (µS cm*$^{-1}$*), NO3=water nitrate concentration (µg l*$^{-1}$*), sed.OM=sediment organic matter content (%).*



Among spectral traits, r515 was not influenced by any of the investigated variables. All other spectral traits were correlated with SPC (p=0.003 for r440, p<0.0001 for r625 and r800), and overall showed a unimodal trend peaking at conductivity around 400 – 450 µS cm^{-1} for r440 and r625, or around 300 – 350 µS cm^{-1} for r800 (Fig 3c-d-g). Besides, r625 was also significantly influenced by sed.TP ($p=0.001$) and NO3 ($p=0.003$), with a trend similar to that observed for SPC (Fig. 3e-f). The relationship seemed particularly driven by the plots in Mantua covering the upper range of these parameters (from 750 to 1789 μg g-1 for sed.TP and from 1.7 to 9.5 μg l^{-1} for NO3). Generally, Chiusi and Pusiano-Annone showed the lowest leaf reflectance traits, and Mantua and Iseo-Torbiere the highest (see detailed results in AIII.2).

Genetic drivers

The variance of genetics metrics explained by GAM models is lower than with leaf traits (<48%), also due to the lower cardinality ($N=28$). Yet, AGD and OUTLIER showed significantly negative correlations with SPC ($p<0.002$) (Fig. 3a-b). A site effect was also detected for AGD, which was significantly lower in Chiusi than in Iseo-Torbiere and Mantua ($p=0.019$ and $p=0.009$ respectively); however, no site differences were highlighted for OUTLIER.

4.4 Discussion

All traits, except r515, and genetic diversity metrics investigated were statistically influenced by at least one of the environmental variables considered. Overall, SPC and nutrient-related sediment features were the most influential variables, with SPC influencing 8 out of 11 investigated traits. The role of SPC is not the same for every trait and shows non-linear responses: it often determined a negative trend in traits values, especially at high SPC levels, but it is not always the case, with SLA as a notable exception to this pattern, showing a positive relation with SPC. This negative trend was observed also for genetic metrics. Especially, the decrease in OUTLIER along with the increase in conductivity suggests that the higher incidence of neutral loci at high SPC is favoured by balanced selection rather than local genetic adaptation (Excoffier et al., 2009). Conversely, sed.TP determined greater traits performance in LA and chlab at high concentrations, however it also determined negative traits response for SLA and r625. Water depth and nutrients content (NO3) appeared to have a marginal role in shaping *N. lutea* leaf traits plasticity at the regional scale (they were significantly related only to chlab and r625, respectively), due to their high spatial and temporal dynamicity in wetland environments.

Environmental trait filters

The investigated lake systems represent a gradient of conditions, and non-linear trends (unimodal patterns) seem to be due to site-specific effects and are therefore informative of processes taking place within the systems. A strong site-specific component was particularly evident for SPC, which exerted a negative influence locally on leaf reflectance traits (r440, r625 and r800) at the site scale for Mantua and Chiusi, where the SPC range cover was wider (from 357 to 454 μS cm^{-1} in Mantua and from 533 to 608 μS cm^{-1} in Chiusi). The specificity of Chiusi was evident also on a genetic base (Table 1b). In fact, the AMOVA highlighted it as separate from other sites regarding genetic diversity. This could further support our previous results of lower genetic diversity in populations with higher SPC, suggesting that peculiar environmental conditions have selected distinct *N. lutea* genotypes over time in this site. Other studies have already confirmed the effect of environmental conditions on genetic variation of aquatic species *Ranunculus subrigidus* (Wu et al., 2019), *Phragmites australis* (Coppi et al., 2018), *Ranunculus baudotii* (Coppi et al., 2015) and *Ceratophyllum demersum* (Li et al., 2022), although literature on this topic remains scarce.

SPC reflects the ions content in the water and can therefore be related to the trophic level of the system (Yuwono et al., 2015), possibly representing a first signal of eutrophication (Beeton, 1965). At the local scale, Dalla Vecchia and Bolpagni (2022) found that SPC was highly correlated with water depth, reflecting the influence of the sediment metabolism on dissolved nutrients in the above water column of a static water body, i.e. Lake Chiusi. By expanding the spatial range of investigation, the links between SPC, Depth and more in general the water trophic conditions weakened. These findings lead to two main considerations. First, nutrients available for plants use are not easily detectable during the growing season, because their actual presence is masked by the fast recycling by photosynthetic organisms (including microalgae), temporarily incorporating them into their biomass (Twinch and Ashton, 1983; Nedwell et al., 1999). Secondly, SPC seems to provide information on basin scale, medium to long term hydrogeological setting (and biogeochemistry) of each site, so relations between traits and SPC can be expected to show site-specific, non-linear patterns. After all, SPC is one of the most used descriptors for water chemistry and is known to show a much wider variation among sites than within sites (Borowiak et al., 2020).

Sediments are characterized by nutrient concentrations of at least an order of magnitude higher than those measured in the water column (Hopkins et al., 2018), as a result of human impacts (e.g., pollution) accumulated in time and space, but also as a natural consequence to sedimentation and decomposition, determining a slow release of nutrient to the water column (Tammeorg *et al.*, 2013). The effect of nutrients availability on *N. lutea* traits variability was visible when considering sediment quality. In fact, besides sed.TP, sed.OM is also a proxy of the trophic level, because it reflects the productivity of the water column and the decomposition capability of benthic organisms, as it was previously found to be related with phosphorus availability (House and Denison, 2002). A higher nutrient availability is often associated to higher trait performance, notably so for LES traits like LA, SLA and pigments (Fan et al., 2013; Zervas et al., 2019; Dalle Fratte et al., 2019). Our results are generally in line with this trend with regard to LA and chlab, which showed a positive response to sed.TP increase at high phosphorus concentrations, although chlab seemed to be less sensitive for sed.TP < 1500 $\mu g\ g^{-1}$. This behavior could be related to a temporary N limitation, hampering the sed.TP – traits relation in plots where sediment P scores are in the low-medium range; indeed, in our data nitrogen in water was almost undetectable in Chiusi (< 0.15 $\mu g\ l^{-1}$), as well as in Iseo and Pusiano-Annone (< 1 $\mu g\ l^{-1}$ in most of the plots).

Among the spectral traits, r625 – as synthesis of spectral features linked to foliar pigments and light use efficiency – is inversely related to the chlorophylls content ($R^2 = 0.30$ in our *N. lutea* samples). Therefore, the overall negative trends for r625 with sed.TP and NO3 in GAM models indicate a positive effect of nutrients on plant traits performance, in terms of pigments content. Strengthening this pattern, measured chlab showed a negative a correlation with Depth, which could be interpreted as the effect of less favorable conditions for *N. lutea* individuals growing in deeper waters (Richards et al., 2011) and indicate older leaf age (but also slower turnover rates and therefore lower productivity). Still, chlab did not show any trend with NO3, given the scarcity of this nutrient in the water of most sites, which confirms the importance of sediments as nutrients source for rooted species (Bornette and Puijalon, 2011).

Leaf reflectance in the near infrared range (780-820 nm), i.e. r800, can instead surrogate foliar mesophyll structural complexity and is positively related to leaf structure descriptors ($R^2 = 0.45$ with blade SLA in our *N. lutea* samples). The overall negative trend for r800 with SPC, indicating lower leaf structural investment in environments characterized by higher conductivity, is therefore consistent with the trends highlighted for SLA and LDMC (positive and negative, respectively). As these traits represent opposite sides of the trade-off between "fast and acquisitive" leaves (high SLA) and "slow and conservative" leaves (high LDMC) (Wright et al., 2004), our findings suggest that at the regional scale SPC tends to promote more acquisitive behavior in *N. lutea*. This result in in contrast with the trend found at site scale (Lake Chiusi) by Dalla Vecchia and Bolpagni (2022) of a negative relation between *N. lutea* SLA and SPC, which suggested a stressful effect of SPC. The relationship, however, was highly significant for petiole SLA in Dalla Vecchia and Bolpagni (2022), highlighting on one hand that plant responses can be different changing the observation scale, and on the other hand that distinguishing between petiole and blade responses, in this species, are highly informative of processes happening within the plant. The reduced investment in leaf structure found at higher SPC, namely in Chiusi, suggests that in this site the species is able to exploit the more extreme environmental conditions to implement a more acquisitive resource-use strategy, ending up as the dominant hydrophyte species within this lake. These results are in line with Klok and van der Velde (2017), who observed, instead, a more conservative strategy of *N. lutea* under limited nutrient availability.

Leaf responses described by SLA, LDMC and r800 in Chiusi, where *N. lutea* has practically no competition in its functional group, may be interlinked to the monospecificity of the floating stands of this site. The hyper-eutrophic conditions of the site (also related to the high measured SPC) can in fact favor competitive floating-leaved species (Bornette et al., 1998, 2001), and *N. lutea* in particular (Nowak et al., 2015). In the other sites *N. lutea* populations seem to be either highly stressed and in regression (Iseo-Torbiere) or have to face the competition of other expansive floating and floating-leaved species (e.g., *Ludwigia hexapetala* and *Nelumbo nucifera* in Mantua, or *Trapa natans* in Annone and Pusiano). This is an aspect not covered in the present work, with an intraspecific focus, but which should be considered for a full understanding of the mechanisms regulating the functional responses of *N. lutea*, and of plant species in general.

Even if it constitutes a physical constraint for rooted floating-leaved species, Depth does not emerge as a key driver of *N. lutea* traits variability in this study, carried out at regional scale.

For studies with a spatially limited focus, previous studies have reported for *N. lutea* and confamiliar species a positive correlation of Depth with leaf size (Dalla Vecchia and Bolpagni, 2022) and SLA (Richards et al., 2011), demonstrating that nymphaeids tend to minimize leaves construction costs in deep water (Richards et al., 2011). Fu and colleagues (2014), however, found a negative relation between SLA and Depth in submerged and floating macrophytes. These contrasting results support the idea of the authors that Depth alone should be considered a partial driver of macrophyte functional variability, and the relation between depth and leaf structural investment can be mediated by other important parameters that vary along the depth gradient, like nutrients and light availability.

Information on spectral traits variability – especially in large, horizontally leaved species such as *N. lutea* – can be exploited at synoptic scale to assess intra-site patterns of leaf traits surrogated by reflectance features by deriving continuous, fine scale maps from very-high-resolution multispectral remote sensing images (see AIII.4 for example maps at 2 m spatial resolution over Lake Chiusi), thus allowing to increase the spatial coverage and level of detail of the target macrophyte stands that could be investigated (Villa et al., 2021). Such implementation can be greatly useful in helping to detect patterns and trends that might be difficult to capture following a point-sampling strategy.

4.5 Conclusions

Our findings provide evidence of strong plastic adaptation responses of leaf traits to changing environmental conditions in macrophytes, using *N. lutea* as model species. The wide range of intraspecific variation found highlights the importance of considering traits variability to detect the real effect of habitat filtering, possibly underestimated by research limited to species-level variation (Fu et al., 2014). The addition of genetic insights to functional variability assessment helped us to better explore the high plasticity of this species and explain its competitiveness in aquatic environments. Site-specific conditions in environmental parameters are reflected in the genetic identity of sampled stands, especially in Lake Chiusi, highlighting a long-term effect on *N. lutea* populations. In this regard, we strongly support the inclusion into functional traits collection design of detailed ecological descriptors, accounting for variability in both time and space. Orienting research in this direction could promote a truly understanding of the role of traits in plant strategies. Finally, we stress the advantages of multidimensional, integrated approaches linking different aspects of plant diversity – e.g., functional, spectral and genetic – as they allow the in depth, quantitative investigation of populations responses to environmental drivers at multiple scales. On one hand, the implementation of spectral traits features into remote sensing based, high-resolution maps could widen the spatial coverage and level of detail of plant functional ecology studies. Genetic diversity metrics, on the other hand, could help explain hidden patterns in trait variation, because of mediated responses through generations.

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5. Chapter IV

ECO-FUNCTIONAL NICHES OF NYMPHAEIDS: TROPHIC-MEDIATED FUNCTIONAL RESPONSES ACROSS SPATIAL SCALES[2]

5.1 Introduction

The concept of niche has long been used in ecology studies to explore species specialization and distribution (Devictor *et al.*, 2010). Definitions of niche are various, but they can be summarized both by the theory by Grinnel (1917) and Elton (1927). The Grinnelian niche refers to the ecological breath of a species, or how a species is able to use and adapt to the resources available in its habitat (Grinnel, 1917). The Eltonian niche, on the other hand, focuses on the effect of species on the environment, so it's more related to species characteristics and can be referred to as the functional niche (Elton, 1927, Devictor *et al.*, 2010). Furthermore, Hutchinson (1957) expands the eltonian definition to a hypervolume described by the multivariate space constrained by biotic and abiotic factors that influence a species.

The application of niche theory has raised great interest among biologists, for its potential adaptation to many taxonomic groups and purposes (e.g., Pagani-Núñez *et al.*, 2017; Papuga *et al.*, 2018; Saccò *et al.*, 2020). The ecological niche is by far the most investigated in vegetation science: in fact, it can give valuable information on species specialization, highlighting generalist versus specialist behaviour (Devictor *et al.*, 2010), and it reflects the effect of habitat filtering, the main process determining community assembly (Viana *et al.*, 2016). This can bring important implications for species distribution, requirements and conservation concerns in a changing environment (Papuga *et al.*, 2018; Liu *et al.*, 2021), and help understanding the dynamics of (invasive) species spread and identifying the most exposed habitats to modification or invasion (Montagnani *et al.*, 2022; Robichaud and Rooney, 2022). Indeed, functional traits – i.e., any morphological, physiological or phenological feature measurable at the individual level and affecting plant fitness (McGill *et al.*, 2006) – can

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substantially implement the niche concept by defining the amplitude of the responses of species and the entity of their plasticity (Kearney *et al.*, 2010; Ajal *et al.*, 2022).

Macrophytes, compared to terrestrial species, often exhibit wider trait variation (Pierce *et al.*, 2012; Dalla Vecchia and Bolpagni, 2022), as a response to the peculiarities and steep gradients of the environments they inhabit. For this group of plants, phenotypic plasticity interacts with environmental tolerance and greatly influence the variety of conditions in which they can persist (De Wilde *et al.*, 2014). Macrophytes are facing on one hand a rapid and dramatic decline worldwide due to direct anthropic pressure, climate change and habitat degradation (O'Hare *et al.*, 2018), but on the other hand many macrophyte species are spreading worldwide and represent some of the most impacting invasive species, causing negative effects on ecosystems and nuisance for human activities (Strayer, 2010; Hussner *et al.*, 2017). Therefore, a combined approach integrating ecological with functional niche, can bring to a more complete understanding of the extent of variation of macrophytes resource-use strategies and functional adaptation (Lukács *et al.*, 2017), thus allowing to catch possible mechanistic explanations for species decline, migration or invasive behavior. A common question on the success of invasive species is whether they share the same niche as native species, outcompeting them, or if they are able to occupy a new portion of niche, left unexploited by native species (Loiola *et al.*, 2018; Dalle Fratte *et al.*, 2019). It is hence interesting to evaluate the degree of niche uniqueness – namely the proportion of a species niche not shared with other species – in order to quantify differences among species and catch functional or ecological specialization.

Nymphaeids (water lilies) distinguish themselves among other macrophytes growth forms by having a root system anchored in the sediment and a rosette of leaves which may be submerged, but most commonly floating or emergent, reaching the water surface with elongated petioles. Leaves can vary consistently in size (Pierce *et al.*, 2012), and may entirely cover the water surface, a strategy that helps them outcompete other growth forms and results in mostly monospecific populations (Paillisson and Marion, 2011; Nowak *et al.*, 2015). Nymphaeids are an important component of aquatic ecosystems, as they connect and influence sediments, water and atmosphere, but so far they have received little attention compared to submerged vegetation in ecological studies (Klok and van der Velde, 2017). They provide habitat not only for aquatic organisms like fish, that can use them to lay eggs and raise juveniles (Grenouillet *et al.*, 2002; Dharmono *et al.*, 2022), but also for terrestrial species that live on their floating and emerging leaves (van der Velde and Brock, 1980; Paillisson and Marion, 2001). Dense stands influence the water current and promote sedimentation (Thomaz, 2021), and many species are able to withstand a variety of environmental conditions (Sender *et al.*, 2020) and survive in anoxic conditions thanks to complex ventilation systems (Große, 1996). Nevertheless, some species show a declining trend, like *Nymphaea* spp., though a full understanding of these species responses to changing environments is yet to be achieved (Parveen *et al.*, 2022).

Invasive species are forcing native species to strong regression or even local extinction because they rapidly colonise waterbodies: For example, invasive *Nelumbo nucifera* and *Ludwigia hexapetala* are replacing native stands of *Nymphaea alba* and *Nuphar lutea* in shallow lakes of Northern Italy (Pinardi *et al.*, 2021), even though *N. lutea* is considered a top competitor species (Temmink *et al.*, 2021). Indeed, nymphaeids also include invasive species that cause trouble to ecosystem functioning and human practical utility (Darbyshire and Francis, 2008). With their canopies they can form a barrier between water and atmosphere, favoring anoxia in the water column, a condition fostered also by the high organic matter accumulation associated to nymphaeids stands (Pinardi *et al.*, 2018; Gettys, 2019). Mechanical control of *N. nucifera* has even proved necessary to open water passages in rivers, obstructed by its propagation that caused significant water flow reduction (Pinardi *et al.*, 2021).

However, based on a CSR functional classification, which describes species as Competitive, Stress-tolerant or Ruderal (Grime and Pierce, 2012), Dalle Fratte *et al.*, (2019) did not observe a difference in native or alien species of nymphaeids in their strategies, all clustering together in the most competitive end of the spectrum compared to other aquatic or terrestrial species. Previous studies on nymphaeids have highlighted great plasticity of traits in response to environmental gradients (Dalla Vecchia and Bolpagni, 2022; Dalla Vecchia *et al.*, under review) and there seems to be contrasting results regarding the performance and resource-use of different species under more or less eutrophic conditions (Pełechaty, 2007).

In this study we aim at evaluating the size and uniqueness of the ecological and functional niches (here defined as a multidimensional space given by the set of environmental conditions where a species lives and by the variability of its functional traits, respectively) of four species of nymphaeids, and comparing them based on information on both niches, to explain their mutual links with the colonized environments. We focus on *N. lutea*, *N. alba*, *Nymphoides peltata* and *N. nucifera*, relatively common in Italy. We hypothesize that species showing a stringer declining trend, namely *N. alba* and *N. peltata*, would show smaller niche size and higher niche overlap with other species, and that more competitive species, like *N. lutea* and *N. nucifera*, would show large niche size, with its functional niche highly overlapping with native species (especially for *N. nucifera*).

5.2 Materials and methods

Study area

Sampling of plants and related environmental descriptors were carried out in several locations in Italy (Fig. 1), where the presence of the species of interest was known either by expert-based observation, previous studies (e.g., Lastrucci *et al.*, 2014; Pinardi *et al.*, 2021) or Italian websites reporting updated records (www.actaplantarum.org).

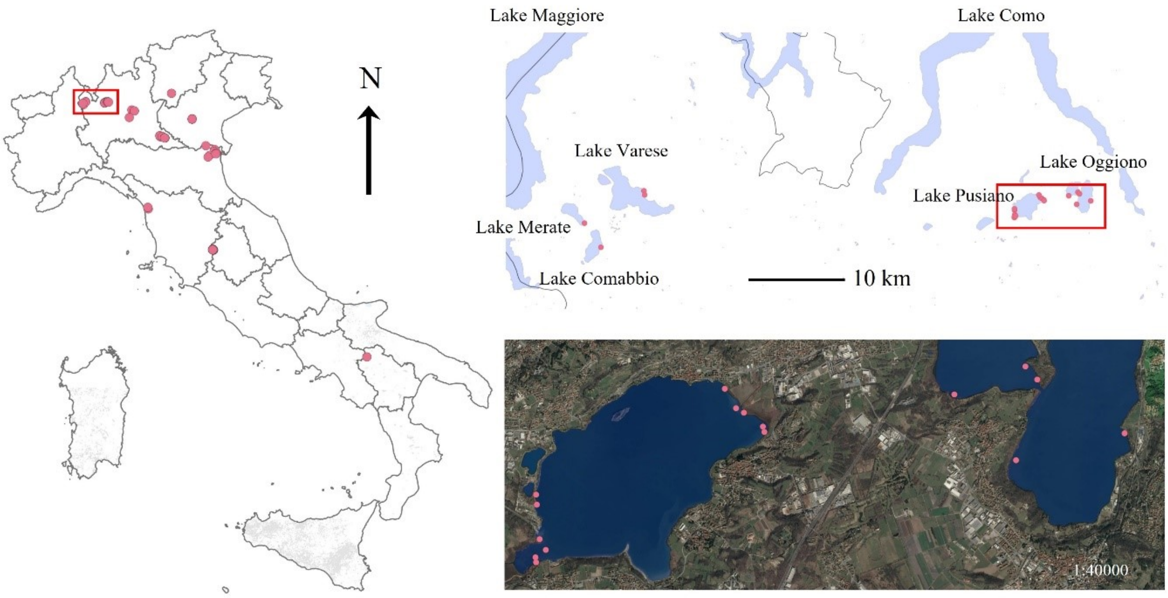


Fig. 1: *Map of the study sites located in Italy, and a focus on the alpine lakes near Lake Maggiore and Lake Como sampling area, showing the spatial arrangement of sampling plots.*

Study sites included several lakes, among which Cei Lake (Trento, 45.9° N 11.0° E), Varese Lake (Varese, 45.8° N 8.7° E), Comabbio Lake (Varese, 45.8° N 8.7° E), Pusiano Lake (Como, 45.8° N 9.3° E), Annone Lake (Lecco, 45.8° N 9.4° E), Iseo Lake (Bergamo/Brescia, 45.7° N 10.0° E) and Fimon Lake (Vicenza, 45.5° N 11.5° E) are sub-alpine lakes, spanning in dimensions between 0.045 km^2 (Cei Lake) to 65.3 km^2 (Iseo Lake). Other lakes were the fluvial lakes system of Mantua (45.2° N 10.0° E), Gorro Lake located in the Northern Appenines (Parma, 44.5° N 9.9° E), Chiusi Lake, a shallow lake remnant of ancient wetlands (Siena, 43.1° N 12.0° E) and the volcanic Monticchio Grande Lake (Potenza, 40.9° N 15.6° E). Samples were collected also from two wetlands, the Torbiere del Sebino, south of Iseo Lake (Brescia, 45.6° N 10.0° E) and the Vallazza, south of the Mantua lakes system (Mantua, 45.1° N 10.8° E), and from a pond in Massarosa (Lucca, 43.9° N 10.3° E). Lastly, we included in the study some artificial large canals along the last stretch of the Po River (in the province of Ferrara, from 45.0° N 11.9° E eastwards). They show minimal to no water flow (< 0.2 m s^{-1}) and can therefore be ecologically attributed to stagnant environments. Altitude of sampling sites varied from 3 m asl (Codigoro, Ferrara) to 920 m asl (Cei Lake, Trento).

Ecological niche sampling

For each population water physical and chemical parameters and sediment quality were characterized. For this study’s purpose, we use the term population to indicate each sampled macrophyte stand, defined by a 4x4 m^2 plot and isolated from other macrophytes stands of the same species by at least 200 m. Depending on the extension and heterogeneity of the study sites, some lakes and canals could host more than one population. *In situ*, water temperature, conductivity, pH and dissolved oxygen were measured with a multiparameter probe (Eureka Manta2), as well as water depth. A sample of water was collected, immediately filtered with 0.7 μm pore size glass fiber filters (Whatman) and kept refrigerated at 4° C until processing. Dissolved inorganic carbon (DIC) was determined by titration with 0.1 N HCl (Anderson *et al.*, 1986), and soluble reactive phosphorus (SRP) was determined spectrophotometrically following Valderrama (1977). Both analyses were carried out within one or two days from sampling. The remaining sample was frozen for further analyses. Dissolved reactive silica (DRSi) was then determined spectrophotometrically (Lienig *et al.*, 1978). After filtering the samples with 0.2 μm pore size nylon filters, dissolved anions and cations were determined by ion chromatography (883 Basic IC plus Metrohm, Herisau, Switzerland). A 50 mL sediment sample was collected from the upper 5 cm sediment layer in each population, stored in a falcon tube and frozen until processing. In the laboratory, it was homogenized, and a subsample of 5 mL was used for determination of sediment density, porosity, water content by weighing the fresh and dry weight after drying in the oven at 50°C until constant weight, and around 0.3 g of dry sediment were used for the determination of organic matter content (sed.OM) after combustion at 450° C for 4 h, using the gravimetric method (Buchanan *et al.*, 1984). After acid extraction of a subsample of the incinerated sediment, total phosphorus (sed.TP) was analyzed spectrophotometrically (Aspila *et al.*, 1976).

Species and functional niche sampling

In this study we investigated four species of nymphaeids: *N. nucifera*, *N. lutea*, *N. alba* and *N. peltata*. *N. nucifera* is an invasive species in Italy, introduced in the 1920s (Tóth *et al.*, 2019) and forms dense populations with floating and emergent leaves, while the latter three species are autochthonous and all with rooted floating-leaved plants. For each species, 12 populations were characterized. We chose to measure leaf traits related to the leaf economics spectrum because they reflect the plants resource-use strategies (Wright *et al.*, 2004), and especially so in nymphaeids, where leaves represent most of the above-ground biomass (Brock *et al.*, 1983a), besides they are widely used in the literature, which would allow our results to be more comparable with previous studies (Pierce *et al.*, 2012; Pan *et al.*, 2019). In each population, eight young, i.e. fully expanded leaves with no sign of damage, or illness, or herbivory were collected, paying attention to collect the full petiole length. The leaves were immediately refrigerated in airtight plastic bags to avoid dehydration. Fresh weight (LFW) as well as leaf area (LA) were determined on five leaves within hours from collection, using a precision scale and a scanner at 300 dpi resolution. LA was successively determined using the software imageJ (Rasband, 1997-2018). At the same time, leaf disks of known area (24.8 mm^2) were cut out of fresh blades from the remaining three leaves and frozen in airtight plastic containers for further pigments analyses. Weighed leaves were then dried in the oven at 50° C until constant weight, to determine dry weight (LDW). LFW, LDW and LA were measured on blades and petioles separately. We then calculated leaf dry matter content (LDMC) and specific leaf area (SLA) following Pérez-Harguindeguy *et al.* (2013). We also calculated two petiole-related traits: proportion of petiole area (pet.propA) and proportion of petiole dry weight (pet.propDW), measured as the ratio between the petiole area or dry weight and the total leaf area or dry weight, respectively. These two traits indicate the resource investment of plants in the structural support of leaves. Dry material of leaf blades was pooled together, grounded to fine powder with liquid nitrogen and stored for elemental composition analyses (nitrogen and carbon). From the pooled material, three aliquots were used for phosphorus content (P) determination, measured spectrophotometrically after acid extraction as described for the sediments, and one more aliquot was used for carbon (C) and nitrogen (N) determination by combustion analysis with an elemental analyzer (Thermo FlashEA 1112). Leaf chlorophyll-*a*, chlorophyll-*b* and carotenoids content were determined on a fresh weight and area basis after extraction for 24 h in 80% acetone, and the solution was read spectrophotometrically according to Wellburn (1994). Ratios between chlorophyll-*a* and chlorophyll-*b* (chla/b), and between chlorophylls and carotenoids (chlab/car) were subsequently calculated.

Statistical analyses

To assess the differences in environmental conditions and functional traits among the four investigated species we used linear mixed models, ANOVA and Kruskal Wallis tests, followed by Tukey post-hoc test or Dunn test for models deriving from Kruskal Wallis. All analyses were carried out in R environment (R Core Team, 2022). Linear mixed models were used for traits measured in more than one replicate in the same population, so population identity was added in the model as a random effect, and traits were log-transformed whenever needed to meet the assumptions. The analysis was carried out using the packages *lme4* (Bates *et al.*, 2015) and post-hoc comparisons using *emmeans* (Lenth, 2022). For traits with one measure per population (i.e., N and C) and environmental variables we used ANOVA or Kruskal Wallis test followed by Dunn test when residuals where not homogeneously distributed.

Then, to summarize the ecological and functional information for the computation of the niches, two principal component analyses (PCA) were carried out, one on the environmental parameters and one on traits. The number of variables included in the PCAs was reduced taking into account redundant variables, with a Pearson’s correlation coefficient >0.7, except for the pairs LA and LDW (showing a correlation of 0.98) and P and N (0.78), which are among the main traits commonly used in the literature (Pierce *et al.*, 2012) (see Table 1 for the complete list of environmental parameters and traits included in the analysis). Overall, the analyses included ten environmental variables (Depth, pH, SPC, DIC, NO3, DRSi, SRP, sed.OM, sed.TP and sed.density; Table 1), and ten functional traits (LA, pet.propA, LDW, pet.propDW, LDMC, SLA, chlab, P, N, and C; Table 1).

All variables included were transformed to have mean of zero and unit variance before running the PCA. The function ggscreeplot from the package *ggplot2* (Wickham, 2016) was used to determine the optimal number of axes to be included in the niche determination. The package *hypervolume* (Blonder *et al.*, 2022) was implemented and integrated to quantify the size and uniqueness of the niche. The niche size; i.e., the volume occupied by a species relative to the volume occupied by all species in the dataset, was used to compare the investigated species. Hypervolumes were built with the gaussian kernel method, being the most appropriate for functional data and fundamental niche application (Blonder *et al.*, 2018). This method uses elliptic random sampling to create clouds of data points around the observed data points, assuming that sampled data points within the hypervolume would be close to observed data points (Blonder *et al.*, 2018). However, the number of observations influences the final hypervolume size, because higher uncertainty (i.e., fewer data points) results in bigger hypervolume size. To perform set operations, we subset hypervolumes of each species to the same random density using the function hypervolume_n_occupancy. Mean absolute error (MAE) and Root mean squared error (RMSE) were used to evaluate the accuracy of the subset hypervolumes. The relative niche size of each species was then calculated as its absolute volume divided by the volume of the union of input hypervolumes. Relative niche uniqueness, on the other hand, was calculated as the absolute niche uniqueness of a species divided by its absolute niche size. To obtain uncertainty measures of niche size and niche overlap, bootstrapping was implemented on 199 permutations of each species niche, which were then used to calculate 199 relative total occupancies. Pairwise niche size comparison among species, as well as niche uniqueness comparison, were tested setting the significance level at 0.05. Graphical representations of all results were created with packages *ggplot2* (Wickham, 2016) and *ggbiplot* (Vu, 2011).

4.3 Results

The analyses included ten environmental variables - Depth, pH, SPC, DIC, NO3, DRSi, SRP, sed.OM, sed.TP and sed.density – and eight functional traits – LA, pet.propA, pet.propDW, LDMC, SLA, chlab, P and C (Table 1). Five out of ten environmental variables did not show any difference among species, namely, Depth, pH, SPC, DIC and sed.TP (Table 2), although wide ranges of these parameters were observed in the investigated populations.

***Table 1**: List of environmental variables and functional traits included in the niches computation. Loadings of the axes used in the analyses are reported.*

	Variable	**Unit of measurement**	**Abbreviation**	**PC1**	**PC2**	**PC3**
ECOLOGICAL NICHE	Depth	m	Depth	-0.342	-0.024	0.024
	pH		pH	-0.172	-0.138	0.578
	Dissolved inorganic carbon	mE l^{-1}	DIC	0.269	-0.053	0.370
	Specific conductivity	µS cm^{-2}	SPC	0.512	0.055	0.011
	Nitrate	µg l^{-1}	NO3	-0.076	0.164	-0.505
	Dissolved reactive silica	µg l^{-1}	DRSi	0.467	0.056	-0.103
	Soluble reactive phosphorus	µg l^{-1}	SRP	0.525	0.082	0.007
	Sediment organic matter content	%	sed.OM	-0.006	0.601	0.285
	Sediment total phosphorus	µg g^{-1}	sed.TP	-0.108	0.491	-0.311
	Sediment density	g ml^{-1}	sed.density	0.080	-0.579	-0.291
FUNCTIONAL NICHE	Leaf area	mm^{2}	LA	0.436	-0.084	
	Proportion of petiole area	%	pet.propA	-0.354	0.014	
	Leaf dry weight	g	LDW	0.433	-0.054	
	Proportion of petiole dry weight	%	pet.propDW	0.294	-0.121	
	Leaf dry matter content	mg g^{-1}	LDMC	0.306	0.129	
	Specific leaf area	mm^{2} mg^{-1}	SLA	-0.263	-0.479	
	Leaf chlorophylls content	µg cm^{-2}	chlab	0.302	0.036	
	Leaf phosphorus content	%	P	0.001	-0.607	
	Leaf nitrogen content	%	N	0.137	-0.600	
	Leaf carbon content	%	C	0.370	0.018	

Depth varied from 0.1 m for *N. lutea* and *N. nucifera* to 2.5 m for *N. nucifera*, pH ranged between 6.45 pH unit for *N. alba* to 9.71 pH unit for *N. lutea*, SPC spanned from 215 µS cm^{-1} for *N. alba* to 2214 µS cm^{-1} for *N. nucifera*, DIC ranged between 1.22 mE L^{-1} for *N. peltata* to 10.75 mE L^{-1} for *N. alba*, and sed.TP varied from 150 µg g^{-1} to 2629 µg g^{-1}, both in *N. alba* populations. Nitrate reached a peak of 8.89 µg L^{-1} in *N. lutea* and was significantly lower for *N. alba* than for *N. peltata* (p-value=0.02). DRSi was significantly lower in *N. alba*, with a minimum of 60 µg L^{-1}, than in *N. nucifera* (p-value=0.04), where it reached 4543 µg L^{-1}. The same trend was observed for SRP (p-value=0.003), with a maximum of 485 µg L^{-1} for *N. nucifera* and a minimum of 0 µg L^{-1} for *N. alba, N. nucifera* and *N. lutea*. Sed.OM was significantly lower for *N. peltata* than for all other species (p-values=0.03 compared to *N. lutea*, 0.001 to *N. nucifera* and 0.007 to *N. alba*) and ranged between 1.2% for *N. peltata* and 63.2% for *N. nucifera*. By contrast, sed.density was higher in *N. peltata* compared to the other species, although a peak of 2 g mL^{-1} was observed for *N. lutea* (p-value=0.03 compared to *N. lutea* and <0.005 to *N. nucifera* and *N. alba*).

Functional traits, on the other hand, all showed some differences among species (Table 2).

***Table 2**: mean values (and standard deviation in brackets) of environmental variables and functional traits in the investigated species. Letters indicate statistical differences among species, if letters are not present no significant difference was found.*

		NL	**NN**	**NP**	**NyA**
ECOLOGY	Depth	1.03(0.52)	0.87(0.72)	0.98(0.3)	1.33(0.46)
	pH	8.41(0.78)	7.86(0.48)	7.82(0.35)	8.11(0.64)
	SPC	390.83(123.47)	674.92(614.88)	535.37(211.35)	326.82(110.47)
	Nitrate	2.26(3.16) [**ab**]	3.37(3.89) [**ab**]	1.37(0.85) [**a**]	0.26(0.45) [**b**]
	DRSi	900.02(376.72) [**ab**]	1482.62(1161.18) [**a**]	1038.06(313.47) [**ab**]	686.03(674.88) [**b**]
	DIC	3.49(0.76)	3.87(1.53)	2.93(0.89)	3.58(2.47)
	SRP	24(20.8) [**ab**]	104.13(143.59) [**a**]	12.37(7.17) [**ab**]	7.99(16.31) [**b**]
	LOI	18.71(10.51) [**a**]	24.63(15.82) [**a**]	5.66(4.44) [**b**]	21.54(11.48) [**a**]
	sed.TP	984.69(459.44)	1260.44(534.6)	700.64(319.79)	868.46(719.28)
	sed.density	1.23(0.26) [**b**]	1.18(0.27) [**b**]	1.53(0.2) [**a**]	1.18(0.22) [**b**]
TRAITS	LA	75610(19339) [**b**]	417330(115826) [**a**]	14934(3722) [**c**]	74119(24912) [**b**]
	pet.propA	0.2(0.05) [**a**]	0.08(0.02) [**b**]	0.21(0.06) [**a**]	0.18(0.03) [**a**]
	LDW	10.6(3.8) [**b**]	58.4(18.43) [**a**]	0.88(0.3) [**c**]	10.85(4.35) [**b**]
	pet.propDW	0.44(0.07) [**b**]	0.53(0.07) [**a**]	0.38(0.08) [**bc**]	0.38(0.06) [**c**]
	LDMC	110.65(15.2) [**b**]	162.26(29.26) [**a**]	109.34(24.59) [**b**]	121.89(24.72) [**b**]
	SLA	7.58(1.59) [**b**]	7.30(1.10) [**b**]	17.95(3.98) [**a**]	7.04(1.25) [**b**]
	chlab (/g)	905.54(372.65) [**b**]	2640.48(634.22) [**a**]	806.44(308.26) [**b**]	798.15(368.52) [**b**]
	chlab (/cm)	37.35(10.64) [**ab**]	41.31(9.77) [**a**]	25.97(5.43) [**c**]	30.37(10.32) [**bc**]
	P	0.28(0.09) [**b**]	0.37(0.11) [**ab**]	0.48(0.13) [**a**]	0.17(0.04) [**c**]
	N	2.97(0.51) [**b**]	3.64(0.56) [**a**]	3.61(0.52) [**a**]	2.2(0.33) [**c**]
	C	42.85(0.5) [**b**]	43.84(1.13) [**a**]	41.31(0.92) [**c**]	42.48(0.43) [**b**]

Results for LDW and N are reported although not included in the functional niche determination because they are highly correlated with LA and P, respectively. Leaf size (LA and LDW) was highest in *N. nucifera* and lowest in *N. peltata* (for complete list of pairwise comparison p-values, see AIV.1), ranging from 6793 mm^2 and 0.5 g in *N. peltata* to 702858 mm^2

and 99.5 g in *N. nucifera*. Pet.propA and pet.propDW were respectively lowest (p-value<0.0001) and highest (p-value<0.0005) in *N. nucifera* compared to other species, and ranged between 3.9% in *N. nucifera* and 39.8% in *N. lutea* for pet.propA, and between 21.9% in *N. lutea* and 68.8% in *N. nucifera*. LDMC was significantly higher in *N. nucifera* (p-value<0.0001) reaching 243 mg g^{-1}, while SLA was significantly higher in *N. peltata* (p-value<0.0001), up to 25.5 mm^2 mg^{-1}. Chlorophylls content on fresh weight basis was omitted from the analyses because highly positively correlated with leaf size (Pearson's correlation index=0.84 with LA), so only chlorophylls content on area basis (chlab) was included and were higher in *N. nucifera* compared to *N. peltata* and *N. alba* (p-values<0.0001 and 0.0018 respectively) and ranged between 16 μg cm^{-2} in *N. peltata* to 68 μg cm^{-2} in *N. nucifera*. Leaves elemental composition also showed marked differences, with P and N higher in *N. nucifera* and *N. peltata*, reaching 0.73% and 4.89% for P and N respectively, and lower in *N. alba* (p-value<0.005), reaching only 0.10% and 1.73% respectively. C, on the other hand, was significantly higher in *N. nucifera* compared to all other species, and significantly lower in *N. peltata*, and varied from 40.2% in *N. peltata* to 45.9% in *N. nucifera*.

Ecological niche

The first three PCA axes were used in the hypervolumes computation, and together explained 60.7 % of the variance in the data (Fig. 2a-b-c). The first axis (26.8% explained variance) was related to water nutrients at the positive end (SRP, DRSi and SPC) and with water depth at the negative end (Table 1). The second axis (20.6% explained variance) was more related to sediment features, with sed.TP and sed.OM associated with positive axis values and sed.density with negative values. The third axis (13.3% explained variance) was again related with water quality, positively correlated with pH and negatively with NO3. *N. nucifera* showed the highest relative niche size (82.0%), followed by *N. lutea* (28.4%), *N. alba* (27.8%) and *N. peltata* (6.6%). However, uncertainty estimates revealed that only *N. nucifera* and *N. peltata* have a statistically different niche size (Fig. 4 and AIV.2). *N. nucifera* also showed the highest relative niche uniqueness, with 74.6% of its ecological niche not shared with any other species, followed by *N. peltata* (29.2%), *N. alba* (14.6%) and *N. lutea* (9.9%). The variability of niche uniqueness, though, was very high, and no species showed significantly different values of niche uniqueness.

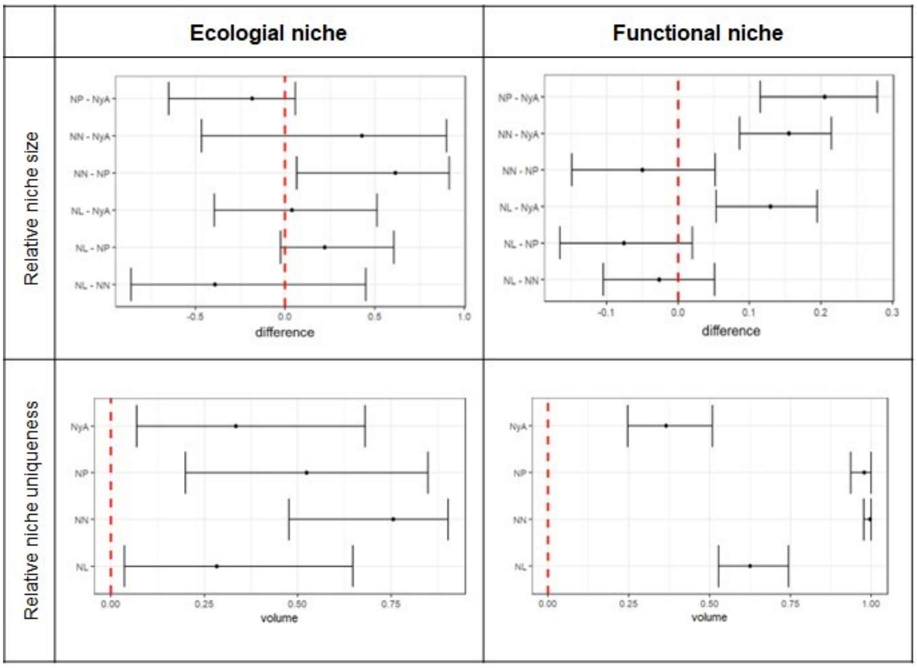


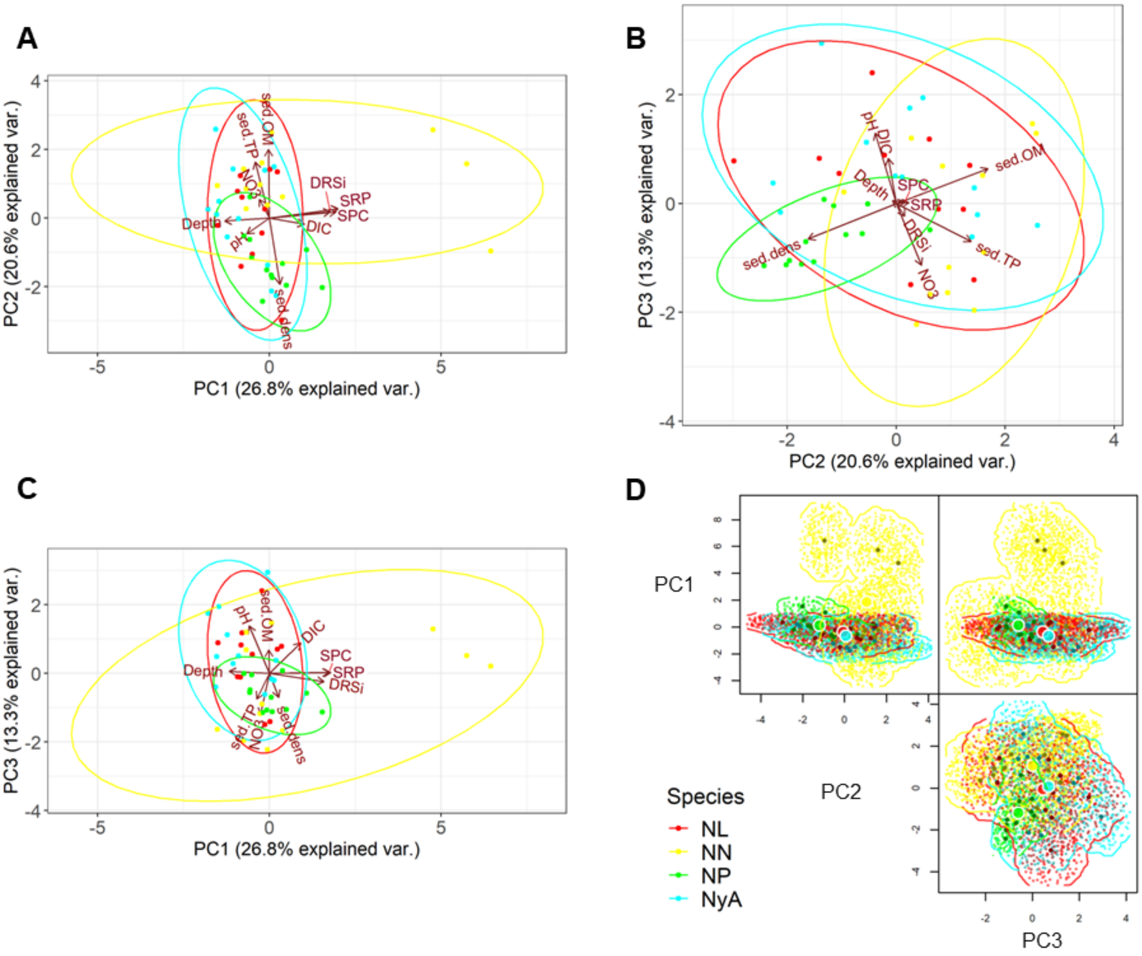
Figure 4. *Uncertainty estimates of hypervolumes size and uniqueness, obtained with bootstrapping on 199 permutated hypervolumes on each species. Relative niche size is the proportion of niche occupied by each species relative to the total niche occupied by all species. Relative niche uniqueness is the proportion of unique niche of each species relative to its total niche volume. Significant level was set at 0.05, mean differences, 2.5 and 97.5 quantiles can be found in SI2. For relative niche size, differences between species are significant if the black line doesn't cross the red dashed line. For relative niche uniqueness, species have a uniqueness significantly higher than 0 if the black line doesn't cross the red dashed line, and species have significantly different niche uniqueness if their black lines do not overlap. NL=N. lutea, NN=N. nucifera, NP=N. peltata, NyA=N. alba.*

Nevertheless, all species showed a niche uniqueness significantly higher than zero (Fig. 4 and AIV.2). The results obtained suggest that all the investigated nymphaeids species share similar ecological conditions, but *N. peltata* tendentially has a smaller niche size, due to its presence in sites with lower sed.OM and higher sed.density. *N. nucifera*, on the other hand, showed a bigger and more unique niche (Fig. 2d) because three of its populations were found at sites with very high SPC (>1300 µS cm^{-1}).

Functional niche

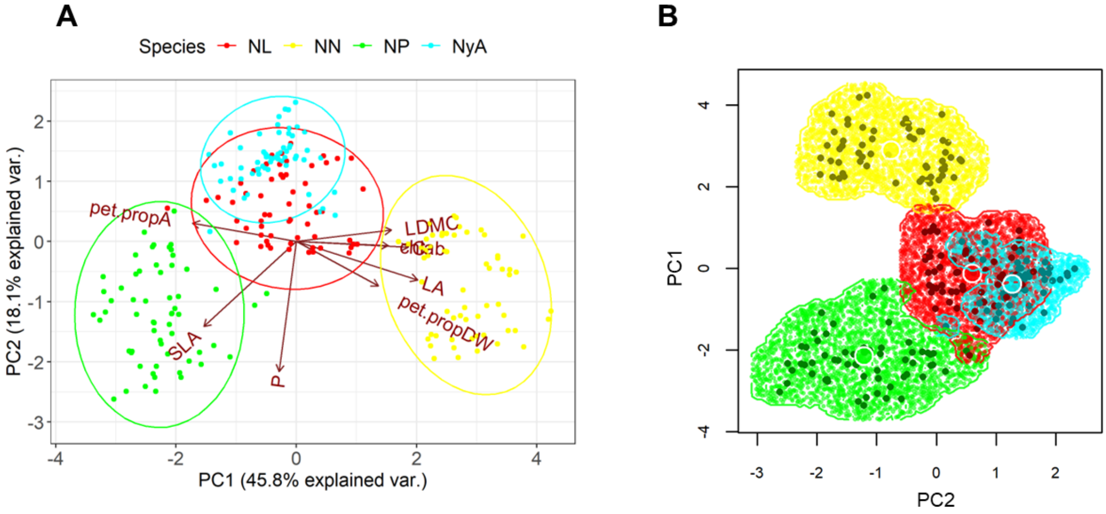
For the functional niche determination, only the first two PCA axes were used, which accounted for 63.8% of the trait variation (Fig. 3a). PC1 (45.8% explained variance) was mainly related to leaf size and structure. Positive values of PC1 were associated to high LA and C, as well as LDMC, pet.propDW and chlab, though with lower loadings (Table 1). Negative values of PC1 were instead associated to high pet.propA. PC2 (18.1% explained variance) was related with leaf resource-use efficiency, and was negatively correlated with P and SLA.

Fig. 2: first three axes of environmental PCA (a-b-c) and graphical representation of the ecological niche (d). Explained var.=explained variance, NL=Nuphar lutea, NN=Nelumbo nucifera, NP=Nymphoides peltata, NyA=Nymphaea alba



The biggest relative niche size was found for *N. peltata* (37.4%) followed by *N. nucifera* (30.8%), *N. lutea* (29.0%) and *N. alba* (16.1%). The uncertainty estimates showed no difference between the first three species niche sizes, but *N. alba* showed a significantly smaller relative niche size compared to the other species (Fig. 4 and AIV.2). Compared to the ecological niches, functional niches of the investigated species were more distinct: *N. nucifera* had 98.9% niche uniqueness, followed by *N. peltata* (96.8%), *N. lutea* (54.3%) and *N. alba* (27.0%). All estimates of niche uniqueness were significantly > 0, but they were not statistically different between *N. nucifera* and *N. peltata* (Fig. 4 and AIV.2). *N. nucifera* had a distinct niche that occupies the functional space described by large leaf size and high P (Fig. 3b). At the other end of the functional space, with higher SLA, pet.propA, leaf nutrients content and lower leaf size lied *N. peltata*. Between these two species there were *N. lutea* and *N. alba*, with intermediate traits values. *N. alba* occupies a subset of *N. lutea* functional niche, slightly differentiated by lower leaf nutrients content.

***Fig. 3**: first two axes of functional PCA (a) and graphical representation of the functional niche (b). Explained var.=explained variance, NL=Nuphar lutea, NN=Nelumbo nucifera, NP=Nymphoides peltata, NyA=Nymphaea alba*



5.4 Discussion

The comparison between the ecological and functional niches of four species of nymphaeids show that all species share most of their ecological niches, suggesting that they occur in similar environments and have similar basic resource requirements. This is in accordance with the general state of very poor quality of the aquatic environments investigated, mainly represented by marginal environments strongly impacted by agriculture and largely used as water reserves (Bolpagni *et al*., 2016, 2020).

Only *N. nucifera* presented a rather big ecological niche, given by its presence in sites with extremely high conductivity, and *N. peltata*, on the other hand, shows a reduced niche size, indicative of a more specialist behavior, which is partly distinguished from other species by the higher sediment density, typical of reclamation canals that are periodically dug for irrigation purposes. With regard to the functional niche, species are clearly more distinguished, indicating a differentiation in the functional response to similar environmental conditions. *N. nucifera*, *N. lutea* and *N. peltata* present similar functional niche size, however both *N. nucifera* and *N. peltata* have highly unique niches, mostly separated from other species. This differentiation is due to leaf size, but not only. Indeed, both species presented high leaf nutrients content (N and P), and they also differentiate by the SLA, C and petiole contribution. Interestingly, *N. alba* has a small relative functional niche size, occupying an intermediate portion of the functional space, which is contrasting with its relatively wide ecological niche, and would suggest a lower ability of this species to adapt to different environmental conditions (e.g., showing lower functional plasticity). The opposite can be said for *N. peltata*, that shows a small relative ecological niche size compared to a functional niche size similar to most other species, suggesting a high plasticity of this species expressed in a reduced ecological space.

N. lutea shows intermediate features both in terms of ecological and functional niche. This confirms its reputation of top-competitor species in aquatic environments (Bornette *et al.*, 2008, Paillisson and Marion, 2011). Indeed, our results indicate a fair plasticity of *N. lutea* – given by a wide functional niche – coupled with wide environmental tolerance. Previous studies have shown that *N. lutea* is able to modulate its response and resource-use strategies to variable conditions and can inhabit also highly eutrophic environments (Dalla Vecchia and Bolpagni, 2022; Dalla Vecchia *et al.*, under review). Therefore, the functional plasticity of this species is triggered by variations in the environment, and this allows it to increase its performance in favorable conditions (Kordyum and Klimenko, 2013; Nowak *et al.*, 2015), or adapt to a more conservative strategy under stress (Richards *et al.*, 2011, Klok and van der Velde, 2017). However, although *N. lutea* populations can occupy a wide range of environmental conditions, with an electrical conductivity range of nearly 400 $\mu S\ cm^{-1}$ and nitrate concentration up to 9.5 $\mu g\ L^{-1}$ (Dalla Vecchia *et al.*, under review), based on our results, it is not able to establish in extreme environmental conditions, represented by very high conductivity and nutrients content, where *N. nucifera* was found.

Similarly to *N. lutea*, *N. alba* also occupies intermediate portions of the ecological and functional space, widely overlapping with *N. lutea*. Szańkowski and Kłosowski (1999) and Pełechaty (2007) also observed a similar and wide environmental tolerance or requirements for both these species. Klok and van der Velde (2017), moreover, observed a comparable functional response of these species to the variation in environmental drivers. However, adding the information on the functional niche, we clearly see that *N. alba* niche is nested in that of *N. lutea*, occupying only a subset of *N. lutea* functional space. It slightly distinguishes only by a lower leaf nutrients content, a feature that might explain for *N. alba* and higher conservative resource-use strategy and lower competitiveness (Ding *et al.*, 2019). In fact, the general decline of *N. alba* populations was imputed to the hybridization with introduced ornamental varieties (Nierbauer *et al.*, 2014) and to habitat degradation (Parveen *et al.*, 2022). Nonetheless, habitat degradation is happening for *N. lutea* as well, given that they share similar environmental conditions, and it does not show the same declining trend. Therefore, we believe that the limited plasticity given by the smaller functional niche and the lower leaf efficiency represented by the lower nutrients content may expand the evidence based on environmental conditions and partly explain the higher susceptibility of *N. alba* populations.

N. peltata, instead, shows its own specificities both in the ecological and functional niche. First of all, this species does not exhibit the same variability in environmental tolerance, and its ecological niche size is shrunk mainly due to particular sediment quality requirements. In fact, we found *N. peltata* mainly in lowland canals, close to the mouth of the river Po, where sediments were poor in organic matter content and presented higher density due to the abundance of fine clay particles. Szańkowski and Kłosowski (1999) also observed a separate ecological niche of *N. peltata* compared to other nymphaeid species, distinguished by a higher sediment mineral content. In contrast to the specificity of its ecological niche, which potentially limits the spread of this species, we observed a wide and highly unique functional niche, that occupies the most acquisitive corner of the functional space. Indeed, *N. peltata* leaves differentiate from other species not only for the small size and high nutrients content (typical of *N. nucifera*, too), but also for a high SLA. This is in line with previous findings that described a high N and P content in *N. peltata* biomass, stored in the root system and moved to the photosynthetic organs during the growing season (Brock *et al.*, 1983b), thus confirming the active role of this rooted floating-leaved species in the nutrient cycling of aquatic habitats. Markovskaya and coauthors (2019) also observed a high SLA in *N. peltata*, a trait that increases the photosynthetic efficiency of the leaves. The relatively low pet.propDW observed in our study further supports the acquisitive strategy of this species, because it indicates a low biomass allocation in petioles than in blades, compared to other nympaeid species. Moreover, it commonly reproduces by seeds and produces persistent seed banks (Smits *et al.*, 1990; Wang *et al.*, 2005), which might increase the genetic diversity of *N. peltata* populations, promoting higher traits variability despite limited environmental ranges (Liao *et al.*, 2013).

Finally, *N. nucifera* represents the only alien and invasive species in our study. Our results mirror the competitiveness and invasive potential of this species, given by a very wide environmental niche coupled with a highly unique functional niche. Its ecological niche embraces most of the niches of the other species, excluding sediments with high density and low organic matter content, and extending to extreme values of conductivity, indicating a generalist behavior. However, our hypothesis of functional niche similarity to native species was not supported, since it was separated from the other investigated species. This is in contrast with findings by Dalle Fratte *et al.* (2019), who did not observe a differentiation of *N. nucifera* from other nymphaeids. This discrepancy is probably due to the fact that Dalle Fratte and coauthors (2019) represented species groups based on their CSR strategy (*sensu* Grime and Pierce, 2012), which was calculated using LA, LDMC and SLA, while here we expanded the number of traits to represent a more complete functional niche. In this study, niche uniqueness of *N. nucifera* was given mainly by the leaf size, but also by the significantly higher LDMC and pet.propDW, both traits indicating a more conservative strategy, and by high leaf N and P. Leaf chlorophylls were significantly higher than other species when considered on a fresh weight basis, and was among the highest also on an area basis, indicating high photosynthetic efficiency. It seems that *N. nucifera* combines both acquisitive and conservative traits, and still manages to exhibit a high invasive potential. In one of our study sites, Mantua lakes system, it has been observed to rapidly colonize available space and outcompete native nymphaeid species (Pinardi *et al.*, 2021). One of the reasons for its ability to survive and thrive even in highly organic sediments might be the sophisticated aeration system, based on a two-way gas flow, that helps maintaining the submerged organs oxygenated even in anoxic conditions (Große, 1996). Besides, the extremely large LA of *N. nucifera* is a strong indicator of high competitiveness (Díaz *et al.*, 2016) allowing it to efficiently shade other species and rapidly gain space forming monospecific stands. Indeed, competitive traits are typical of invasive species (Guo *et al.*, 2022). However, large LA might come at a cost: big emerging leaf blades need strong support, which can explain the high pet.propDW and LDMC observed in our study, once again highlighting the importance of petioles in influencing resource-use strategies in nymphaeids (Dalla Vecchia and Bolpagni, 2022).

5.5 Conclusions

This study demonstrated how the investigated nymphaeid species, though sharing more or less similar environmental conditions in the investigated sites, can be highly differentiated with regard to their functional niche, highlighting not only morphological (leaf size) but also biochemical (leaf efficiency) adaptations. Overall, our data reinforced the main known drivers for functional variation in rooted floating-leaved species, namely water conductivity and sediment quality.

The hypervolume approach permitted to evidence aspects of the species behaviors, from plasticity and specialization to competitiveness. Particularly, three functional types were identified: highly acquisitive leaves with high specific leaf area (SLA), low leaf dry matter content (LDMC) and high nutrients content (*N. peltata*), acquisitive leaves with large leaf size and high chlorophylls and nutrients content, though with conservative traits like high LDMC and low SLA (*N. nucifera*) and intermediate traits (*N. lutea* and *N. alba*). Competitive species occupied intermediate functional and ecological niche portions, though less competitive species showed lower leaf efficiency and plasticity (*N. alba*). More specialized species (*N. peltata*) had a small ecological and highly unique functional niche, while, contrary to our expectations, invasive species enhanced its competitiveness by distinguishing its functional niche rather than by just enlarging it.

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6. Synthesis and final conclusions

The aim of this PhD thesis was to summarize the available information on the use of functional traits in macrophytes studies and to investigate the environmental drivers of traits variability in nymphaeids with regard to their resource-use strategies, using field data.

6.1 Synthesis of main activities

In the review (Dalla Vecchia et al., 2020) presented in chapter I, we analyzed nearly 300 scientific papers published between 2010 and 2020 that included around one third of all the world macrophyte species. The main evident trend within the analyzed studies is that most research has been carried out in Europe and Asia, was focused on lakes, and aimed at investigating the effect of environmental gradients on morphology and productivity traits (cfr. Fig. 5 – chapter I).

Although most of the categories of information on methodology, traits and research questions were all quite well represented in the literature, some gaps were identified. Regarding the geographical range of studies, important regions of the world are underrepresented, like South America, still they host hotspots of macrophytes biodiversity. Moreover, most of the studies focused on submerged and emergent species in shallow lakes (7/10 of the most studied species are submerged macrophytes), dedicating little space to floating species and deeper lakes, possibly leading to a limited understanding of the full gradient of macrophytes functional response, by neglecting some growth forms and omitting the extremes of their habitats (i.e., deep water). The choice of traits should always be the best fitting to the research questions, though indeed this often conflicts with the time and resources available. Therefore, it is no surprise that soft traits describing the plants morphology and productivity are the most commonly used. Besides, although field and laboratory studies are equally represented in the literature, field studies often avoid a direct measure of traits and rely on already available traits. This could obviously lead to misleading results if the scale adopted is not big enough to cross that threshold represented by the interspecific/intraspecific trait variability ratio. Nonetheless, we observed an effort to potentially increase the spatial and temporal scale considered, by implementing new tools like remote sensing techniques, exploiting the result of plants interactions with light to derive traits data on aboveground – leaves – organs. Certainly, another substantial knowledge gap is belowground traits. Around a third of the analyzed studies included root traits, but, actually, most of them were limited to biomass ratio between belowground and aboveground organs. Hence, our current knowledge on macrophytes roots functions is extremely limited, in spite of their confirmed role in macrophytes establishment and fitness. Related to this, sediments also received little attention as an environmental driver, despite their importance as nutrient source (and potential stressful factor) demonstrated in this thesis. One additional remark on the proper choice of functional traits regards the study of invasive species, which was the third most investigated topic after environmental gradients and anthropic pressure. However, we believe that, to better understand the dynamics of invasive species spread, the most common approach using morphology and productivity traits should expand to include other important traits, like reproduction-related traits. Finally, we strongly promoted the consideration of interactions with other biotic components of the ecosystems, in order to fully understand the ecological role of macrophytes. In light of the findings from the literature analysis, and depending on the macroDIVERSITY project purposes, we focused our attention on a combination of poorly investigated topics: the intraspecific variation of resource-use strategies in rooted floating-leaved species, based on measured traits from field collection.

In chapter II (Dalla Vecchia and Bolpagni, 2022), we investigated the leaf traits variation of a nymphaeid species, *N. lutea*, in relation to local environmental variability. We demonstrated the presence of processes taking place at a very local scale, within a lake system (e.g., Lake Chiusi). This species showed high traits plasticity in response to changes in water depth and sediment features (cfr. Fig. 2 – chapter II).

In particular, at the local scale the influence of sediments metabolism on the water column was clearly evident and resulted in correlated water parameters (like conductivity and nutrients). Nymphaeids are structurally different from other macrophytes, and the construction costs of their long petioles reflected in the efficiency of their resource-use. We observed a relatively constant ratio between the biomass allocated to petioles and to leaf blades, resulting in bigger leaf blades in deeper water that can compensate for the petiole construction costs with a larger photosynthetic surface. Besides, some correlations with environmental variables were visible at the local scale only including petiole-related traits. Common handbooks on the measure of functional traits for plants are generally based on terrestrial species, thus they do not take into account or give specific indications for the structural differences of aquatic plants, so the information on petioles is not always included in macrophytes studies. Based on our results, we encourage the consideration of petiole-related traits for rooted, floating-leaved macrophytes, as they provide important information on plants performance.

Then, we extended the scale of the investigation by including more lakes and by integrating functional traits with leaf reflectance features and genetic diversity metrics, in chapter III (Dalla Vecchia et al., under review *a*). Again, *N. lutea* showed a plastic response to changing environmental conditions. The main environmental driver was represented by electrical conductivity, which was explicative of site-specific conditions (cfr. Fig. 2 – chapter III). In general, non-linear relationships between traits and environmental drivers were due to a site effect.

Genetic diversity metrics also responded to conductivity, showing a negative relationship. Usually, genetic information is compared to functional traits measures to understand to understand the importance of phenotypic plasticity over genotype, so this is one of the few attempts to investigate the effect of the environment on genetic diversity. Specifically, one of our study sites – characterized by high conductivity – hosted a distinctly lower genetic diversity, suggesting that extreme environmental conditions might affect the genetic structure of populations by selecting genotypes over time. At the regional scale, we observed a limited effect of water nutrients on traits. This is mainly because nutrients are seldom detectable during the growing season, due to a fast recycling. However, the effect of nutrients was evident by considering sediment quality, emphasizing the importance of sediments for rooted macrophytes. An increase in nutrients availability was able to boost *N. lutea* performance, favoring traits related to a more acquisitive strategy, though very high nutrients levels seem to determine stressful conditions, leading to a switch to more conservative strategies. Moreover, leaf reflectance features, related to leaf structure and anatomy, also showed strong correlation with environmental variables, and revealed good indicators of leaves responses. This result itself advises a successful use of remote sensing-derived traits to study the effect of environmental drivers on plants performance.

In the IV chapter we eventually widened the study to include three other nymphaeid species (Dalla Vecchia et al., under review *b*). We used a hypervolume approach (Blonder et al., 2018) to discern between ecological and functional niche of the species, in order to discuss their environmental requirements and functional expression. We observed that all these species share similar ecological requirements, but the invasive species *Nelumbo nucifera* is able to extend its niche and survive in extreme conditions, described by a very high electrical conductivity (>1300 μS cm^{-1}). Instead, species differentiated with regard to their functional niche (cfr. Fig. 3 – chapter IV), reflecting both leaf size and photosynthetic efficiency (described by leaf nutrients content, specific leaf area – SLA – and leaf dry matter content – LDMC).

The main drivers highlighted in chapter III were confirmed here, hence conductivity and sediment quality, together with water nutrients, were the most important determinants of ecological requirements of nymphaeids. Most species have statistically similar ecological niche size, except for *N. peltata*, which showed a more specialist behavior having a reduced niche size. By contrast, it had the biggest functional niche, and highly unique, suggesting a marked functional adaptation ability to a reduced set of conditions, expressed also by highly acquisitive leaf features. A rather opposite behavior was observed for *N. alba*, that showed a large ecological niche size – indicative of more generalist requirements – opposing to a small functional niche, nested within *N. lutea*, suggesting a limited capability to functionally differentiate from other competitor species and to adapt to variable environmental conditions. This possibly explains the general decline observed in *N. alba* populations. As for *N. lutea*, it exhibited proportionally similar ecological and functional niche size, located in the middle of the multivariate space, which could confirm its role as a top competitor species, and once again highlights its functional plasticity in response to a wide range of environmental conditions. Finally, *N. nucifera* didn't show a functional niche bigger than other species, however, quite like *N. peltata*, it was nearly completely separated from other nymphaeids. Therefore, its invasive potential is induced by the ability to occupy a different portion of the functional niche, coupled with a wide environmental tolerance. Nonetheless, its leaves curiously possess both acquisitive and conservative features: extremely big leaves with high nutrients and chlorophyll content present, at the same time, high LDMC and low SLA. This probably indicates a structural limit represented by large leaf area, which in turn needs more support by strong petioles, overall increasing the LDMC and lowering the SLA.

6.2 Final conclusions

The results obtained in this thesis reveal important insights in a group of macrophytes which was so far not well represented in the literature with regard of functional traits. We were able to demonstrate the high functional plasticity of nymphaeids to environmental conditions, and to identify main drivers of this variability. In all our studies, sediment quality and electrical conductivity were the most important variables influencing nymphaeids traits and distribution, highlighting how water physical and chemical parameters alone – indeed the most used in previous studies – may be only partial descriptors. The importance of sediments, confirmed by our studies, urges a change of perspectives, with the aim of regularly including root traits in future functional research, as they most likely mediate the response observed in leaves. The focus on the intraspecific trait variability, provided by this thesis, proves that there is a lot happening below the species level, too, and questions whether the use common use of mean trait values is really able to adequately catch community level responses. Besides, one more issue emerging from this thesis is the problem of standardization in functional traits measures, especially for aquatic plants. On one hand, a standard and comparable measure of traits for all species is desirable, but on the other hand it is sometimes impossible not to take into account species-specific adaptations which could twist the meaning of the trait itself. For this reason, some of the side activities of my PhD were directed to finalizing *ad hoc* protocols for the measure of functional traits in particular groups of species (*Characeae*, *Utricularia* spp., *Salvinia* spp.). Moreover, the application of functional traits both to native and invasive species proved useful to understand the extent of their environmental tolerance and the characteristics that make these species good or poor competitors. In light of the results of my side research on pioneer vegetation, we expect that the application of functional traits to these communities will allow to better understand the changes taking place in these ecosystems, indicating whether or not their functionality is still preserved, regardless of the taxonomic modification. Finally, we believe that this thesis provided valuable perspectives also from the methodological point of view. In fact, we incorporated new statistical approaches to the study of macrophytes ecology, namely the use of combined functional and ecological hypervolumes and the mMCA analysis for the investigation of spatial structure, and we tried to demonstrate the potential of remote sensing tools to enlarge a classical functional traits approach.

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Appendix I

Supplementary materials to chapter I.

AI.1: Revision matrix summarizing the information obtained from the 296 papers analyzed. The absolute and relative representativeness of each category within each feature is presented.

Features	Tag	Explanation	Papers	%
Continent	*Eur*	Europe	122	41.4
	Asi	Asia	93	31.5
	AmS	South America	24	8.1
	AmN	North America	24	8.1
	Oce	Oceania	7	2.4
	Afr	Africa	4	1.4
	Glo	Global	21	7.1
Habitat type	*Lak*	Lake	84	30.0
	Pon	Pond	34	12.1
	WetL	Wetland	56	20.0
	Mar	Marine	17	6.1
	Lot	Lotic	59	21.1
	AnyH	Any	30	10.7
Field/Lab	*L*	Lab	124	42.3
	F	Field	129	44.0
	LF	Field and lab	16	5.5
	R	Review	24	8.2
Macrophyte type	*Sub*	Submerged	124	39.0
	RoEm	Rooted emergent/floating leaved	95	29.9
	FFlo	Free floating	28	8.8
	AnyM	Any	71	22.3
Species number	*1to3*		169	57.3
	4to6		30	10.2
	>6	more than 6	96	32.5
FTs category	*GroF*	Growth form	48	6.8
	Mor	Morphology	195	27.7
	Prod	Productivity	159	22.6
	Phys	Physiology	86	12.2
	BioC	Biochemistry	75	10.7
	Rep	Reproduction	96	13.7
	EcoP	Ecological preferences	16	2.3
	Mec	Biomechanics	18	2.6
	Bint	Biotic interactions	4	0.6
	OthFT	Other	6	0.9
FTs root-shoot	*Sho*	Shoot	166	57.4
	Ro	Root	12	4.2
	SR	Shoot and Root	111	38.4
Main environmental variable	*Wat*	Water	126	34.2
	Sed	Sediment	52	14.1
	Clim	Climate	42	11.4
	Ant	Anthropic	27	7.3
	DeLig	Depth/light	65	17.7
	HydTop	Hydrology/topography	56	15.2
Main topic	*EnvG*	Environmental gradients	97	30.4
	ComS	Community structure	42	13.2
	AntPr	Anthropic pressure	58	18.2
	BioI	Biotic interactions	35	11.0
	Inv	Invasiveness	47	14.7
	SpCh	Specific characteristics	29	9.1
	OthTop	Other	11	3.4

AI.2: List of the 296 papers analyzed for the review, reported in chronological order. Reviews are highlighted in bold.

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AI.3: Extension of Table 1 showing the funcional traits and applications for the ten most studied species, including references. On the same line, traits and corresponding ecological questions addressed are marked. Only papers studying *1to3* species were included in this table.

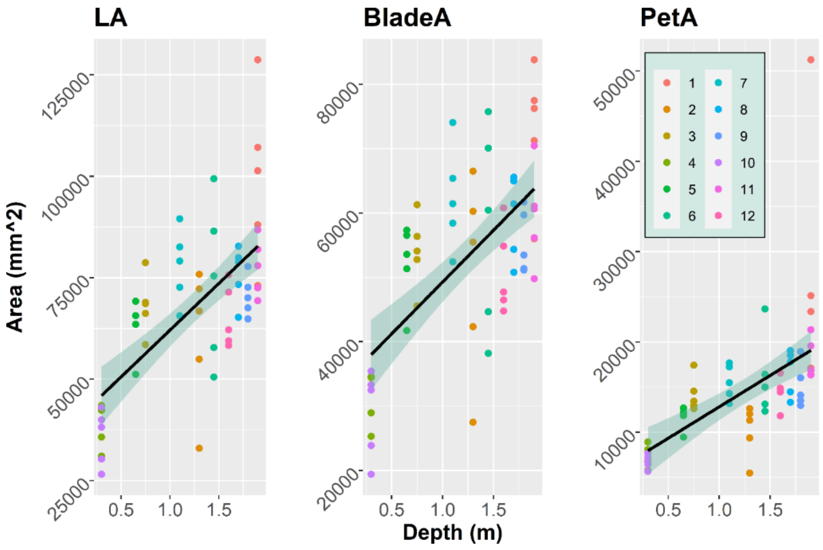
Targeted species	**Functional traits**								**Application**									
	Morphology	Elemental composition, DMC	Productivity	Physiology	Biomecanics	Reproduction	Root anatomy	Contaminants accumulation	Herbivory, palatability	Invasive species, differences/interactions with natives	Environmental conditions (water, climate, landscape)	Sediment characteristics	Eutrophication and pollution	Mechanical stress	Competition and community structure	Interactions other organisms (algae, bacteria, animals)	Intraspecific variability	**References**
Myriophyllum spicatum		x							x	x					x			Wong et al., 2010; Thouvenot et al., 2019
	x		x									x		x	x			Xie and Yu 2011b; Xie et al., 2013; Richter and Gross 2013; Li et al., 2016; Cao et al., 2016
		x	x	x							x					x		Hussner and Jahns 2014; Li et al., 2019a
			x					x					x					Roubeau Dumont et al., 2020
Ceratophyllum demersum	x	x		x						x	x		x				x	Hyldgaard et al., 2012; Hyldgaard and Brix 2012; Eller et al., 2015; Ellawala Kankanamge et al., 2019; Roubeau Dumont et al., 2019
	x		x							x								Thouvenot et al., 2013; Thiébaut et al., 2018
		x							x				x					Xing et al., 2016; Sun et al., 2018
					x								x	x				Sun et al., 2018
Phragmites australis				x							x							Liu et al., 2011
			x	x			x					x				x		Sutton-Grier and Megonigal 2011; Nakamura et al., 2012
	x		x								x		x					Chmura and Molenda 2012; Lastrucci et al., 2016
								x					x					Anjum et al., 2012
		x							x									Yam et al., 2016
Potamogeton crispus						x						x	x			x		Xie and Yu 2011b; Qian et al., 2014; Xie et al., 2015
			x	x												x		Zuo et al., 2016
	x				x												x	Łoboda et al., 2018a; Łoboda et al., 2019

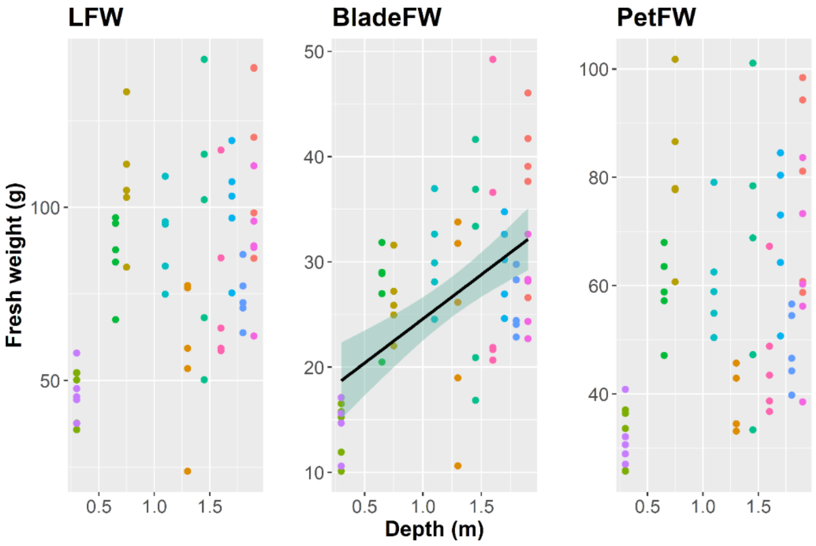
Potamogeton pectinatus						x	x		x							Hidding et al., 2012; Li et al., 2015
	x				x						x	x			x	Zhu et al., 2018; Łoboda et al., 2018a; Łoboda et al., 2019
	x	x	x				x		x							Sebilian-Wittyngham et al., 2019
Elodea canadensis			x	x						x				x		Mermillod-Blondin and Lemoine 2010; Lemoine et al., 2012
		x					x									Boiché et al., 2011
	x		x					x	x				x			Kočić et al., 2014; Thouvenot and Thiébaut 2018; Szabó et al., 2018; Riis et al., 2018
			x	x				x	x						x	Eller et al., 2015; Thouvenot et al., 2015; Ellawala Kankanamge et al., 2019
	x				x										x	Łoboda et al., 2018a-b
Potamogeton perfoliatus				x					x							Amano et al., 2012
			x										x			Riis et al., 2018
Lemna minor	x		x								x					Tabou et al., 2014
				x		x					x		x			Barks and Laird 2015; Pietrini et al., 2019; Roubeau Dumont et al., 2019
			x						x		x		x			McCann et al., 2016; Armitage and Jones 2019; Hart et al., 2019; Radulović et al., 2019
Hydrilla verticillata	x		x					x	x	x						Su et al., 2019; Dong et al., 2017; Wei et al., 2013; Xie et al., 2010
		x					x									Sun et al., 2018
	x	x			x					x						Zhu et al., 2012
Polygonum amphibium	x		x										x			Pätzig et al., 2020

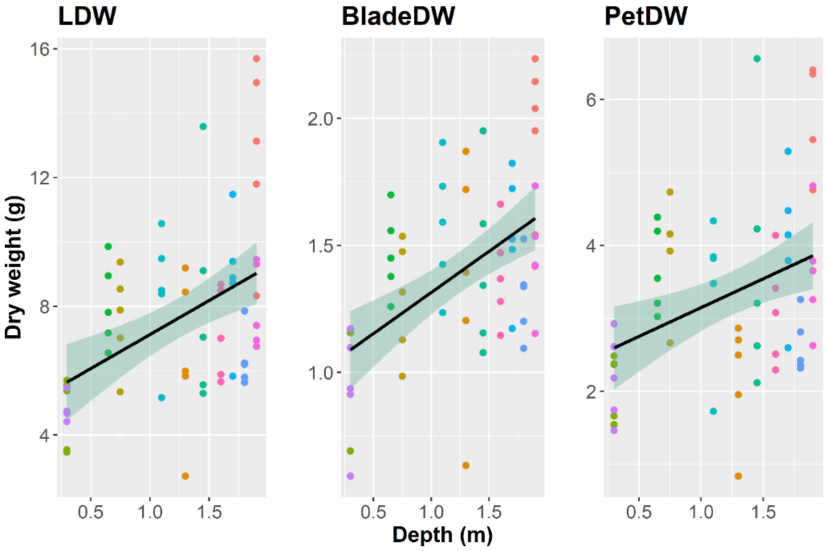
Appendix II

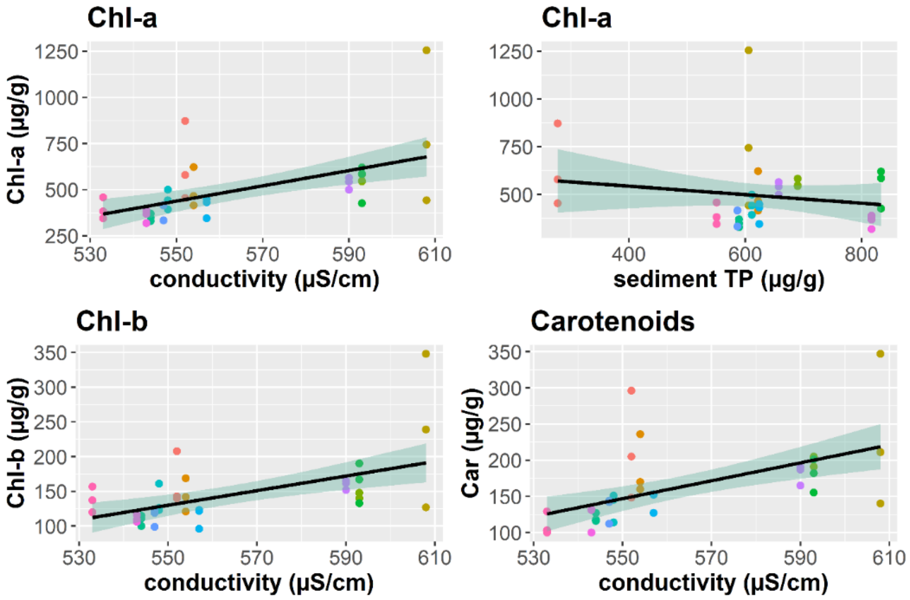
Supplementary materials for chapter II.

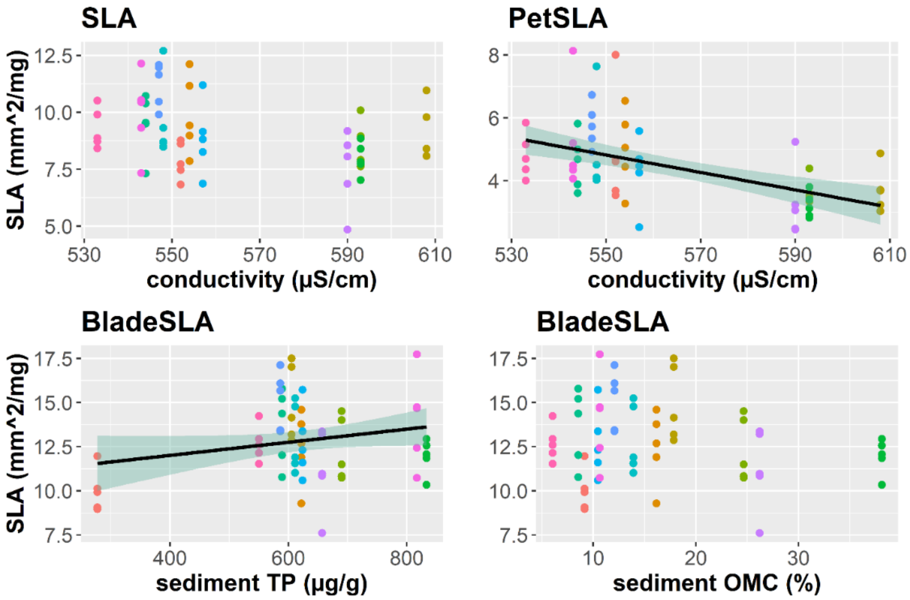
AII.1: Scatterplots show the relationships between traits and environmental variables resulting from the linear mixed models. Different colors for points identify the plot. Trend lines are drawn only for significant relations, and the buffer shows the standard error (trait tags: LA = Leaf Area; BladeA = Blade area; PetA = Petiole area; LFW = Leaf fresh weight; BladeFW = Blade fresh weight; petFW = Petiole fresh weight; LDW = Leaf dry weight; bladeDW = Blade dry weight; petDW = Petiole dry weight; SLA = Specific leaf area; bladeSLA = Specific blade area; petSLA = Specific petiole area; LDMC = Leaf dry matter content; bladeDMC = Blade dry matter content; petDMC = Petiole dry matter content; leafTP = Total phosphorus content; Chl-a = Chlorophyll-a content; Chl-b = Chlorophyll-b content; Car = Carotenoids content; Ca/Cb = Chlorophyll-a to Chlorophyll-b ratio; Chl/Car = Chlorophyll to carotenoids ratio).

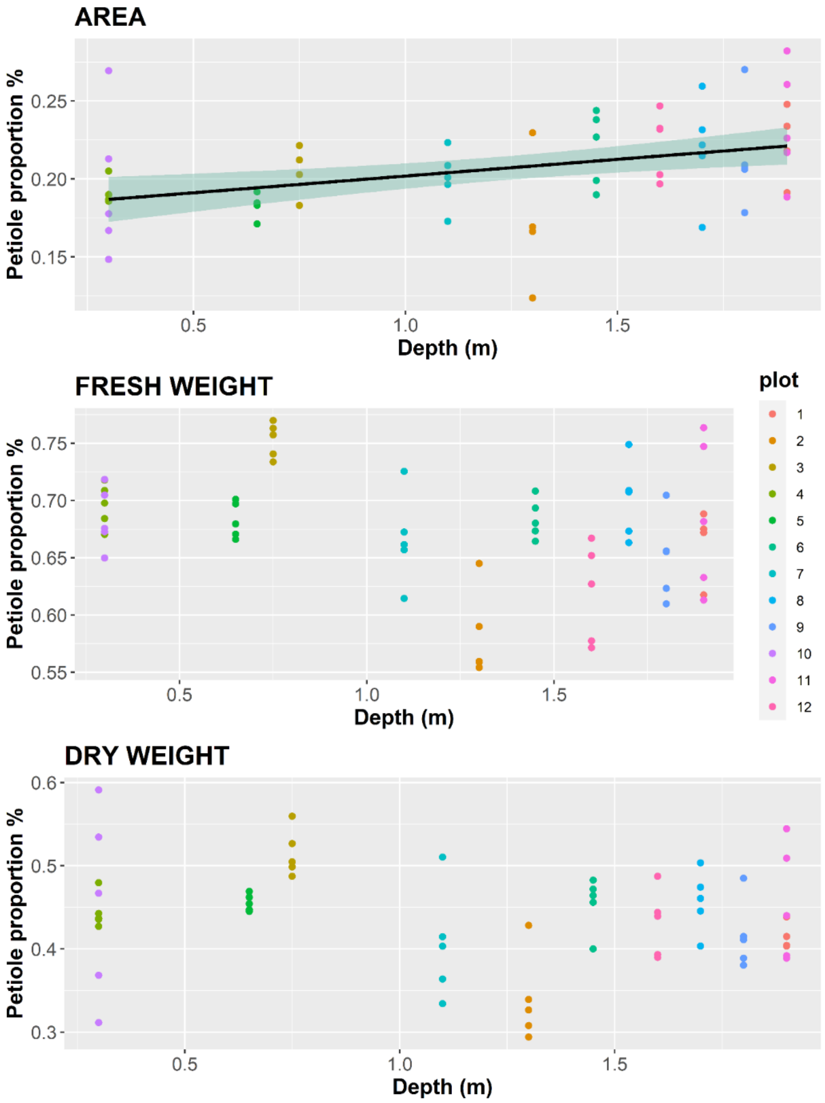





LDW
BladeDW
PetDW
Dry weight (g)
Depth (m)
16
12
8
4
2.0
1.5
1.0
6
4
2
0.5
1.0
1.5


Chl-a
Chl-a
Chl-a (µg/g)
1250
1000
750
500
250
530
550
570
590
610
conductivity (µS/cm)
400
600
800
sediment TP (µg/g)
Chl-b
Carotenoids
Chl-b (µg/g)
Car (µg/g)
350
300
250
200
150
100
conductivity (µS/cm)
conductivity (µS/cm)


SLA
PetSLA
SLA (mm^2/mg)
12.5
10.0
7.5
5.0
8
6
4
530
550
570
590
610
conductivity (µS/cm)
conductivity (µS/cm)
BladeSLA
BladeSLA
SLA (mm^2/mg)
17.5
15.0
12.5
10.0
7.5
400
600
800
sediment TP (µg/g)
10
20
30
sediment OMC (%)


AREA
Petiole proportion %
0.25
0.20
0.15
0.5
1.0
1.5
Depth (m)
FRESH WEIGHT
Petiole proportion %
0.75
0.70
0.65
0.60
0.55
0.5
1.0
1.5
Depth (m)
plot
1
2
3
4
5
6
7
8
9
10
11
12
DRY WEIGHT
Petiole proportion %
0.6
0.5
0.4
0.3
0.5
1.0
1.5
Depth (m)

AII.2: environmental parameters considered for the analyses, including plots coordinates. Cond = conductivity, DIC = Dissolved Inorganic Carbon, SRP = Soluble Reactive Phosphorus, Sed. = sediment, OMC = Organic Matter Content, TP = Total Phosphorus

PLOT	**x**	**y**	**Depth**	**Cond**	**pH**	**DIC**	**SRP**	**Sed. density**	**Sed. water content**	**Sed. porosity**	**OMC sed.**	**TP sed.**
			m	µS cm^{-1}		meq L^{-1}	µgP L^{-1}	d (g ml^{-1})	β (%)	β*d/100	%	µgP g^{-1}
1	11.95842	43.06605	1.9	552	8.51	2.91	2.14	1.35	53.04	0.71484	9.17	277.47
2	11.95312	43.05903	1.3	554	8.43	2.78	3.53	1.22	67.65	0.81690	16.16	622.06
3	11.97910	43.04560	0.8	608	7.53	3.29	10.51	1.16	72.77	0.84611	17.85	605.60
4	11.97412	43.04470	0.3	593	7.7	2.97	10.51	1.19	68.56	0.81504	24.65	690.04
5	11.97487	43.04610	0.6	593	7.64	3.44	11.90	1.03	85.14	0.87742	38.11	833.42
6	11.97675	43.05270	1.4	544	7.99	3.08	3.53	1.28	62.92	0.80458	8.56	589.38
7	11.97633	43.05008	1.1	548	8.27	2.54	3.53	1.18	70.17	0.83064	13.92	611.26
8	11.97240	43.04960	1.7	557	7.41	2.58	17.48	1.26	62.70	0.78764	10.47	623.97
9	11.96037	43.05107	1.8	547	8.36	2.97	3.53	1.23	66.73	0.82222	12.08	586.46
10	11.94963	43.05303	0.3	590	7.47	3.17	4.93	1.15	78.20	0.89898	26.22	657.31
11	11.95137	43.05535	1.9	543	8.6	2.70	6.32	1.24	67.46	0.83726	10.65	817.18
12	11.95655	43.05330	1.6	533	8.55	3.00	4.93	1.33	56.44	0.75122	6.06	550.62

Appendix III

Supplementary materials to chapter III.

AIII.1: Environmental PCA to detect sites differences. Environmental variables included in the analysis are: Depth, electrical conductivity=SPC, nitrate=NO3, sediment organic matter content=sed.OM and sediment total phosphorus=sed.TP. Sites are Chiusi=CH (n=12), Iseo-Torbiere=IS (n=4), Mantova=MN (n=7) and Pusiano-Annone=PA (n=5). The first two PCA axes are represented in a biplot.

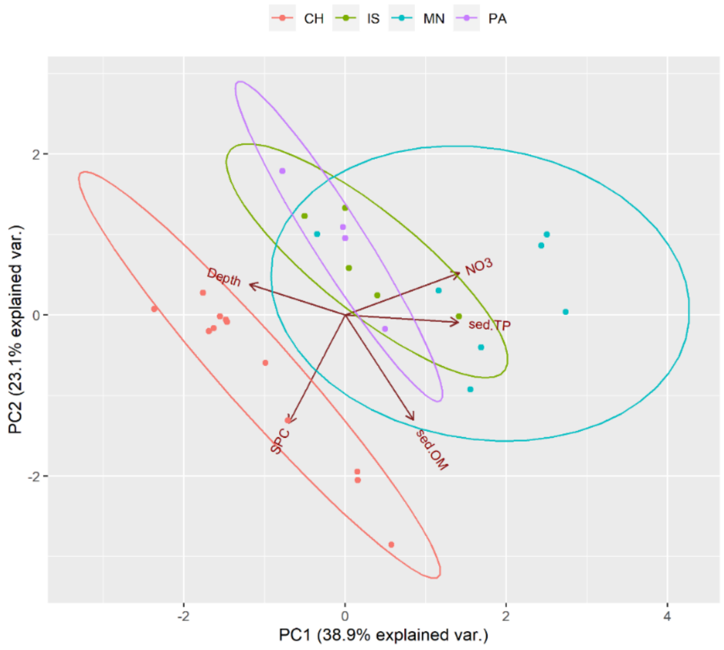
Importance of components:

	PC1	PC2	PC3	PC4	PC5
Standard deviation	1.395	1.076	1.001	0.743	0.587
Proportion of variance	0.389	0.232	0.200	0.110	0.069
Cumulative proportion	0.389	0.621	0.821	0.931	1.000

Loadings:

	PC1	PC2	PC3	PC4	PC5
Depth	-0.46	0.190	-0.610	0.439	-0.432
SPC	-0.273	-0.677	0.385	0.564	0.022
NO3	0.552	0.265	0.357	0.365	-0.604
sed.OM	0.331	-0.657	-0.401	-0.339	-0.428
sed.TP	0.55	-0.05	-0.440	0.490	0.515

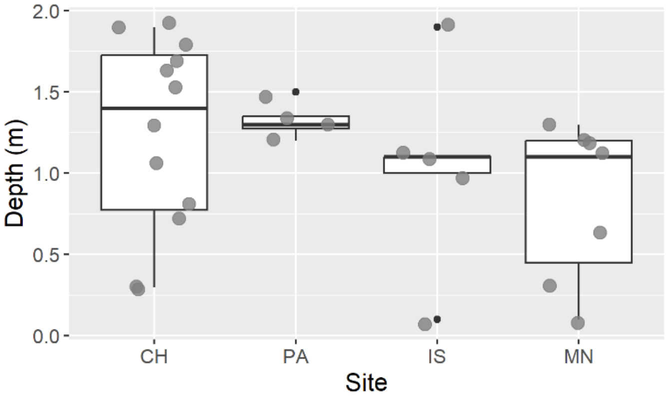
Biplot:



AIII.2: Tukey post-hoc test on environmental variables and functional traits showing differences among sites. Sites are Chiusi=CH (n=12), Iseo-Torbiere=IS (n=4), Mantova=MN (n=7) and Pusiano-Annone=PA (n=5). “diff”=mean difference between sites, “lwr”=lower difference, “upr”=highest difference, “p adj”=adjusted p-value. Significance threshold set at 0.05. Letters in the plots indicate significant differences.

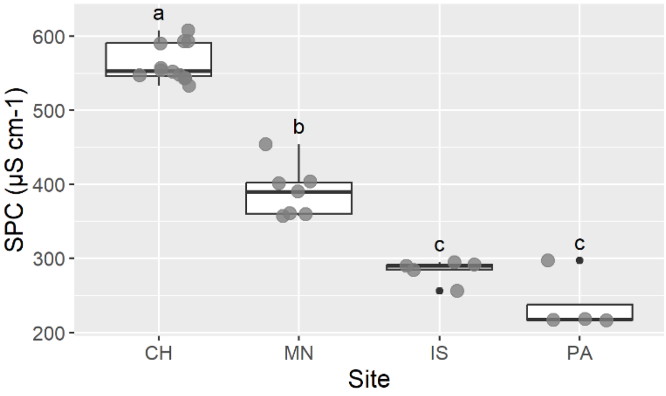
Depth

	diff	lwr	upr	p adj
IS-CH	-0.202	-0.994	0.590	0.8951
MN-CH	-0.413	-1.121	0.295	0.3920
PA-CH	0.083	-0.776	0.942	0.9931
MN-IS	-0.211	-1.083	0.660	0.9076
PA-IS	0.285	-0.713	1.283	0.8593
PA-MN	0.496	-0.436	1.429	0.4713



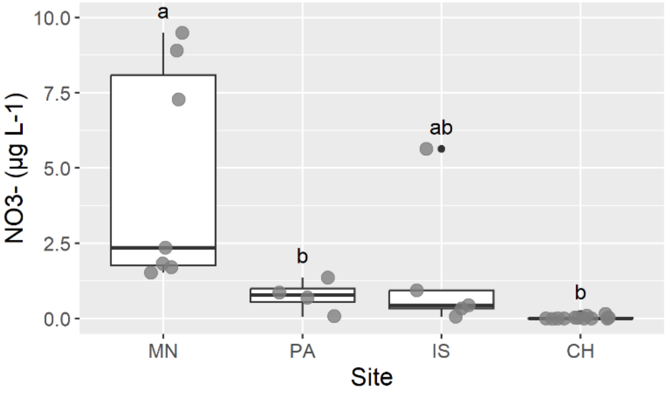
Electrical conductivity

	diff	lwr	upr	p adj
IS-CH	-279.90	-322.27	-237.53	0.0000
MN-CH	-173.93	-211.78	-136.07	0.0000
PA-CH	-326.50	-372.45	-280.55	0.0000
MN-IS	105.97	59.37	152.58	0.0000
PA-IS	-46.60	-99.99	6.79	0.1026
PA-MN	-152.57	-202.46	-102.68	0.0000



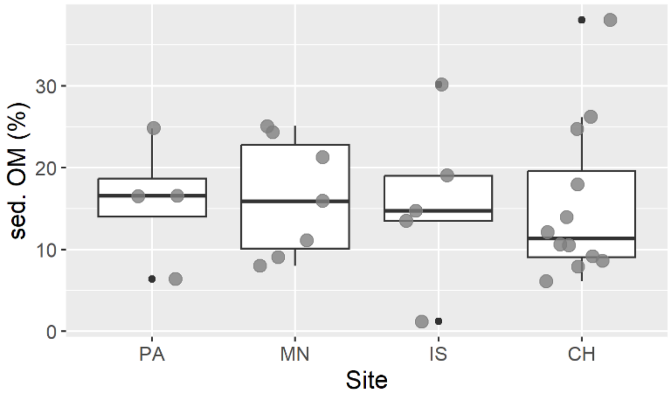
Nitrate

	diff	lwr	upr	p adj
IS-CH	1.46	-1.58	4.49	0.5581
MN-CH	4.69	1.98	7.41	0.0004
PA-CH	0.72	-2.57	4.02	0.9295
MN-IS	3.24	-0.10	6.58	0.0600
PA-IS	-0.73	-4.56	3.09	0.9512
PA-MN	-3.97	-7.55	-0.39	0.0257



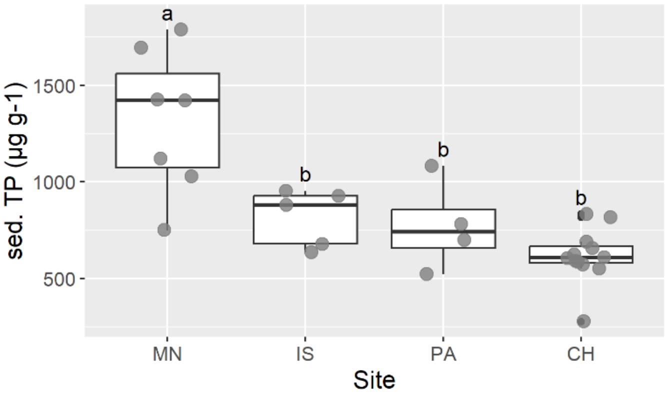
Sediment organic matter content

	diff	lwr	upr	p adj
IS-CH	0.24	-12.94	13.42	1.0000
MN-CH	0.92	-10.86	12.69	0.9964
PA-CH	0.59	-13.71	14.89	0.9995
MN-IS	0.68	-13.82	15.18	0.9992
PA-IS	0.36	-16.26	16.97	0.9999
PA-MN	-0.33	-15.85	15.20	0.9999



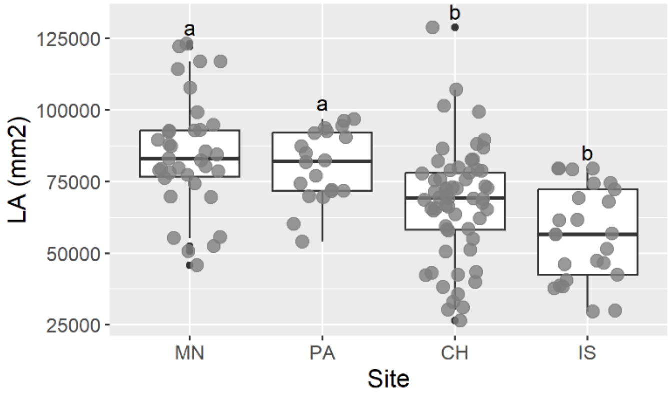
Sediment total phosphorus

	diff	lwr	upr	p adj
IS-CH	197.13	-144.13	538.39	0.4010
MN-CH	700.21	395.30	1005.12	0.0000
PA-CH	154.18	-215.97	524.32	0.6636
MN-IS	503.08	127.68	878.48	0.0058
PA-IS	-42.96	-473.03	387.12	0.9925
PA-MN	-546.03	-947.87	-144.19	0.0051



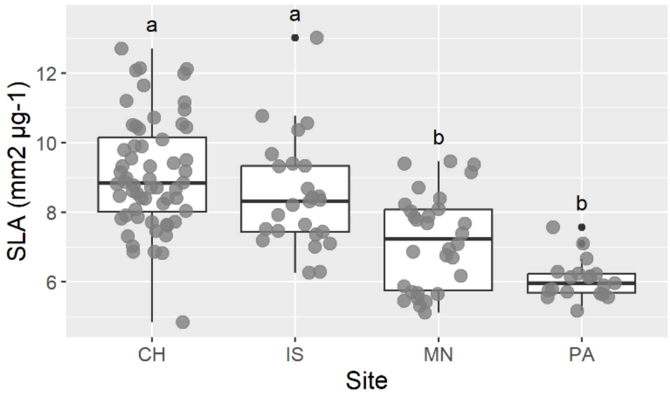
Leaf area

	diff	lwr	upr	p adj
IS-CH	-10623	-21942	697	0.0743
MN-CH	17433	7319	27547	0.0001
PA-CH	13285	1007	25563	0.0284
MN-IS	28056	15604	40508	0.0000
PA-IS	23908	9642	38174	0.0001
PA-MN	-4148	-17477	9181	0.8499



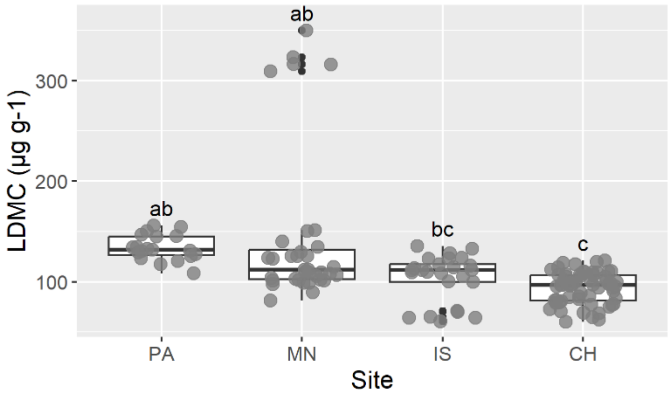
Specific leaf area

	diff	lwr	upr	p adj
IS-CH	-0.615	-1.581	0.351	0.3508
MN-CH	-2.463	-3.326	-1.600	0.0000
PA-CH	-3.031	-4.100	-1.963	0.0000
MN-IS	-1.848	-2.911	-0.785	0.0001
PA-IS	-2.416	-3.651	-1.181	0.0000
PA-MN	-0.568	-1.724	0.588	0.5785



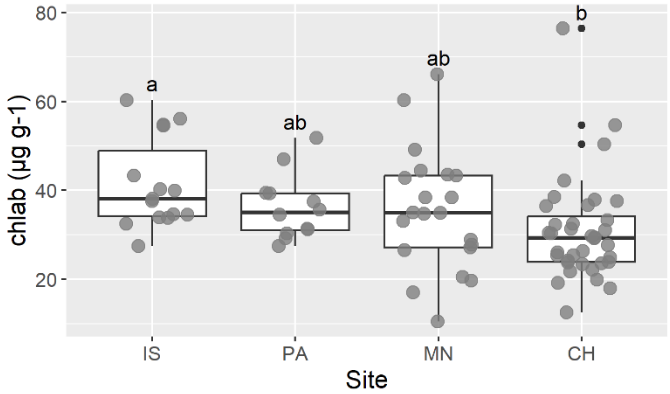
Leaf dry matter content

	diff	lwr	upr	p adj
IS-CH	9.48	-15.97	34.93	0.7672
MN-CH	49.06	26.32	71.80	0.0000
PA-CH	39.81	11.66	67.95	0.0019
MN-IS	39.58	11.58	67.57	0.0019
PA-IS	30.33	-2.21	62.86	0.0773
PA-MN	-9.25	-39.72	21.21	0.8589



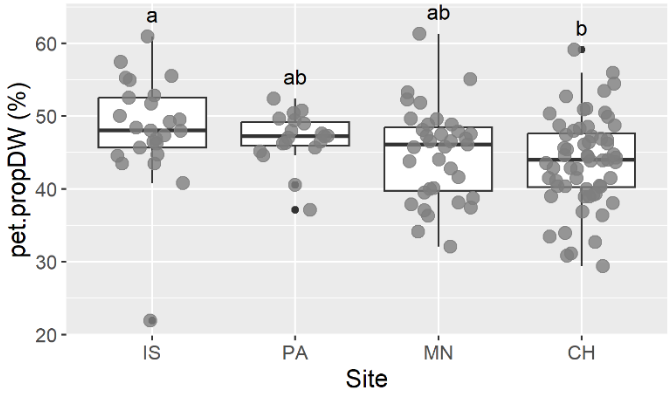
Chlorophylls (*a*+*b*)

	diff	lwr	upr	p adj
IS-CH	10.648	1.445	19.851	0.0167
MN-CH	4.557	-3.666	12.779	0.4699
PA-CH	5.435	-4.546	15.417	0.4853
MN-IS	-6.091	-16.215	4.032	0.3965
PA-IS	-5.212	-16.810	6.386	0.6417
PA-MN	0.879	-9.958	11.715	0.9966



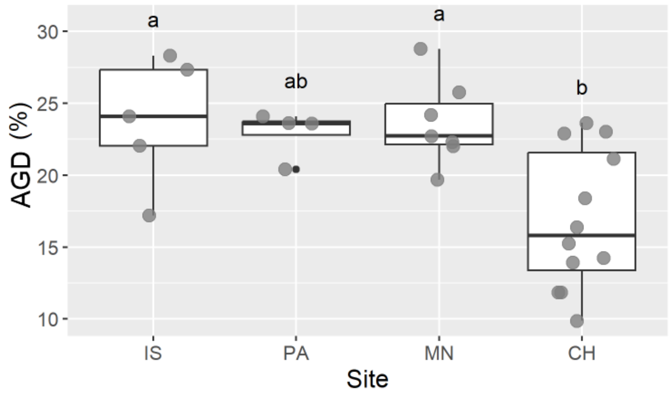
Petiole dry weight proportion

	diff	lwr	upr	p adj
IS-CH	0.050	0.010	0.080	0.0138
MN-CH	0.011	-0.023	0.046	0.8226
PA-CH	0.032	-0.011	0.074	0.2154
MN-IS	-0.034	-0.076	0.008	0.1637
PA-IS	-0.013	-0.062	0.035	0.8900
PA-MN	0.020	-0.026	0.066	0.6602



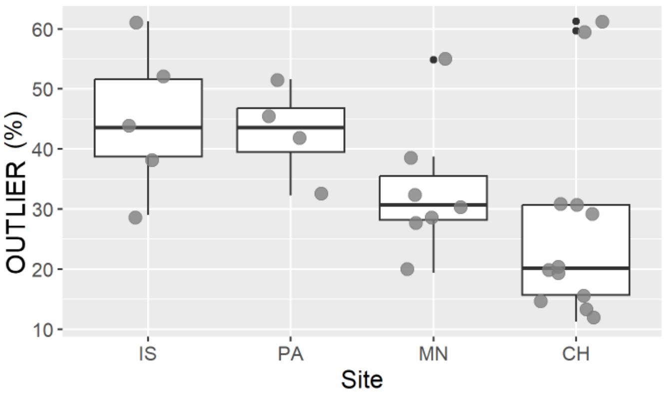
Genetic diversity

	diff	lwr	upr	p adj
IS-CH	0.0693	0.0094	0.1292	0.0190
MN-CH	0.0677	0.0142	0.1212	0.0095
PA-CH	0.0606	-0.0043	0.1255	0.0734
MN-IS	-0.0016	-0.0674	0.0643	0.9999
PA-IS	-0.0087	-0.0841	0.0667	0.9886
PA-MN	-0.0071	-0.0776	0.0634	0.9923



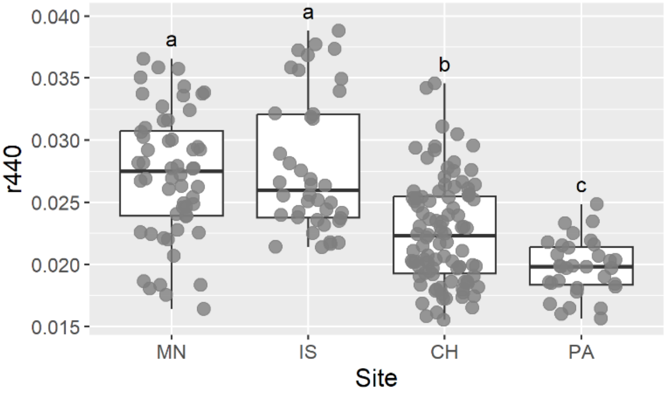
Percentage of outlier loci

	diff	lwr	upr	p adj
IS-CH	17.69	-2.85	38.23	0.1092
MN-CH	6.03	-12.32	24.38	0.8016
PA-CH	15.59	-6.69	37.87	0.2423
MN-IS	-11.66	-34.25	10.94	0.4976
PA-IS	-2.10	-27.98	23.79	0.9960
PA-MN	9.56	-14.62	33.75	0.6985



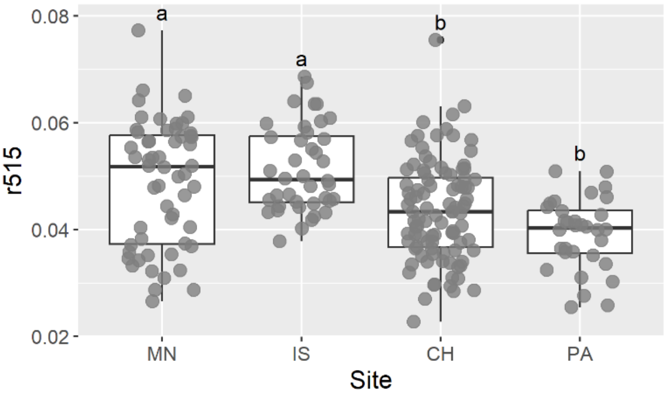
r440

	diff	lwr	upr	p adj
IS-CH	0.0054	0.0032	0.0076	0.0000
MN-CH	0.0046	0.0027	0.0066	0.0000
PA-CH	-0.0028	-0.0052	-0.0004	0.0136
MN-IS	-0.0008	-0.0032	0.0016	0.8390
PA-IS	-0.0082	-0.0110	-0.0055	0.0000
PA-MN	-0.0074	-0.0100	-0.0049	0.0000



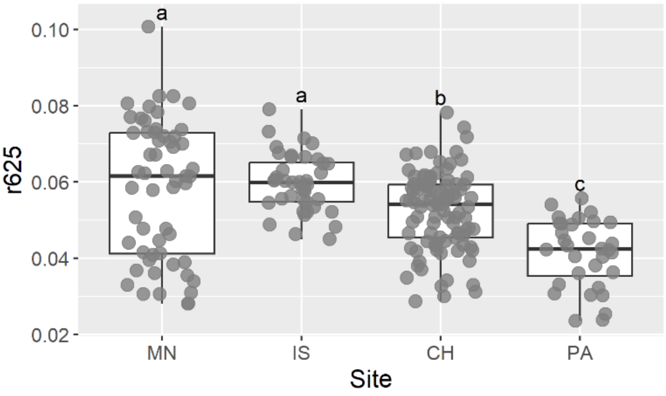
r515

	diff	lwr	upr	p adj
IS-CH	0.0078	0.0032	0.0124	0.0001
MN-CH	0.0051	0.0010	0.0093	0.0077
PA-CH	-0.0044	-0.0094	0.0006	0.1065
MN-IS	-0.0027	-0.0077	0.0024	0.5285
PA-IS	-0.0122	-0.0180	-0.0064	0.0000
PA-MN	-0.0095	-0.0150	-0.0041	0.0001



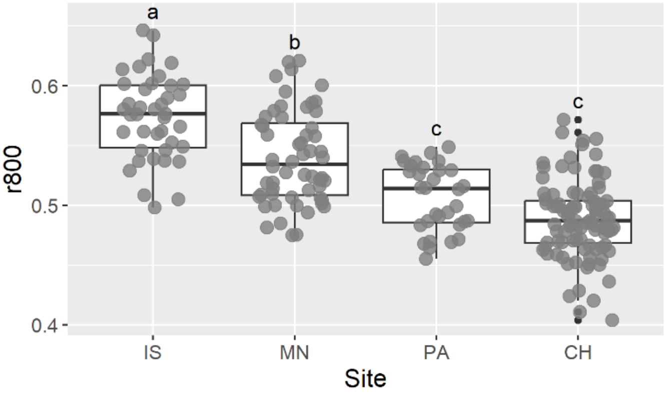
r625

	diff	lwr	upr	p adj
IS-CH	0.0072	0.0013	0.0131	0.0097
MN-CH	0.0056	0.0003	0.0109	0.0328
PA-CH	-0.0112	-0.0176	-0.0048	0.0001
MN-IS	-0.0016	-0.0081	0.0049	0.9180
PA-IS	-0.0184	-0.0259	-0.0110	0.0000
PA-MN	-0.0168	-0.0238	-0.0099	0.0000



r800

	diff	lwr	upr	p adj
IS-CH	0.0853	0.0688	0.1018	0.0000
MN-CH	0.0512	0.0364	0.0660	0.0000
PA-CH	0.0177	-0.0002	0.0357	0.0536
MN-IS	-0.0341	-0.0523	-0.0159	0.0000
PA-IS	-0.0675	-0.0884	-0.0467	0.0000
PA-MN	-0.0335	-0.0529	-0.0140	0.0001



AIII.3: statistical output of GAM models used to test the effect of environmental drivers on traits variability. Response variables were log- or sq. root-transformed when necessary to meet the model's assumptions. Environmental variables are Depth, electrical conductivity=SPC, nitrate=NO3, sediment organic matter content=sed.OM and sediment total phosphorus=sed.TP. "plotID" was introduced as random effect to take into account the non-independence of replicates within a plot. "s" before the environmental variables in the model indicate smooth terms. Model output of r515 is not shown, as no correlation with any environmental variable was found.

Leaf area

```
Formula:
log(LA) ~ s(SPC) + s(sed.TP) + s(sed.OM) + s(plotID, bs = "re")

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 11.13374    0.04502   247.3   <2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Approximate significance of smooth terms:
             edf Ref.df     F p-value
s(SPC)      1.000  1.000 1.699  0.1951
s(sed.TP)   2.245  2.289 3.659  0.0372 *
s(sed.OM)   3.622  3.683 3.613  0.0278 *
s(plotID) 18.614 24.000 8.943  <2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

R-sq.(adj) =  0.792   Deviance explained =   83%
-REML = -23.886  Scale est. = 0.021405  n = 140
```

Specific leaf area

```
Formula:
log(SLA) ~ s(SPC) + s(sed.TP) + s(plotID, bs = "re")

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  2.07020    0.02367   87.45   <2e-16 ***

Approximate significance of smooth terms:
             edf Ref.df     F  p-value
s(SPC)      3.277  3.440 6.455 0.000213 ***
s(sed.TP)   2.458  2.598 5.413 0.003779 **
s(plotID) 15.722 24.000 2.465  < 2e-16 ***

R-sq.(adj) =  0.661   Deviance explained = 71.5%
-REML = -52.904  Scale est. = 0.016821  n = 134
```

Leaf dry matter content

```
Formula:
log(LDMC) ~ s(SPC) + s(sed.TP) + s(plotID, bs = "re")

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  4.63984    0.03174   146.2   <2e-16 ***

Approximate significance of smooth terms:
            edf Ref.df      F p-value
s(SPC)     1.00      1 10.535 0.00155 **
s(sed.TP)  1.00      1  0.032 0.85753
s(plotID) 21.38     24  8.182 < 2e-16 ***

R-sq.(adj) =  0.687   Deviance explained = 74.2%
-REML = -56.485  Scale est. = 0.014735  n = 134
```

Proportion of petiole dry weight

```
Formula:
pet.propDW ~ s(sed.TP) + s(SPC) + s(plotID, bs = "re")

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.452440   0.006974   64.88   <2e-16 ***

Approximate significance of smooth terms:
            edf Ref.df     F p-value
s(sed.TP)  1.00  1.000 1.815 0.18030
s(SPC)     2.42  2.692 3.819 0.03508 *
s(plotID) 13.51 25.000 1.247 0.00106 **

R-sq.(adj) =  0.285   Deviance explained = 37.3%
-REML = -185.56  Scale est. = 0.0028862  n = 139
```

Chlorophylls (*a*+*b*)

```
Formula:
chlab ~ s(Depth) + s(NO3) + s(sed.OM) + s(sed.TP) + s(plotID,
    bs = "re")

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   34.617      1.279   27.07   <2e-16 ***

Approximate significance of smooth terms:
            edf Ref.df      F  p-value
s(Depth)  1.000  1.000 12.943 0.000615 ***
s(NO3)    1.000  1.000  2.279 0.135865
s(sed.OM) 1.000  1.000  0.625 0.431971
s(sed.TP) 4.354  4.827  2.671 0.026658 *
s(plotID) 9.478 23.000  0.772 0.019491 *

R-sq.(adj) =  0.495   Deviance explained = 59.8%
-REML = 302.84  Scale est. = 71.072    n = 84
```

Genetic diversity

```
Formula:
sqrt(gene.div) ~ s(SPC)

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.450730   0.008583   52.52   <2e-16 ***

Approximate significance of smooth terms:
         edf Ref.df     F p-value
s(SPC) 2.051   2.55 7.809 0.00133 **

R-sq.(adj) =  0.414   Deviance explained = 45.8%
-REML = -39.366  Scale est. = 0.0020625  n = 28
```

Percentage of polymorphic loci

```
Formula:
log(pol.loci) ~ s(SPC)

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  3.42304    0.07675    44.6   <2e-16 ***

Approximate significance of smooth terms:
       edf Ref.df     F p-value
s(SPC)   1      1 12.12 0.00178 **

R-sq.(adj) =  0.292   Deviance explained = 31.8%
-REML = 16.797  Scale est. = 0.16494   n = 28
```

r440

```
Formula:
r440 ~ s(SPC) + s(plotID, bs = "re")

Parametric coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0242716  0.0006816   35.61   <2e-16 ***

Approximate significance of smooth terms:
             edf Ref.df     F p-value
s(SPC)     2.872  2.944 4.868 0.00386 **
s(plotID) 21.555 26.000 7.280 < 2e-16 ***

R-sq.(adj) =  0.614   Deviance explained = 65.6%
-REML = -915.05  Scale est. = 1.1098e-05  n = 224
```

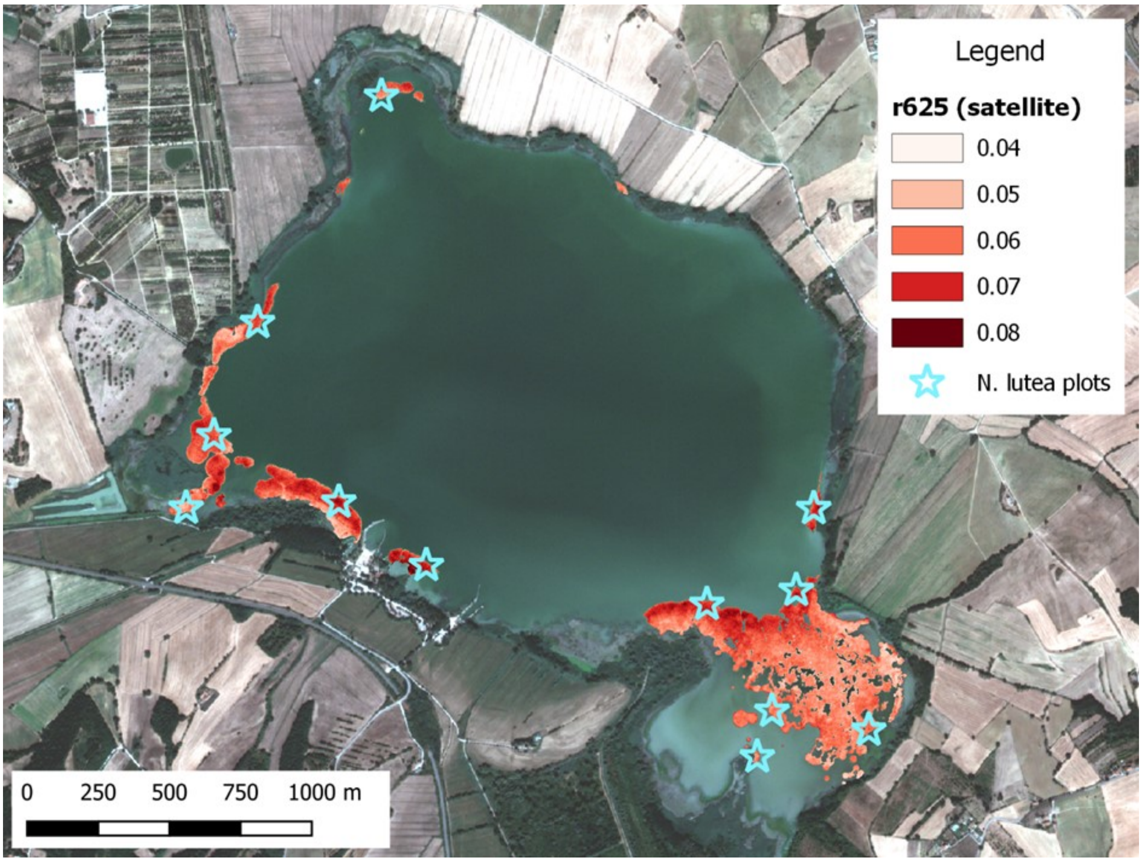
r625

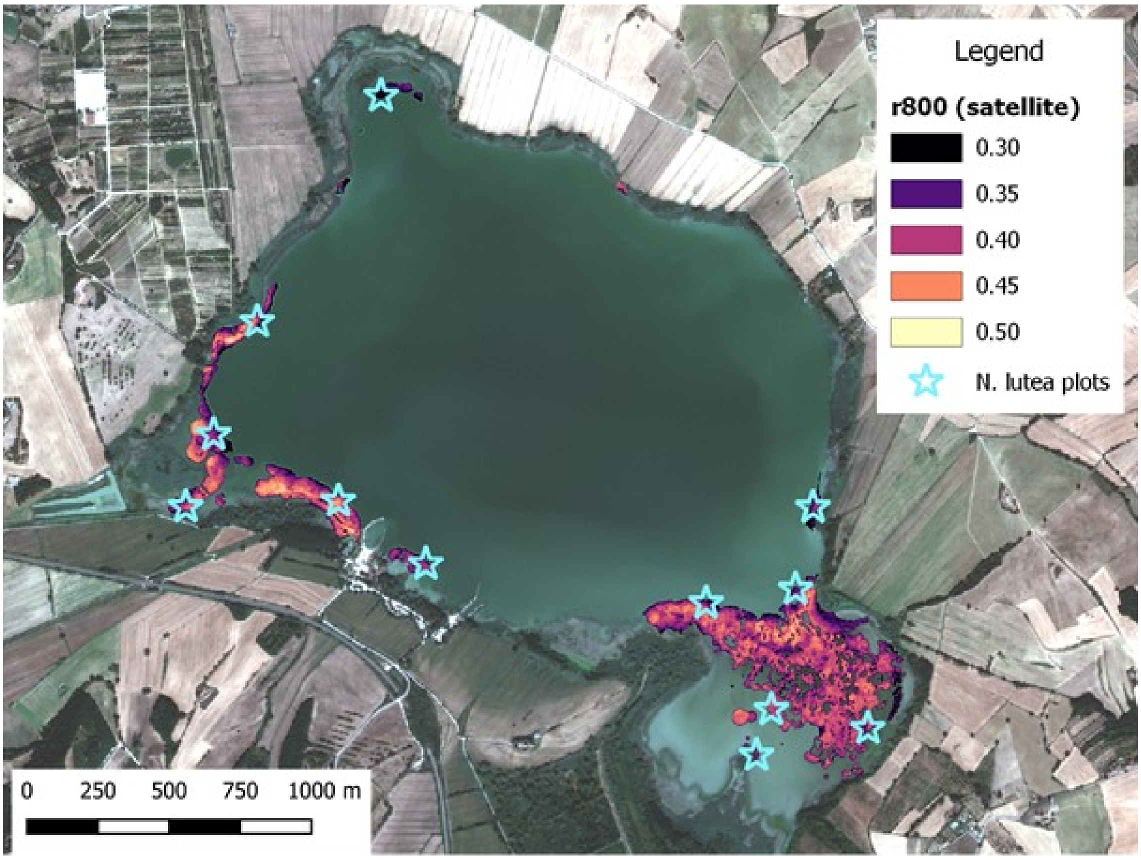
```
Formula:
r625 ~ s(Depth) + s(NO3) + s(SPC) + s(sed.TP) + s(plotID, bs = "re")

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.053605   0.001307      41   <2e-16 ***

Approximate significance of smooth terms:
             edf Ref.df     F p-value
s(Depth)   1.000  1.000 0.066 0.79760
s(NO3)     2.875  2.935 4.509 0.00337 **
s(SPC)     4.050  4.143 9.545 8.9e-07 ***
s(sed.TP)  2.602  2.675 5.920 0.00104 **
s(plotID) 14.370 23.000 3.014 < 2e-16 ***

R-sq.(adj) =  0.727   Deviance explained = 75.7%
-REML = -731.52  Scale est. = 4.8896e-05  n = 224
```

r800

```
Formula:
r800 ~ s(NO3) + s(SPC) + s(plotID, bs = "re")

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.519070   0.005333   97.33   <2e-16 ***

Approximate significance of smooth terms:
             edf Ref.df     F p-value
s(NO3)     1.000  1.000 1.926   0.167
s(SPC)     3.231  3.308 9.021 2.9e-05 ***
s(plotID) 20.341 25.000 6.745 < 2e-16 ***

R-sq.(adj) =  0.692   Deviance explained = 72.6%
-REML = -454.11  Scale est. = 0.00067933  n = 224
```

AIII.4: Maps reproducing spatialized information on r625 and r800 at 2 m spatial resolution over Lake Chiusi, derived from a Pleiades scene acquired on 18 August 2021. Sampled *N. lutea* plots are marked in light blue stars.





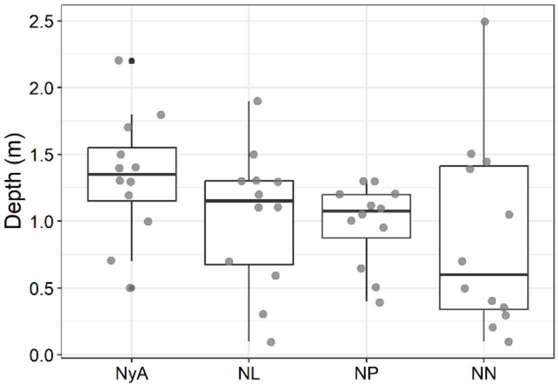
Appendix IV

Supplementary materials to chapter IV.

AIV.1: post-hoc pairwise comparisons between species in environmental variables and functional traits. Parametric or non-parametric test and post-hoc test choice are indicated. “diff”=mean difference between species; “lwr”=lowest difference; “upr”=highest difference; “p adj”=adjusted p-value; LMM=linear mixed models; NL=*Nuphar lutea*; NN=*Nelumbo nucifera*; NP=*Nymphoides peltata*; NyA=*Nymphaea alba*.

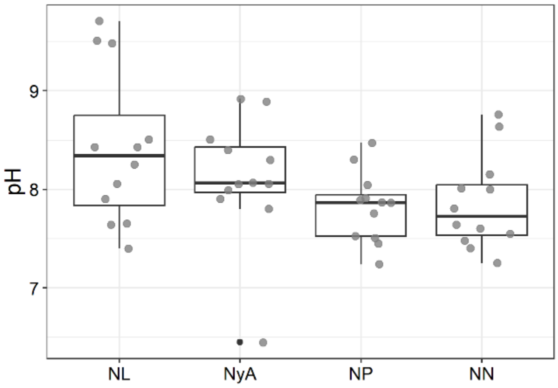
Depth (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	-0.163	-0.733	0.408	0.8715
NP-NL	-0.053	-0.623	0.518	0.9947
NyA-NL	0.300	-0.270	0.870	0.5031
NP-NN	0.110	-0.460	0.680	0.9550
NyA-NN	0.463	-0.108	1.033	0.1488
NyA-NP	0.353	-0.218	0.923	0.3616



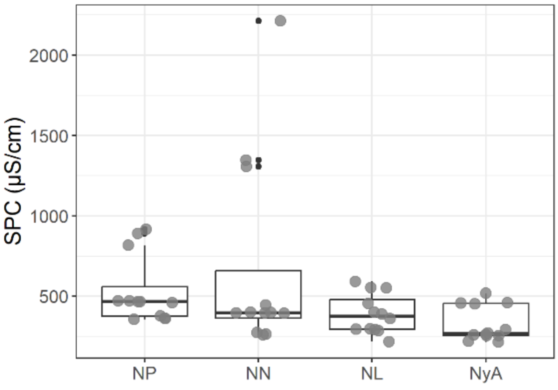
pH (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	-0.557	-1.193	0.079	0.1052
NP-NL	-0.594	-1.230	0.042	0.0748
NyA-NL	-0.303	-0.939	0.334	0.5867
NP-NN	-0.038	-0.674	0.599	0.9986
NyA-NN	0.254	-0.382	0.890	0.7112
NyA-NP	0.292	-0.344	0.928	0.6150



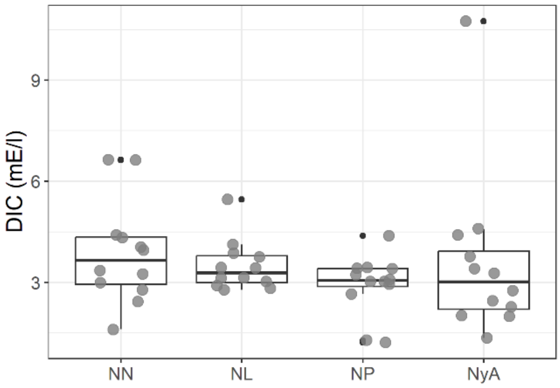
Electrical conductivity (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	284.1	-81.6	649.8	0.1775
NP-NL	144.5	-221.2	510.2	0.7181
NyA-NL	-64.0	-429.7	301.7	0.9658
NP-NN	-139.6	-505.2	226.1	0.7393
NyA-NN	-348.1	-713.8	17.6	0.0673
NyA-NP	-208.5	-574.2	157.1	0.4330



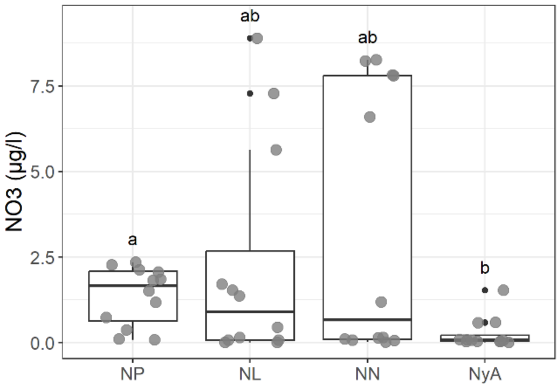
Dissolved inorganic carbon (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	0.382	-1.322	2.085	0.9320
NP-NL	-0.563	-2.266	1.141	0.8143
NyA-NL	0.097	-1.607	1.800	0.9987
NP-NN	-0.944	-2.647	0.759	0.4579
NyA-NN	-0.285	-1.988	1.418	0.9699
NyA-NP	0.659	-1.044	2.362	0.7310



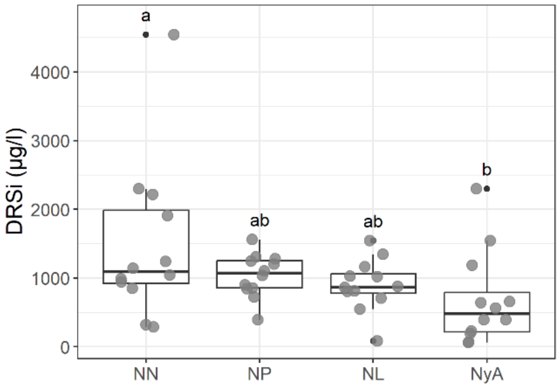
Nitrate (Kruskal-Wallis + Dunn)

	diff	p adj
NL-NN	-0.510	1.0000
NL-NP	-0.941	1.0000
NN-NP	-0.430	0.6670
NL-NyA	1.976	0.1925
NN-NyA	2.487	0.0645
NP-NyA	2.917	0.0212



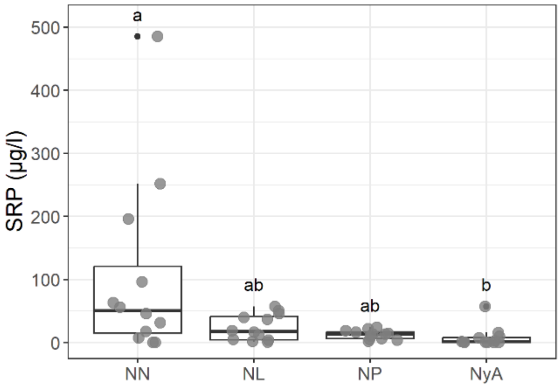
Dissolved reactive silica (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	582.6	-196.6	1361.8	0.2049
NP-NL	138.0	-641.1	917.2	0.9646
NyA-NL	-214.0	-993.2	565.2	0.8832
NP-NN	-444.6	-1223.8	334.6	0.4326
NyA-NN	-796.6	-1575.8	-17.4	0.0434
NyA-NP	-352.0	-1131.2	427.2	0.6262



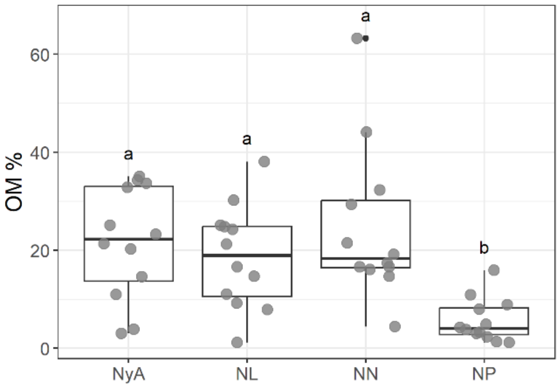
Soluble reactive phosphorus (Kruskal-Wallis + Dunn)

	diff	p adj
NL-NN	-1.080	0.5601
NL-NP	0.650	0.5160
NN-NP	1.730	0.2510
NL-NyA	2.387	0.0850
NN-NyA	3.467	0.0032
NP-NyA	1.737	0.3295



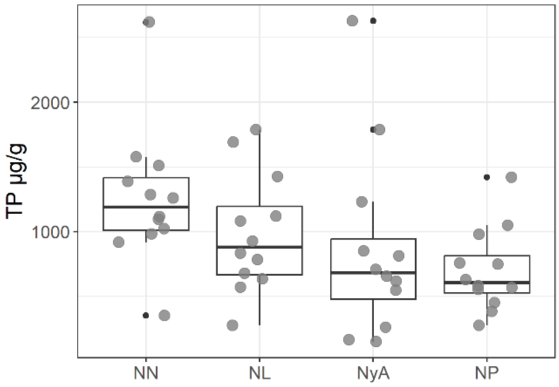
Sediment organic matter content (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	5.92	-6.42	18.26	0.5796
NP-NL	-13.05	-25.39	-0.72	0.0345
NyA-NL	2.83	-9.51	15.17	0.9277
NP-NN	-18.97	-31.31	-6.63	0.0010
NyA-NN	-3.09	-15.43	9.25	0.9082
NyA-NP	15.88	3.54	28.22	0.0069



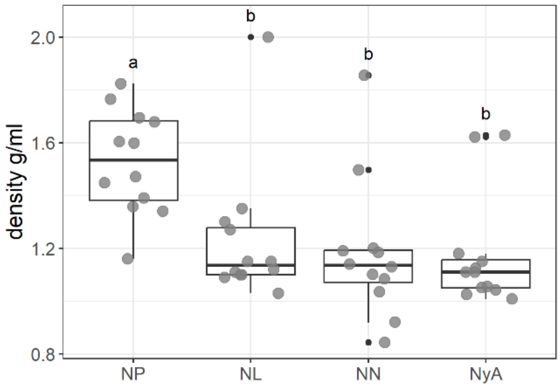
Sediment total phosphorus (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	275.7	-300.1	851.6	0.5813
NP-NL	-284.1	-859.9	291.8	0.5573
NyA-NL	-116.2	-692.1	459.7	0.9490
NP-NN	-559.8	-1135.7	16.1	0.0595
NyA-NN	-392.0	-967.9	183.9	0.2790
NyA-NP	167.8	-408.1	743.7	0.8640



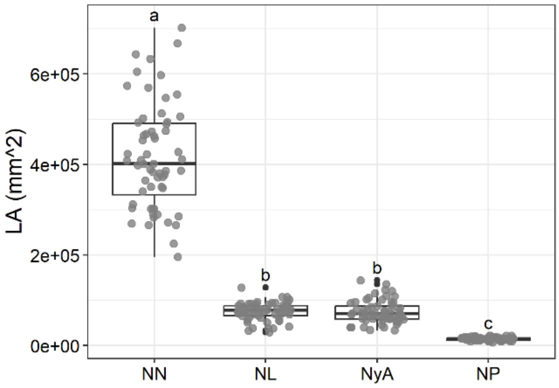
Sediment density (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	-0.0494	-0.3076	0.2089	0.9562
NP-NL	0.2965	0.0382	0.5547	0.0188
NyA-NL	-0.0554	-0.3137	0.2029	0.9397
NP-NN	0.3458	0.0876	0.6041	0.0046
NyA-NN	-0.0060	-0.2643	0.2522	0.9999
NyA-NP	-0.3519	-0.6101	-0.0936	0.0039



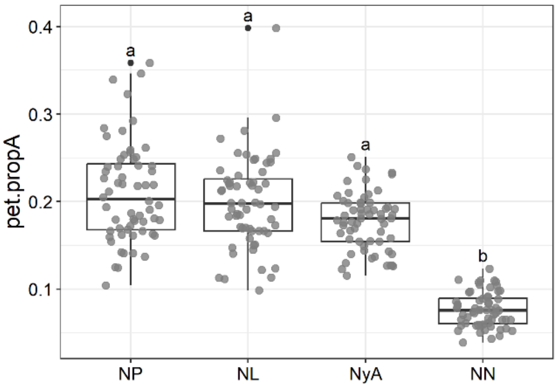
Leaf area (LMM + Tukey)

	diff	p.value
NL-NN	-341720	0.0000
NL-NP	60676	0.0245
NL-NyA	1632	0.9998
NN-NP	402396	0.0000
NN-NyA	343351	0.0000
NP-NyA	-59044	0.0298



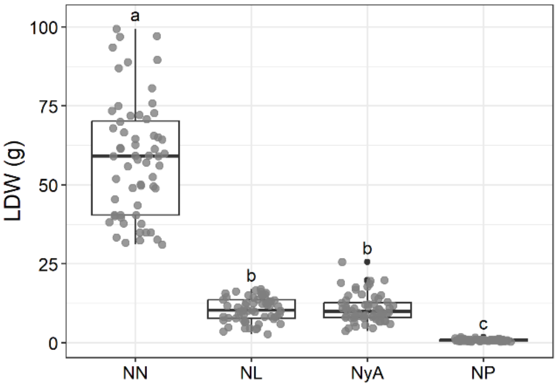
Proportion of petiole area (LMM + Tukey)

	diff	p.value
NL-NN	0.955	0.0000
NL-NP	-0.054	0.9070
NL-NyA	0.097	0.6212
NN-NP	-1.009	0.0000
NN-NyA	-0.857	0.0000
NP-NyA	0.151	0.2484



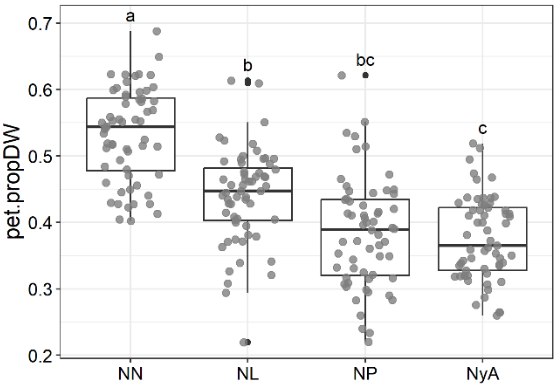
Leaf dry weight (LMM + Tukey)

	diff	p.value
NL-NN	-1.735	0.0000
NL-NP	2.472	0.0000
NL-NyA	-0.025	0.9978
NN-NP	4.207	0.0000
NN-NyA	1.710	0.0000
NP-NyA	-2.497	0.0000



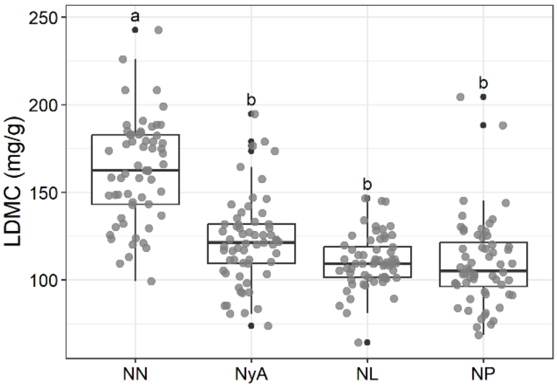
Proportion of petiole dry weight (LMM + Tukey)

	diff	p.value
NL-NN	-0.0934	0.0004
NL-NP	0.0549	0.0588
NL-NyA	0.0636	0.0214
NN-NP	0.1483	0.0000
NN-NyA	0.1570	0.0000
NP-NyA	0.0087	0.9763



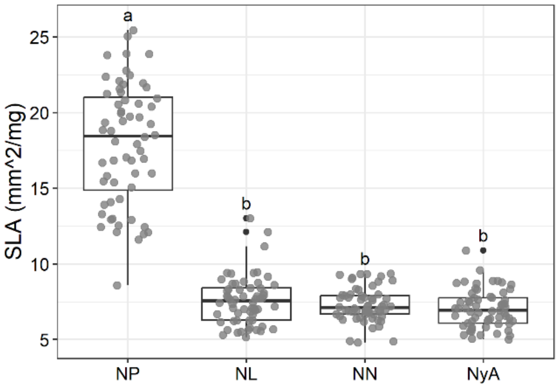
Leaf dry matter content (LMM + Tukey)

	diff	p.value
NL-NN	-0.3760	0.0000
NL-NP	0.0248	0.9762
NL-NyA	-0.0864	0.4834
NN-NP	0.4007	0.0000
NN-NyA	0.2896	0.0001
NP-NyA	-0.1112	0.2646



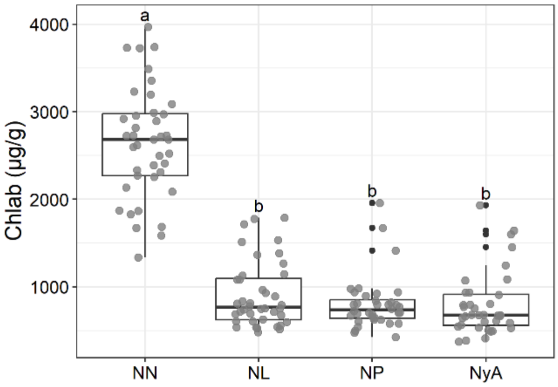
Specific leaf area (LMM + Tukey)

	diff	p.value
NL-NN	0.0280	0.9718
NL-NP	-0.8562	0.0000
NL-NyA	0.0692	0.7041
NN-NP	-0.8842	0.0000
NN-NyA	0.0412	0.9178
NP-NyA	0.9253	0.0000



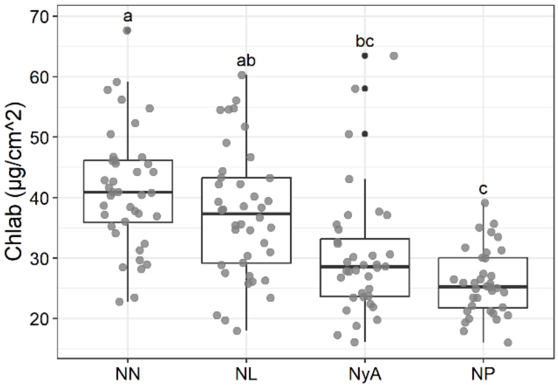
Chlorophylls *a+b*, fresh weight based (LMM + Tukey)

	diff	p.value
NL-NN	-1.115	0.0000
NL-NP	0.092	0.8515
NL-NyA	0.133	0.6472
NN-NP	1.207	0.0000
NN-NyA	1.248	0.0000
NP-NyA	0.042	0.9831



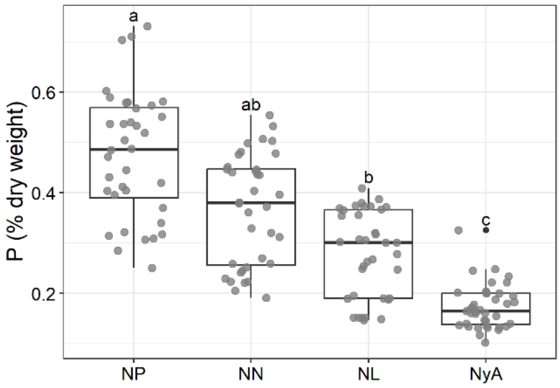
Chlorophylls *a+b*, area based (LMM + Tukey)

	diff	p.value
NL-NN	-0.125	0.4729
NL-NP	0.341	0.0015
NL-NyA	0.211	0.0818
NN-NP	0.466	0.0000
NN-NyA	0.336	0.0018
NP-NyA	-0.130	0.4462



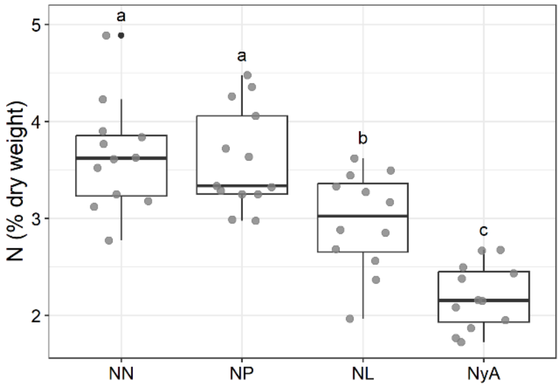
Leaf phosphorus content (LMM + Tukey)

	diff	p.value
NL-NN	-0.277	0.0980
NL-NP	-0.558	0.0001
NL-NyA	0.444	0.0025
NN-NP	-0.281	0.0931
NN-NyA	0.721	0.0000
NP-NyA	1.002	0.0000



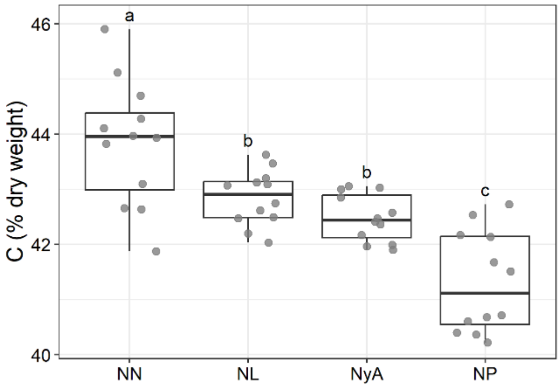
Leaf nitrogen content (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	0.672	0.141	1.203	0.0081
NP-NL	0.638	0.117	1.159	0.0108
NyA-NL	-0.774	-1.305	-0.242	0.0018
NP-NN	-0.034	-0.555	0.487	0.9981
NyA-NN	-1.445	-1.977	-0.914	0.0000
NyA-NP	-1.412	-1.932	-0.891	0.0000



Leaf carbon content (ANOVA + Tukey)

	diff	lwr	upr	p adj
NN-NL	0.998	0.127	1.869	0.0191
NP-NL	-1.534	-2.405	-0.662	0.0001
NyA-NL	-0.363	-1.234	0.509	0.6843
NP-NN	-2.532	-3.403	-1.660	0.0000
NyA-NN	-1.361	-2.232	-0.489	0.0008
NyA-NP	1.171	0.299	2.042	0.0045



AIV.2: uncertainty estimates of hypervolumes size and uniqueness, obtained with bootstrapping on 199 permutated hypervolumes on each species. Relative niche size is the proportion of niche occupied by each species relative to the total niche occupied by all species. Relative niche uniqueness is the proportion of unique niche of each species relative to its total niche volume. "Mean" is the mean difference between species for relative niche size, and mean species uniqueness for relative niche uniqueness, followed by the 2.5 and 97.5 quantiles (Q 2.5 and Q 97.5 respectively). Significant level was set at 0.05. NL=*N. lutea*, NN=*N. nucifera*, NP=*N. peltata*, NyA=*N. alba*.

ECOLOGICAL NICHE

Relative niche size

	MEAN	Q 2.5	Q 97.5
NL-NN	-0.3916	-0.859	0.4487
NL-NP	0.2225	-0.0254	0.6047
NL-NYA	0.038	-0.3944	0.514
NN-NP	0.6141	0.0658	0.9153
NN-NYA	0.4296	-0.4654	0.9008
NP-NYA	-0.1846	-0.6501	0.0547

Relative niche uniqueness

	MEAN	Q 2.5	Q 97.5
NL	0.2835	0.0366	0.6471
NN	0.7551	0.4758	0.9025
NP	0.5241	0.2000	0.8482
NYA	0.3350	0.0700	0.6810

FUNCTIONAL NICHE

Relative niche size

	MEAN	Q 2.5	Q 97.5
NL-NN	-0.026	-0.105	0.051
NL-NP	-0.076	-0.165	0.020
NL-NYA	0.129	0.053	0.194
NN-NP	-0.050	-0.149	0.052
NN-NYA	0.155	0.086	0.214
NP-NYA	0.205	0.115	0.279

Relative niche uniqueness

	MEAN	Q 2.5	Q 97.5
NL	0.6263	0.5278	0.7447
NN	0.9959	0.9773	1.0000
NP	0.9778	0.9363	1.0000
NYA	0.3655	0.2474	0.5093

Appendix V

Submitted manuscript on deep lakes submerged vegetation structure, introduced in section 1.4.

SPATIAL LINKAGES BETWEEN MACROPHYTE ASSEMBLAGES, WATER AND SEDIMENT FEATURES IN DEEP LAKES

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Introduction

Despite occupying a relatively small portion of the Earth's surface, large, deep freshwater lakes have disproportionally high biodiversity compared to terrestrial ecosystems, and provide key services like fisheries, drinking water and recreational activities (Hayford et al., 2015; Salmaso et al., 2018; Heino et al., 2021; Lewerentz et al., 2021). Unfortunately, a worldwide decline in large lake conditions has been observed, in terms of pollution, water temperature, reduction of biodiversity and resources (Zhang et al., 2017; Jenny et al., 2020).

This is due to direct anthropogenic overexploitation, as well as to the close relationship between watershed conditions and the status of large, deep lakes (Heino et al., 2021) which makes them in any case susceptible to environmental changes across their watersheds. Deep lakes present steep environmental gradients along the depth profile, in terms of light availability, temperature, nutrients, oxygen and wind/wave disturbance (Bornette and Puijalon, 2011). These gradients influence the distribution of submerged plants and vegetation composition, in turn affecting the functions and services provided by these ecosystems (Spence, 1972; Thomaz, 2021). Depth increases of a few meters are sufficient for the environmental conditions and availability of resources to undergo significant variations (e.g., light attenuation follows a logarithmic function; see Wetzel, 2001).

Notwithstanding the general recognition of the key role played by light intensity in regulating the presence and distribution of macrophytes in deep waters (Spence, 1972), we lack systematic studies on the interlinks between sediment and water column features and the spatial structure of macrophyte communities in deep lentic systems. Indeed, most of the current knowledge on lake ecology and functioning has focused on shallow lakes (Tarkowska-Kukuryk and Mieczan, 2017; Giles et al., 2018; Pinardi et al., 2021), e.g., for modelling description of the regime shift theory (Scheffer et al., 1993, Scheffer, 1999, Janssen et al., 2014), and wetland plant zonation (Campbell and Keddy, 2022).

The first warning signal of lake degradation is a change in primary producers, including phytoplankton blooms and/or the disappearance of submerged macrophytes (Azzella et al., 2014) due to a shift towards floating and emergent plant life forms or microalgae with increasing trophic status (Baattrup-Pedersen et al., 2016; Zervas et al., 2019). Macrophytes inhabit a challenging environment (O'Hare, 2015) and their spatial structure is determined by a variety of factors acting at different spatial scales. Broad descriptors include latitude, altitude and temperature (Rooney and Kalff, 2000; Lacoul and Freedman, 2006). At the lake scale, multiple abiotic and biotic factors such as topography, turbidity, water chemistry and sediment characteristics are listed as important spatial drivers of macrophyte community structure (Bornette and

Puijalon, 2011), but also competition, herbivory and disease play a major role (Lacoul and Freedman, 2006; Van Onsem and Triest, 2018). Nevertheless, there is a paucity of literature dedicated to intra-lake ecological processes. The aforementioned abiotic factors have a differential effect on various macrophyte growth forms at the local scale (Trindade et al., 2018), with emergent species less affected by environmental changes than submerged species under stable water level conditions (Alahuhta et al., 2016). Light availability is considered one of the most limiting factors for submerged macrophyte growth (Spence, 1972; Wen et al., 2022). It decreases along the depth gradient due to absorption by water, suspended particles and humic substances, therefore determining a zonation of hydrophytes along the littoral area of lakes (Lehmann et al., 1997; Azzella et al., 2014).

Deeper areas are often colonized by charophytes in clear lakes, because of the lower light requirements attributed to these macroalgae, while vascular species occupy the shallower areas (Léonard et al., 2008; Bolpagni et al., 2016; Murphy et al., 2018). Accounting for spatial components of macrophyte structure may allow us to integrate interactions between abiotic factors with dispersal processes (Léonard et al., 2008; Alahuhta et al., 2021; Lobato‐ de Magalhães et al., 2023). However, spatial investigations have been carried out mainly at broad scales, focusing on regional patterns and between-lake comparisons (Alahuhta et al., 2021 and references therein). The importance of environmental variables in structuring communities varies with the spatial scale considered (Alahuhta et al., 2016) yet lakes are often assumed to be homogeneous ecosystems, and little is known about intra-lake processes (but see Ferreira et al., 2015; Lewerentz et al., 2021).

Spatial processes (e.g., dispersal) can confound interpretation of the effect of environmental variables on species distributions because they may influence the community structure regardless of local environmental conditions (Clappe et al., 2018; Török et al., 2020). It is thus desirable to include spatial information in lake macrophyte community studies, when not limited exclusively to littoral contexts, often up to ~5 deep (Lewerentz et al., 2021). Focusing on the appropriate scale can improve the quality of lake macrophyte research because communities respond differently to environmental conditions according to the spatial scale considered (Alahuhta et al., 2016). Capers et al. (2010) suggest that environmental variation and spatial processes (e.g., dispersal) contribute similarly to macrophyte structure, both at local and regional scales, although a great amount of stochasticity is still involved.

The aim of this study is to explore the spatial links between submerged vegetation community structure and its environmental drivers (i.e., water and sediment features), considering both vascular and charophyte components, in five deep Mediterranean lakes up to 20 m depth. Specifically, these lakes show light and nutrient availability gradients ranging from near pristine to highly impacted (Azzella et al., 2014, 2017). To do this we apply a spatial-based approach known as multivariate Multiscale Codependence Analysis (mMCA) (Guénard and Legendre, 2018) with the aim of facilitating the understanding of complex processes such as the re-assembly of macrophyte communities in response to trophic changes. We hypothesize that more pristine lakes show stronger spatial structure driven by community-environment relationships at a wider range of spatial scales than more impacted lakes. Increasingly impacted lakes suffer from the progressive loss of potential colonization areas due to a reduced light availability in deeper areas, which can trigger a spatial rearrangement of *taxa* (i.e., a progressive migration towards shallower depths) with a consequent increase in competition among species.

Methods

Study Sites

The present study was carried out in five volcanic deep lakes of Central Italy (Bolsena, Bracciano, Martignano, Nemi and Vico lakes; Fig. 1), varying in terms of dimension (from 1.7 to 114.5 km$_2$), maximum depth (from 33 to 165 m), and trophic status [from 12.4 to 41.3 μg L^{-1} of total phosphorus (TP), with 10 and 30 μg L^{-1} being the thresholds between oligotrophic, mesotrophic and eutrophic conditions] (Azzella et al., 2017). Indeed, they were selected to represent a trophic gradient resulting from anthropogenic impact: Bracciano and Bolsena lakes represent near-pristine conditions (with TP values close to oligotrophic conditions), Nemi and Vico lakes are the most impacted sites (with TP in the range of 37 to 41 μg L^{-1}), while Martignano Lake has intermediate conditions (17.1 μg TP L^{-1}). These lakes greatly differ also in dissolved oxygen (DO) patterns along the depth gradient (Fig. A.1). During light time, in Martignano and Nemi lakes the DO peaked at 12 m depth with values of 155.7% (±5.6) and 130.2% (±4.2), to then rapidly decrease as it approached and overpassed the maximum depth of macrophytes growth (*Zcmax*). At 20 m depth, DO showed values respectively of 81.0% (±17.5), 46.0% (±12.5) and 0% in Martignano, Vico and Nemi lakes, respectively. Similarly, trophic parameters showed appreciable differences among lakes, highlighting an increase in trophic status towards Martignano and more prominently Nemi and Vico. Water total nitrogen (TN) reached the highest concentrations in Nemi (515 μg L^{-1}) and Vico (473 μg L^{-1}) at 20 m depth and the lowest in Bracciano (50 μg l L^{-1}). Concerning sediment features, an increasing trend was evident in sediment organic matter content from more pristine to more impacted lakes. For further insights on the methodological procedures of water and sediment sampling and analysis and physical and chemical conditions of the target lakes see Azzella et al. (2017).

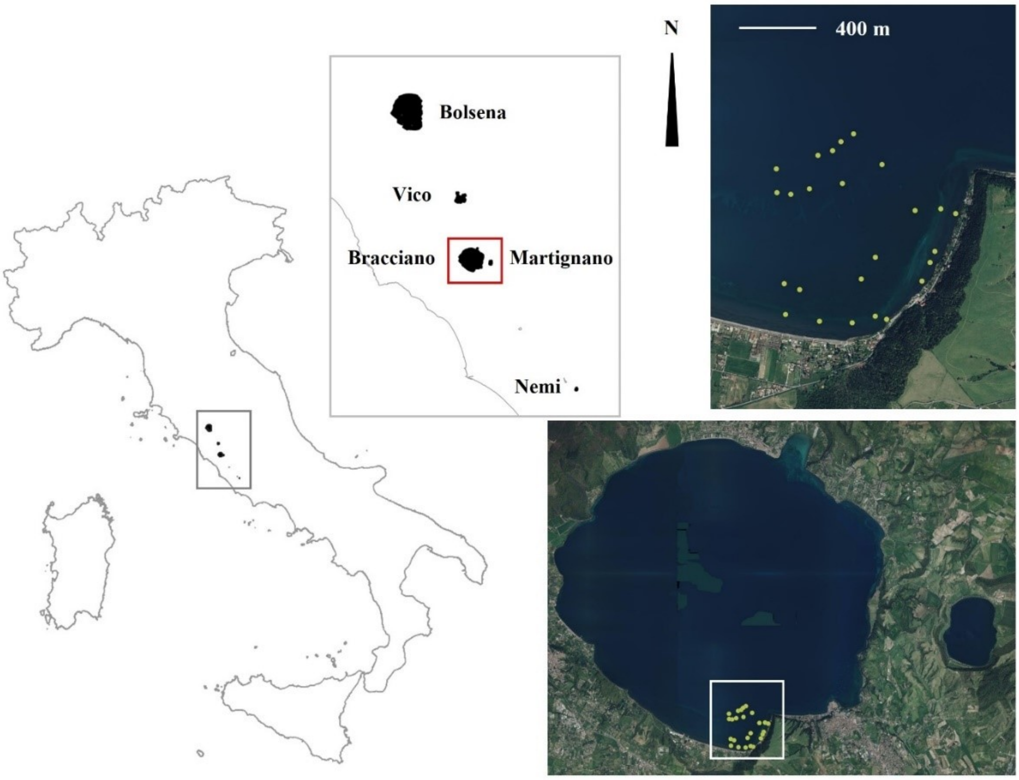

Figure 1*: Map of the study area with a zoom on Bracciano Lake as an example of the sampling design. Yellow dots represent the surveyed plots.*

Vegetation features of studied lakes

In total 24 macrophyte species were recorded, of which 13 are vascular, with one bryophyte and 10 *Characeae* species (Table A.2, see preprint). The most common species in terms of number of colonized plots is *Ceratophyllum demersum* (present in 27 plots out of 125), followed by *Chara polyacantha* (24) and *Myriophyllum spicatum* (24). Vegetation mainly covered the shallowest littoral areas of lakes (in the range 1.5 to 12.0 m of depth), whereas it was normally absent at the depth of 20 m (except for Bracciano) and below 12 m in Nemi and Vico (Azzella et al., 2017). Vascular species were poorly represented in Bolsena and Bracciano (Fig. A.2, preprint), while they occupied the shallowest littoral vegetation belt in Martignano and Nemi. In Vico, instead, vascular species occupied only the 6 m belt. Charophytes were dominant in Bracciano and Bolsena (Fig. A.2, preprint), occupying part of the littoral and the deepest plots in Martignano and were completely absent from Nemi. In Vico, charophytes were present in the shallowest plots, in contrast with vascular species' distributions (for further insights see Azzella et al., 2017).

Sampling design and data collection

In each lake, a homogeneous littoral sector of about 1 km (linear distance) parallel to the shore was selected (Fig. 1). These areas have been identified based on data collected in previous detailed macrophyte surveys

(Azzella et al., 2013) opting for sectors without evident artificial alteration of littorals or point-like sources capable of altering the submerged vegetation (i.e., no or minimum disturbance). At the same time, the selected sectors were not significantly affected by fetch, showing the maximum potential vegetation (i.e.,

representative of lakes macrophyte communities as a whole, in terms of density, consistency of submerged vegetation up to the maximum depth of growth). Within each target sector, in the depth range from 0 to 20 m, 25 plots of 4 m^2 were randomly selected and surveyed, for a total of 125 plots. The sampling design was arranged to have five plots randomly selected at five pre-defined different water depths (centered at 1.5, 3, 6, 12 and 20 ± 0.5 m of depth) which correspond to the core areas occupied by the vegetation bands characterizing Bracciano, the reference site among those explored. In the absence of significant chemical and physical perturbations, each of the five target lakes (Bracciano, Bolsena, Martignano, Nemi and Vico) should be characterized by all five vegetation belts (currently present only in Bracciano) (for further insights see Azzella et al., 2017).

All the species present in the target plots were identified and their relative cover-abundance recorded using percentage classes from 0 to 100% at 5% intervals. Up to 6 m depth, the sampling plots were explored by snorkeling. At greater depths, the plots were investigated combining different approaches, including the use of an underwater camera, a modified rake (as often suggested in the methods implemented within the Water Framework Directive; Azzella et al., 2013) and scuba diving. Each plot was also characterized in terms of water (conductivity, pH, DO, nitrate and ammonia ions, soluble reactive phosphorous, chlorophylla, TN, TP and light attenuation expressed as the proportion of incoming radiation reaching the plot depth = Lperc) and sediment (total phosphorous = TPsed, organic matter = OM expressed as LOI = dry weight Loss on Ignition, density and porosity) features. Standard approaches and methods were followed to collect the physical and chemical data, details are reported by Azzella et al. (2017).

Statistical analyses

To test our hypothesis an mMCA was performed (Guénard and Legendre, 2018) using the software R (R Core Team, 2021). This analysis uses three types of information: the response variables consist of a matrix containing the species abundances in the sites (plots); the explanatory variables are the matrix of environmental variables for each site; and lastly some spatial information of the sites is needed, which can be one-dimensional or bi-dimensional. In this case we used plot coordinates. Before running the mMCA, we used linear mixed effect models to test the effect of vegetation on pH and DO, which we considered to be the most relevant variables that could be influenced by the presence of submerged macrophytes, and which show wide daily variations. This step was necessary to understand if these variables reflected the lake conditions and could therefore be considered explanatory variables of the macrophyte structure, and not response variables. We tested the combined effect of water depth and presence of plants on the chosen variables. We used data from vegetated and non-vegetated plots surveyed in this study, together with data measured in the center of the lakes (in a non-vegetated location) at the corresponding water depth, derived from Azzella et al. (2017). We included random slopes described by the interaction between lakes and vegetation presence.

The significance of predictor variables was explored by calculating the confidence intervals with the function `confint`, and the best model was selected using ANOVA and comparing the AIC criterion. The R package `lme4` was used to perform the analyses (Kuznetsova et al., 2017). There are three main steps in the processing of mMCA analysis. First, given the plot coordinates, the space is organized into a number of spatial Eigenvectors, called MEMs (Moran's eigenvector maps; Dray et al., 2006), that describe the given space from the largest (lower order of MEM) to the smallest scale (higher order of MEM) (Grimaldo et al., 2016). The eigenmap function in the R package `codep` was used to obtain eigenvector maps (Guénard et al., 2018). Then, a PCA is run with the species abundance data, reducing community structure to the first two PCA axes which are used in the analysis. Finally, spatial structure is defined based on the covariation of community composition and environment at each successive spatial scale investigated, represented here by the micro scale (comparison between the different vegetation bands) to the large one (at the whole lake scale). For each lake two analyses were carried out: one mMCA using all species' abundances to investigate the whole community structure, and one mMCA using the relative proportion of vascular species cover compared to charophyte cover, to assess the contribution of these two components. In both cases, collinearity in the environmental variables was checked and redundant variables (linear correlation coefficient r>0.7) were omitted. Plots with 0% vegetation cover were also omitted from the analysis, and species abundances were Hellinger transformed to mitigate the broad differences between total abundances within plots. Finally, a permutation test was performed to test for significance of the mMCA model outcomes for each lake. The R packages `ggplot2` (Wickham, 2016) and `ggrepel` (Slowikowski, 2021) were implemented to graphically represent the community structure PCA as well as the lakes environmental descriptors.

Results

Due to collinearity, the environmental variables included in the mMCA analyses were: DO (mg L^{-1}), pH, Lperc (%), water TP (µg L^{-1}), TN (µg L^{-1}), sediment OM (%) and TPsed (mg g^{-1}). The linear mixed effects models revealed no significant correlation between vegetation and DO nor pH. In fact, the ANOVA revealed no significant difference between the model with plant information and without (p >0.1 in both cases). Besides, AIC values were lower for the models without plants information included (468.15 in the model excluding plants presence compared to 469.64 in the model including plants presence for DO, and 30.43 compared to 30.68 for pH). Therefore, we assumed we could keep DO and pH as explanatory variables in the analyses because they were not significantly influenced by the presence of macrophytes in the investigated lakes.

Micro- to macro-spatial macrophyte structure

Our results indicate that environmental drivers exert significant effects on underwater macrophyte patters almost exclusively at larger scales (Table 1). This is supported by the findings from all the studied lakes except for Nemi. The latter lake is the most impacted system in this study; here no spatial structure in the macrophyte community was found. In this lake the vegetation included only vascular species and no charophytes were observed.

***Table 1**: Summary of the mMCA results. "Species" refers to the analyses on the community including all species, "Band" refers to the vascular vs. charophyte bands comparison. PC1 and PC2 indicate the two species with higher loadings for each PC axis, with loadings values indicated in brackets. Only significant environmental drivers are reported, together with the relative p-value and the scale at which they affect the community (lower MEM number means broader scale); consequently, no information relating to Lake Nemi is reported in the table.*

	Species					Bands		
Lake	**PC1**	**PC2**	**Scale**	**Driver**	**p-value**	**Scale**	**Driver**	**p-value**
Bolsena	CH_TOM (0.67)	CH_ASP (0.71)	MEM2	TPsed	0.0049			
	CH_GLO (-0.69)	CH_TOM (-0.56)						
Bracciano	CH_POL (-0.75)	NIT_OPA (0.60)	MEM2	Lperc	0.0049			
	CH_ASP (0.55)	CH_POL (-0.48)						
Martignano	CH_GLO (0.75)	CH_INT (-0.76)	MEM1	TPsed	0.0049	MEM1	TPsed	0.0098
	CER_DEM (-0.37)	CH_POL (-0.39)	MEM3	pH	0.0130	MEM4	DO	0.0130
			MEM4	DO	0.0041	MEM5	Lperc	0.0093
			MEM5	Lperc	0.0047			
			MEM16	TN	0.0039			
Vico	CER_DEM (-0.87)	NIT_HYA (-0.52)	MEM2	Lperc	0.0049	MEM1	OM	0.0046
	NIT_OBT (0.35)	CH_ASP (-0.51)				MEM2	Lperc	0.0049

Lperc (proportion of incoming radiation reaching the plot depth) emerged as the key large-scale driver of macrophyte community structure in Bracciano and Vico, for the whole macrophyte community, and only in Vico for the vascular versus charophyte models (MEM2, p<0.01 in all cases). OM (sediment organic matter content) was also a significant driver at the lake scale (MEM1, $p<0.05$). Similarly, at a large scale, TPsed (sediment total phosphorous) structured the community of Bolsena (MEM2, $p<0.01$). For Bracciano and Bolsena none of the environmental variables included in the analysis were significant in distinguishing between belts dominated by large charophytes (e.g., *C. tomentosa*, *C. polyacantha*) and vascular species.

Martignano presented a highly structured macrophyte community both at large and at small scales. The environmental variables involved in significant (all $p<0.01$) spatial-ecological relationships were TPsed at MEM1, pH at MEM3, DO (dissolved oxygen concentration) at MEM4, Lperc at MEM5 and TN (total nitrogen concentration) at MEM16. In this lake, we found that TPsed ($p<0.05$), DO ($p<0.01$) and Lperc ($p<0.01$) were also significant in the models of vascular versus *Characeae* community structure at larger scales (MEM1, MEM4 and MEM5, respectively).

Site-specific macrophyte spatial patterns

For Bracciano, the mMCA analysis identified 19 significant ($p<0.05$) spatial Eigenvectors. The first two PCA axes together explained 83% of the variation in community structure. *Characeae* were the dominant species driving the variation; *C. aspera* and *C. polyacantha* respectively had high positive and negative loadings on PC1, while *Nitella opaca* and *C. globularis* had both high positive scores on PC2 (Fig. 2a).

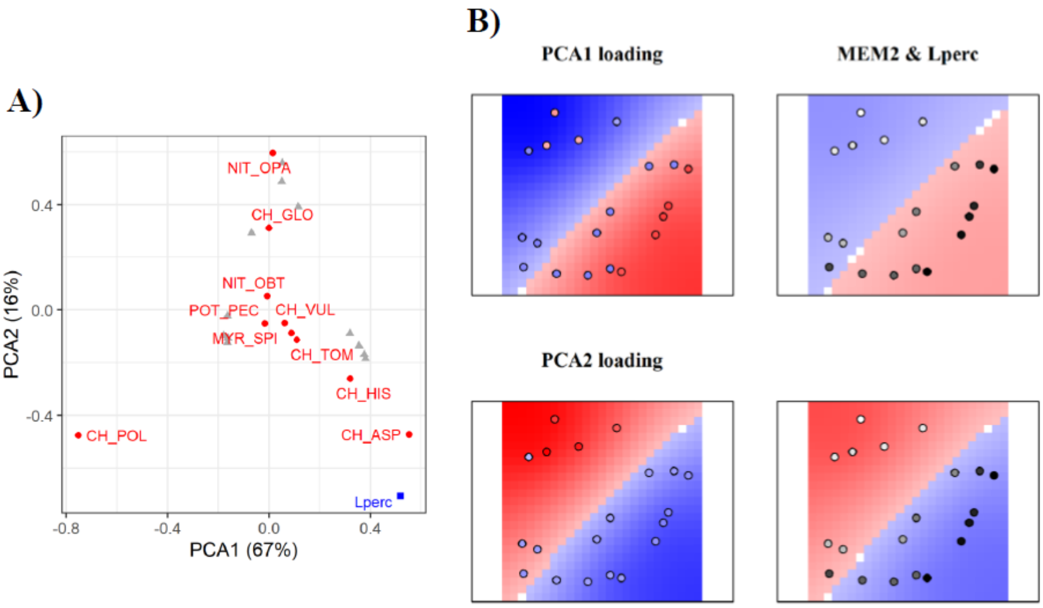

***Figure 2**. Output of the mMCA analyses for Bracciano Lake. On the left the first two principal components of the community structure (A): species are in red, significant environmental variables in the mMCA are in blue, plots are indicated with grey triangles. On the right the representation of significant spatial Eigenvectors (B). Red background and dots correspond to positive loadings, blue to negative loadings. Colored dots in the first column of maps represent plots and their loadings in the corresponding PCA, while dots in greyscale in the right column indicate the value of the environmental variable in that plot (darker dots indicate higher values). Background represents the loadings of the spatial Eigenvector. Intensity of color indicates higher loadings. White dots or areas correspond to loadings around zero.*

High values of Lperc corresponded to positive loadings on PC1 and negative loadings on PC2. Therefore, *C. aspera* was found in shallow plots where Lperc was higher, whereas species like *C. polyacantha*, *C. globularis* and *N. opaca* were found in deeper plots with lower Lperc (Fig. 2b). In Bolsena, 16 significant ($p<0.05$) spatial Eigenvectors were identified. The first two PCA axes explained 79% of the species variation, and here charophytes were also the most important group in the community space. PC1 was positively related to *C. tomentosa* and negatively to *C. globularis*, while PC2 was positively related to *C. aspera* and negatively to *C. tomentosa* (Table 1). High TPsed corresponded to negative loadings on both PC1 and PC2 (S5, preprint). Species like *C. tomentosa* and *C. aspera* grew in shallow plots with low TPsed, while *C. globularis* was present in deeper plots with high TPsed. Vascular species were found nearest to the shore.

In Martignano we obtained 19 significant ($p<0.05$) spatial Eigenvectors. The first two PCA axes described 62% of the variation, and we can observe a higher importance of vascular species in the community structure. The species with higher loadings on PC1 and PC2 were mainly *C. globularis*, *C. intermedia* and *C. demersum* (Fig. 3a).

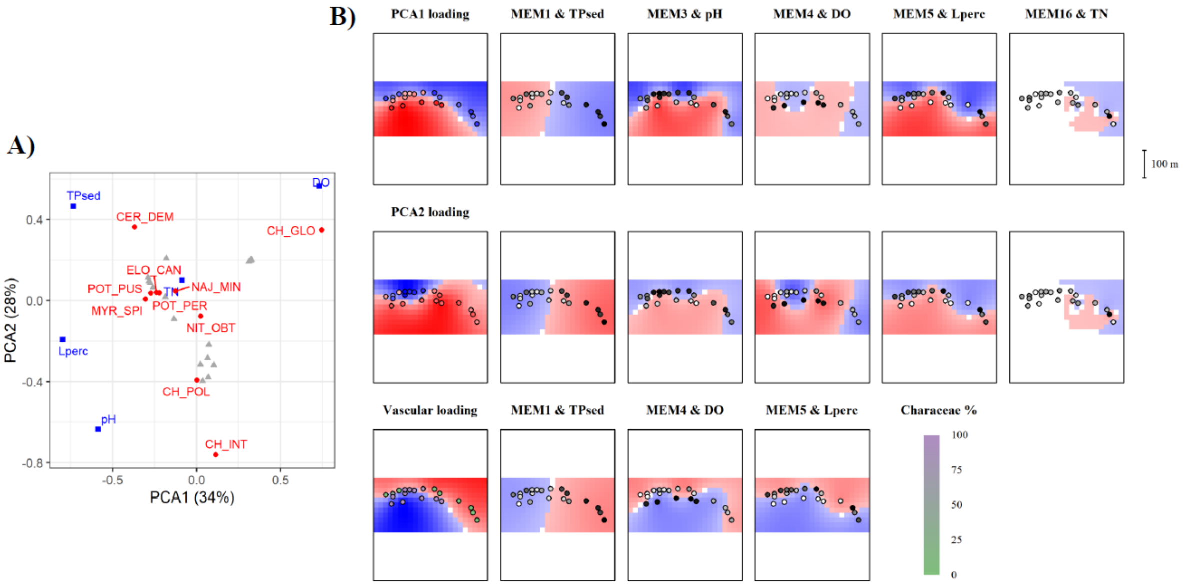

***Figure* 3**. *Output of the mMCA analyses for Martignano Lake. On the left the first two principal components of the community structure (A): species are in red, significant environmental variables in the mMCA are in blue, plots are indicated with grey triangles. On the right the representation of significant spatial Eigenvectors (B). Red background and dots correspond to positive loadings, blue to negative loadings. Colored dots in the first column of maps represent plots and their loadings in the corresponding PCA, while dots in greyscale in the right column indicate the value of the environmental variable in that plot (darker dots indicate higher values). Background represents the loadings of the spatial Eigenvector. Intensity of color indicates higher loadings. White dots or areas correspond to loadings around zero. The last row of maps represents the mMCA on groups (vascular vs. charophytes). Shading of green in the dots indicate the presence of vascular species, and positive loadings refer to the abundance of vascular species.*

Positive loadings on PC1 were related to low TPsed, pH and Lperc, and high DO. Positive loadings on PC2 were more related to high TPsed, and DO, and low pH and Lperc (Fig. 3b). Water TN, though significant, was not very descriptive. In this case, *C. globularis* was found in deeper plots with high DO and low pH and Lperc whereas *C. intermedia* was found in approximately opposite conditions, including plots with low TPsed. *C. demersum* grew mainly in shallow plots located on the East side of the sampling site, where TPsed was higher. Vascular species were present near the shore where plots presented high TPsed and Lperc or low DO.

In Vico, one of the two most impacted lakes, we obtained 16 significant ($p<0.05$) spatial Eigenvectors. PC1 and PC2 accounted for 74% of the variation altogether, and we could observe a clear distinction between charophytes and vascular species. Species like *N. obtusa* and *C. globularis* had positive loadings on both PC1 and PC2, *C. demersum* had negative loadings on PC1 and *N. hyalina* and *C. aspera* were negatively related to PC2 (Table 1 and Fig. A.3, preprint). Compared to other lakes, here we found vascular species (*C. demersum*) in the deepest plots, where Lperc was lowest, and charophytes in the littoral area, with higher Lperc (S5, preprint).

Discussion

In this study we used vegetation data from five deep lakes to assess spatial-ecological relationships within lakes with a range of trophic conditions. Our hypothesis was partially supported by the data. Weak or absent spatial structure was reported for the two most impacted lakes (Vico and Nemi). However, we also found weak spatial structure in the two most pristine lakes (Bracciano and Bolsena). Nevertheless, a well-defined structure was observed in Martignano Lake, an intermediate site both in terms of trophic status (17.1 μg TP L_{-1}, as annual average value) and size (2.02 km_2). Here, several environmental parameters were important at different spatial scales. In general, in the sites investigated, patterns in the macrophyte community structure were found almost exclusively at the largest scales, which often reflected a distinction between shallow (up to 3 m) and deep bands (>3 m). Moreover, no spatial organization of vascular and large *Characeae* species groups was observed in Bracciano and Bolsena, where the vascular component was very poorly represented, nor in Nemi, were charophytes were absent.

Our findings are certainly influenced by the spatial scheme adopted for analyzing both macrophyte communities and environmental drivers, purposely designed to allow a robust comparison between the investigated lakes and testing macrophyte assemblage rules. In future investigations, it could be interesting to explore the micro-scale too, for example by reducing the distance (in terms of depth) between the investigated vegetation bands.

Ecological drivers of macrophyte vegetation in deep lakes

Light availability (expressed as percentage) was the most common driver among our sites. It significantly acted on vegetation patterns in Bracciano, Martignano and Vico. It was associated with variation in community structure at large scales, mainly distinguishing deeper and more shallow bands. Interestingly, light was the only significant driver both in near-pristine and more impacted lakes. Indeed, light is the most important environmental filter in lentic environments, where it can determine very rapid and substantial changes in conditions both in space and time, hence the establishment of light-demanding rather than low light-tolerant submerged species (Rodriguez and Thomaz, 2010; Luhtala et al., 2016; Zhang et al., 2020). A higher reduction in light availability along the depth gradient induces a limitation in the suitable area for colonization by submerged macrophytes, which can be observed in the lower number of vegetated plots in the eutrophic lakes investigated in this study.

Sediment total phosphorus (TPsed) was the second most common driver in our study, confirming the importance of sediment features for macrophyte assemblages (Jones et al., 2012; Bolpagni and Pino, 2017; Dainez-Filho et al., 2019; Marzocchi et al., 2019). It influenced the community structure at large scales in Bolsena and Martignano, distinguishing deep from shallow plots in Bolsena and eastern from western plots in Martignano. This pattern reflects local sediment characteristics as well as a higher influence of sediment metabolism on the water column in shallower areas compared to deeper ones (Dalla Vecchia and Bolpagni, 2022).

Among the several environmental variables found to be significantly related to spatial-ecological structure in Martignano, dissolved oxygen (DO) was important both in defining the general community structure and in determining the vascular versus charophytes composition. The availability of DO depends on the photosynthetic activity of submerged macrophytes (Vilas et al., 2017). However, the oxygen content can also be indicative of the general metabolism of a lake, reflecting not only the activity of autotrophic organisms, but also the consumption by decomposition processes, especially so in deep water (Misra, 2010). On the other hand, pH was only important in the general community structure, but not in the spatial arrangement of the two macrophyte groups (vascular vs. charophytes) in the lake. pH is a strong descriptor of macrophyte distribution because it is related to their carbon acquisition strategy (Rørslett, 1991). Higher pH determines a higher availability of bicarbonate compared to carbon dioxide, therefore favoring the presence of species adapted to use this form of carbon for photosynthesis (Iversen et al., 2019).

The low predictive power of nitrogen in this lake is probably related to an unbalanced stoichiometry of nitrogen and phosphorus in the water; although nitrogen is relatively abundant, the concentration of phosphorus remains relatively low, dampening its effect (de Baar, 1994; Xia et al., 2014).

Spatial insights into macrophyte vegetation in deep lakes

Only relatively few drivers are involved in regulating the depth distribution of macrophytes in deep lakes: above all light availability (Sculthorpe, 1971; Spence, 1972). Based on our data, this means that pristine (or near-pristine deep lakes, with TP<15 μg L$_{-1}$) and impacted lakes (>35 μg L$_{-1}$) have the same main (almost exclusively) driver at the largest spatial scales. In both these two extreme cases there are no other limiting factors as important as light. In near-pristine lakes, the contextual low availability of other nutrients besides phosphorus (TN<300 μg L^{-1}) allows a progressive depth arrangement of macrophyte species according to their different adaptation to submergence (Stross et al., 1988; Schwarz et al., 2002). Along the depth profile, the key challenge is the capability to use progressively lower light intensities to maintain an efficient cellular metabolism. This reflects the classical depth succession of macrophytes, including mid-depth and deeper growths of larger *Chara* species such as *C. tomentosa* and *C. polyacantha*, and *C. globularis* and *N. opaca* in European deep lakes (Azzella et al., 2017). Conversely, in impacted lakes the reduction of light availability is so critical that the effects of other drivers (mainly nutrients) are probably masked.

However, especially for pristine lakes we did not find a strong spatial structuring of macrophyte communities, although under these conditions a typical concentric arrangement of macrophytes is considered expected (Schwarz et al., 2002; Azzella et al., 2017). The impossibility to point out clear macrophytes spatial drivers under oligo-mesotrophic conditions is probably an effect of the low species diversity of submerged assemblages. They are typically dominated by only one *taxon* and often characterized by relatively few species (Tanner et al., 1985), in turn capable of transgressing their optimal depths of growth. This could represent a confounding behavior for evaluating the spatial structure of species poor macrophyte assemblages. In fact, evidence is accumulating on the intrinsic high spatial dynamism of submerged macrophytes, which is much greater than previously expected (Bresciani et al., 2012; Bolpagni et al., 2016; Ghirardi et al., 2019, 2022).

Only Martignano exhibited strong spatial structuring of macrophyte assemblages at multiple scales with many environmental drivers acting together. This lake is characterized by a meso-eutrophic status, and the results may support the intermediate environmental stress hypothesis, which generally predicts higher positive interaction levels under intermediate nutrient conditions (Bakker et al., 2013). The several drivers described by the analysis for this lake may define unique combinations of conditions and niches that can be occupied by various representatives of both *Characeae* and vascular species, as well as by herbivore communities - fundamental in influencing the diversity, patterns and abundance of freshwater macrophytes (Sheldon, 1987; Bakker et al., 2016). Stress, for macrophytes, has often been considered in terms of mechanical disturbance (wind/wave action) or fluctuation in water level (Kilar and McLachlan, 1989; Rørslett, 1991; Gomes de Moura Júnior et al., 2017).

Here, however, stress is related to the combination of factors that define intermediate (rather than optimal or unsuitable) conditions for both macrophytes and the heterotrophic assemblages associated, which do not allow the establishment of a community dominated by only top competitors (Kilar and McLachlan, 1989). The spatial arrangement of plants and charophytes in this meso- to eutrophic lake is therefore well defined and could be detected by the analysis, because the community is composed of a balanced abundance of a good number of representative species.

Conclusions

Findings from the present study indicate distinctive macrophyte community structuring at the largest spatial scales (i.e., whole lake scale), highlighting a clear partitioning between shallow (up to 3 m) and deep vegetated bands (>3 m) colonized by vascular macrophytes and large charophytes (e.g., *C. tomentosa*, *C. polyacantha*), respectively. On the contrary, no robust spatial structure was detected at the microscale (i.e., across vegetation bands), both in pristine and impacted deep lakes. This evidence could be attributed both to the intrinsic low species richness of the explored macrophyte communities and the constant presence of generally only one dominant species. This study highlights the need to further explore the complex mechanisms underlying macrophyte depth arrangement, in addition to reaffirming the true spatial and ecological significance of the depth gradient. In just a few meters of depth substantial ecosystem variations emerge which are comparable to those that characterize the succession of vegetation belts along entire mountain ranges. Knowledge must be implemented in relation to the functional traits of submerged macrophytes, including charophytes, to pursue a better contextualization of the mutual and competitive links among diverse groups of primary producers (vascular vs. macroalgae vs. microalgae). Different investments into biogeochemical and structural features, and their tridimensional arrangement can mirror different adaptive responses, offering interpretation keys of the spatial structuring and temporal responses of macrophytes to environmental change (both local – related to direct anthropogenic pressures – and global by climate change). Secondly, the dynamics of deep macrophyte stands must be better described.

When it comes to extreme environments for plant growth (e.g., cold environments, high salinity habitats and deserts; Bechtold, 2018), lake depths are rarely mentioned. We must urgently change our perception of the aquatic life of macrophytes and understand their ecological-evolutionary mechanisms to offer effective actions to recover lake macrophyte meadows to maintain adequate levels of ecosystem service provision from large, deep lakes. Not only is the survival of a fundamental facet of biodiversity at stake, but also many human communities that depend on these ecosystems.

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