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Drivers of amphibian species richness in European ponds

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Amphibians are commonly occurring inhabitants of most lentic freshwater ecosystems, yet their global populations are in alarming decline. Ponds in particular play a crucial role in supporting amphibian biodiversity. In this study, we identified the main drivers influencing amphibian species richness by conducting a comprehensive ecological characterization in 201 ponds across seven European countries spanning a large latitudinal and longitudinal gradient. The amphibian species richness in each of these ponds was assessed using environmental DNA metabarcoding on water samples. The relative influence of climatic, local abiotic and biotic, and land use variables on variation in species richness across ponds was quantified using boosted regression trees. Our results suggest that local factors, particularly chlorophyll-a concentration, but also pond area and depth, are the main drivers of amphibian richness, together with climatic variables such as annual mean precipitation and temperature. The highest richness was observed in low-nutrient, fishless, intermediate-sized, shallow ponds, located in warmer regions with higher precipitation rates. These potential drivers of amphibian richness should be considered in the planning and implementation of amphibian conservation and management actions.

Keywords: biodiversity, environmental DNA, eutrophication, fish, freshwater, machine learning

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Introduction

Ponds are typically classified as shallow lentic water bodies smaller than five hecares that can be natural or artificial (Oertli et al. 2005, Deacon et al. 2018, Richardson et al. 2022). These ecosystems can have very diverse origins and are generally important sources of fresh water in landscapes (Deacon et al. 2018). Estimations of their global abundance varies from 547 million to 3.19 billion (Holgerson and Raymond 2016). These small and shallow ecosystems are often characterized by high local diversity usually harbouring multiple rare species and, usually, providing an exceptionally strong contribution to regional diversity (Oertli et al. 2005, Biggs et al. 2017). However, ponds are disappearing in Europe, with often more than 50% of European ponds lost in the last century (Oertli et al. 2005, Smith et al. 2022).

Currently, animals in freshwater ecosystems experience extinction rates five times higher than those in terrestrial ecosystems (Eiler et al. 2018, Gozlan et al. 2019). In particular, amphibians are facing severe worldwide declines (Albert et al. 2021, Hill et al. 2021, van Rees et al. 2021, Reyes-Moya et al. 2022), rendering them the most endangered vertebrate group on the planet (Beebee and Griffiths 2005, Luedtke et al. 2023). Amphibians are affected by many threats, including habitat loss, climate change, invasive species and disease (Ribeiro et al. 2018, Beja and Alcazar 2003, Hansen et al. 2019, Luedtke et al. 2023). In Europe, nearly a quarter of amphibian species are currently listed as threatened (included in the categories of 'Critically endangered', 'Endangered' and 'Vulnerable') by the International Union for Conservation of Nature – IUCN (Temple and Cox 2009, Gozlan et al. 2019, IUCN 2023, Luedtke et al. 2023), emphasizing the urgent need for a better understanding of the factors influencing amphibian populations to inform conservation plans (Knutson et al. 2004), and help stablish protected areas (Luedtke et al. 2003).

Climate is one of the strongest drivers of amphibian species richness patterns across Europe. This influence is twofold: first, many amphibian species depend on water for reproduction and thrive in humid environments, being most active during wet periods (Ficetola and Maiorano 2016). Second, due to their ectothermic physiology, amphibians exhibit heightened sensitivity to climatic conditions (Tsianou et al. 2016, Tsianou and Kallimanis 2019). Amphibian biodiversity in Europe follows a clear north-south gradient, influenced by both contemporary climatic conditions and historical climate stability during the Quaternary. Postglacial colonization, following the retreat of glaciers more than 16 000 years ago, has been particularly important in shaping amphibian species richness patterns, with many species limited in their ability to track rapid climatic shifts (Araújo et al. 2008, Zeisset and Beebee 2008). However, at both continental and smaller biogeographical scales, climate is not the only determinant of species richness (Mouchet et al. 2015).

Agricultural intensification (including aquaculture), as a main land use, has been identified as a major threat to amphibians worldwide (Luedtke et al. 2023). Indeed, intensive agriculture can significantly affect local abiotic and biotic characteristics of ponds by causing direct habitat destruction and by enhancing nitrogen and phosphorus influxes (Houlbrooke et al. 2004, Nyenje et al. 2010, Merz et al. 2023), which promotes eutrophication and stimulates algal blooms (Manny et al. 1994, Sfriso et al. 2017). Eutrophication has been found to decrease pond amphibian richness (Schmutzer et al. 2008, Calderón et al. 2019, Ruso et al. 2019), partly because it helps to promote tadpole pathogenic infections (Johnson and Chase 2004, Belden 2006, Johnson et al. 2007, Peltzer et al. 2008).

Two biotic characteristics are also of key importance in explaining amphibian richness. On one hand, the presence of vegetation inside and on the perimeter of the pond. Macrophytes generally have a positive impact on amphibians as they support periphyton growth and constitute food resources for tadpoles, increase water clarity, provide protection from predators, and promote habitat heterogeneity, which are critical factors for amphibian survival (Arribas et al. 2014). On the other hand, the presence of fish in ponds, which can be detrimental for amphibians because of direct predation and competition, especially at their larvae stage (Hecnar and M'Closkey 1997, Kats and Ferrer 2003, Knutson et al. 2004, Semlitsch et al. 2015) and because planktivorous fish predation on zooplankton can strengthen eutrophication (Jeppesen et al. 2012).

Traditionally, amphibian presence in ponds, but not necessarily breeding, has been assessed through established monitoring methods. However, the use of environmental DNA (eDNA) metabarcoding has emerged as a powerful alternative tool to reliably assess amphibian species richness and community composition in ponds (Brys et al. 2021a, Everts et al. 2024). This approach detects DNA released by organisms into their environment through secretions, excretions, blood, or decomposition. Notably, it achieves comparable, and sometimes superior, accuracy when compared to traditional methods (Ruppert et al. 2019, Li et al. 2021, Moss et al. 2022). Ponds are particularly well suited for eDNA studies because they are small, enclosed, have low shoreline erosion levels, and experience minimal water flow dynamics, which enhances DNA detectability and inferences on local species occurrences (Harper et al. 2019a, Brys et al. 2021a). Most amphibian species breed in standing freshwater, rendering ponds as ideal media for amphibian DNA detection (Svenningsen et al. 2022). Moreover, compared with conventional survey methods, eDNA is particularly relevant for the large-scale monitoring of amphibian communities, since eDNA-based techniques are both cost- and time efficient (Eiler et al. 2018, Svenningsen et al. 2022).

Measures of biodiversity are used to understand ecological processes (Blowes et al. 2022) and, despite its growing use in assessing local diversity, the application of eDNA to test ecological hypotheses, such as the impact of environmental stressors and drivers, is still relatively uncommon (Harper et al. 2019a), although Everts et al. (2024), recently studied the impact of American bullfrog *Lithobates catesbeianus* on native pond amphibian communities using eDNA.

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The effectiveness of species richness assessments, for example for conservation actions, can be improved by incorporating information on species abundance patterns (Magurran 2004). However, using eDNA metabarcoding to assess species abundance presents significant challenges and potential biases, which may result in discrepancies from true population sizes, as explained in the meta-analysis of Yates et al. (2019). Despite these challenges, several studies have demonstrated significant positive correlations between eDNA concentrations and species abundance, leading to accurate estimates of species richness and evenness (Nakagawa et al. 2022, Tsuji et al. 2022). In the present study, we focused on assessing species richness rather than abundance, recognizing that eDNA metabarcoding is particularly well-suited for detecting the presence of multiple species within an environment (Taberlet et al. 2012, Brys et al. 2021a).

Despite their crucial role in amphibian conservation (Griffiths 1997, Curado et al. 2011), ponds have been disproportionately neglected in prevailing water, nature, and conservation policies, as well as in freshwater research (Ilg and Oertli 2017, Hill et al. 2021). Yet, this perception is changing. Currently there is a rapidly growing awareness for the high importance of pond conservation, restoration, and for amphibian conservation (Moor et al. 2022). Existing research on the drivers of amphibian species richness has typically considered only a limited set of local pond characteristics (Hecnar and M'Closkey 1996, Werner et al. 2007, Semlitsch et al. 2015, Sauer et al. 2022), assessed a small number of ponds within a single geographical region (Knutson et al. 2004, Werner et al. 2007, Lescano et al. 2015), or concentrated on the effects of a single type of stressor (Knutson et al. 2004, Hamer and Parris 2011, Jeliazkov et al. 2014, Sauer et al. 2022, Yang et al. 2022).

Our research aims to disentangle the relative contribution of multiple climatic, land use, and local abiotic and biotic pond characteristics underpinning variation in amphibian species richness at a large geographical extent across Europe. We conducted a comprehensive and standardized ecological characterization of 21 different variables for 201 ponds in seven European countries and assessed amphibian species richness in each pond using an eDNA metabarcoding approach. Our hypothesis posits that the climatological conditions of a pond will play an important role in driving amphibian species richness, but that local abiotic and biotic characteristics of the pond will also have a strong influence. Therefore, climate and local factors will interact in determining species occurrences and richness. We expected higher amphibian species richness in ponds located in warmer regions, as well as in regions with higher precipitation rates, due to ecological and biogeographical drivers, as mentioned above. We also hypothesised that amphibian species richness would be highest in shallow and fishless ponds, with low nutrient concentrations and high vegetation cover. Ultimately, we aim to shed light on the most relevant pond characteristics driving amphibian species richness to contribute to effective future management and conservation actions.

Material and methods

Pond selection and data collection

Data were collected from 201 ponds distributed across six European countries, namely Belgium (30), Denmark (29), Germany (28), Spain (30), Switzerland (29), United Kingdom (27), and the Asian part of Turkey (28) (Fig. 1). Ponds were selected within each country following an agricultural land use intensity gradient criterion and span a latitudinal and longitudinal geographic range of 40°07′ 12.0″ to 56°14′24.0″ and 02°51′36.0″ to 32°43′48.0″. They are located at altitudes below 800 m, with water column depth ranging from 0.12 to 5.30 m, and with pond surface area ranging between 12.9 m^2 and 1.47 ha. Ponds in each country were distributed in pondscapes, with one to six ponds present in each, depending on the country. Ponds were selected to cover gradients of land use and hydroperiods. Only open-canopy ponds were considered. Ponds were sampled in April–May 2021, except for 12 Spanish ponds and 28 Turkish ponds, which were sampled in May 2022. Each pond was characterized by 21 geographic and climatic, land use, and local abiotic and biotic quantitative and qualitative variables (Table 1). Minimum, maximum and average values for all quantitative variables used can be found in the Supporting information (Table 1).

Climatic and geographic variables

Mean annual air temperature and precipitation, aridity index, and precipitation seasonality data were extracted from the Copernicus Climate Change Service (Wouters 2021) from the 1 × 1 km cell nearest to each pond.

Land use variables

The land use surrounding each pond was visually assessed in the field at a 100 m radius, creating one variable of arable land use that included the percentage of land used for agriculture, and one variable of urban land use that included the percentage of land cover occupied by urban infrastructure. The presence of paved or unpaved roads at a 100 m distance of the pond, and if the pond was in a protected area of any kind, were also considered.

Local abiotic variables

A whole-water column depth-integrating water sample was collected using a tube sampler from each pond at eight locations. Samples from different locations were pooled, leading to the collection of a 10-l sample where possible. Subsamples were subsequently taken from this pooled sample for further analysis. Approximately 250 ml of unfiltered water was collected for the analysis of total nitrogen and total phosphorus, with samples kept in cool and dark conditions in the field prior to freezing at −20°C in the laboratory until further analysis. Turbidity (nephelometric turbidity unit, NTU) was

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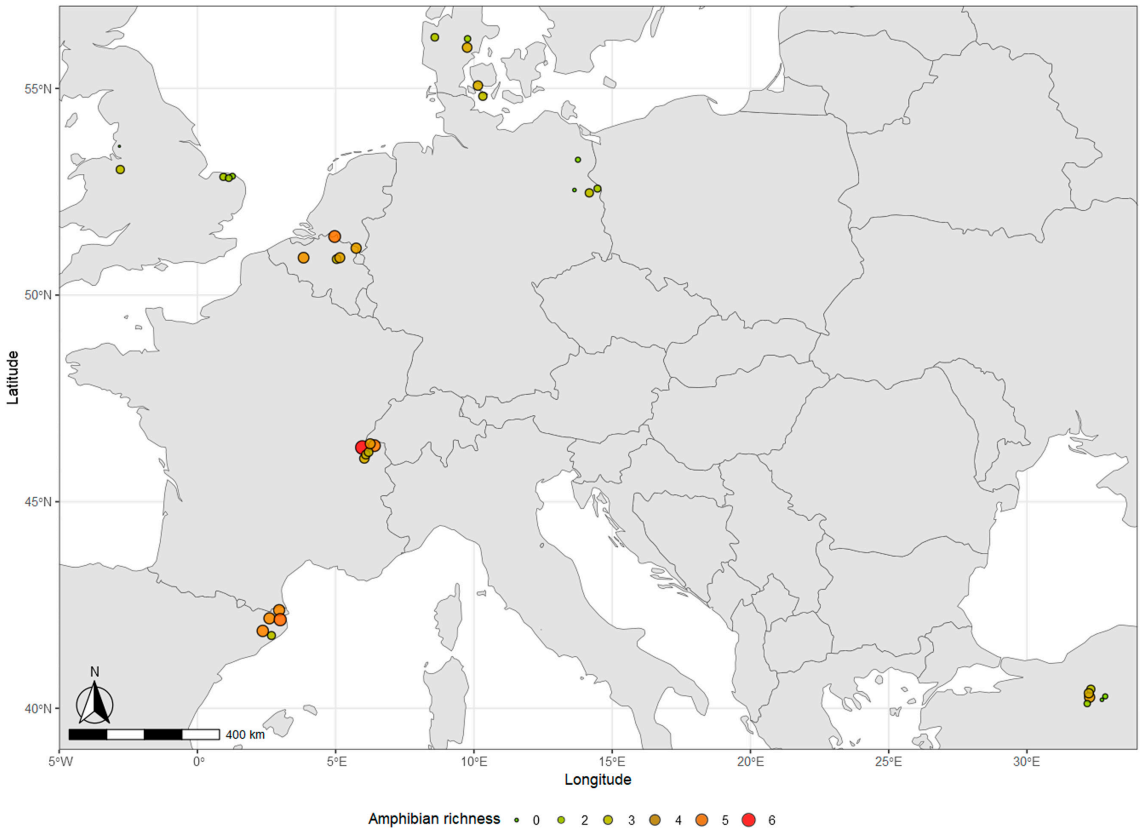


Figure 1. Geographical distribution of the 36 pondscapes comprising the 201 Western Palearctic ponds included in the study. Circles indicate the average number of amphibian species for each pondscape, ranging from small and green (low species richness) to large and red (high species richness).

estimated with a portable fluorometer at a central location in the pond (or in the middle of the largest open water patch in the case of macrophyte-covered ponds) at a depth of 20 cm below water surface.

Pond depth was measured at 2 m intervals using a graduated pole inserted perpendicularly to the base ground of the pond, by foot or boat, depending on the depth, morphology, and substrate of each pond, and a mean depth value was calculated. Pond area was measured using a metric line. Pond hydroperiod was defined as permanent, temporary, or semi-permanent (in principle, permanent but dries out once every several years) based on periodic observations of each pond in combination with data collected by temperature and/or water level loggers (Hobo U2OL-0x, Onset Brands). One temperature logger was installed at the deepest area of each pond and another logger (measuring air temperature) was installed outside the water close to each pond. When both temperature records were similar for both of these loggers, it was assumed that the pond was dry.

Local biotic variables

We used a portable fluorometer to quantify in vivo concentrations of chlorophyll-a (mg l^{-1}). We also performed visual assessments of the percentage of emersed vegetation inside the pond and on its perimeter, as well as the percentage of open water. Fish species richness was assessed by eDNA metabarcoding (methodology in the next section and see also the Supporting information and Table 2 for a detailed description on the occurrence of each fish species for each country).

eDNA data collection

Amphibian and fish species richness was assessed using eDNA metabarcoding. Water samples were collected from each pond in early spring (April–May) at multiple locations using a pole with a sterile Whirl-Pak sampling bag attached to it. Between 15 and 20 subsamples were taken from just below the water surface, covering the entire pond and covering all microhabitats. All subsamples of each pond were merged into a 30-l bucket containing a sterile plastic bag. From this pooled sample, a known volume of water was then filtered on a sterile encapsulated 0.8 µm pore size PES filter (50 mm diameter), containing a 5 µm glass fibre prefilter (NatureMetrics). The filter was air-dried using the syringe and the capsule subsequently filled with 1.5 ml of Longmire's buffer for preservation (for more details see Mauvisseau et al. 2021). Filters were stored in the dark at room temperature prior to DNA extraction at the INBO (Research Institute for Nature and Forest) eDNA laboratories at Geraardsbergen (Belgium), which was implemented in less than four months after sampling.

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Table 1. Quantitative and qualitative independent variables considered for the analysis of the drivers of amphibian species richness in European ponds. Latitude and longitude were not considered for the BRT models.

Variable	Units/categories	Definition
Latitude	degrees (°)	distance of the pond from the Equator
Longitude	degrees (°)	distance of the pond from the Greenwich Meridian
Mean annual temperature	Kelvin degrees (K)	annual mean of the monthly mean temperature at 2 m above the surface
Mean annual precipitation	meters per second (m s^{-1})	annual mean of the monthly mean precipitation rate (both liquid and solid phases)
Aridity index	dimensionless	monthly potential evaporation divided by the monthly mean precipitation, averaged over the year
Precipitation seasonality	percentage (%)	annual coefficient of variation of the monthly precipitation sums
Hydroperiod	permanent/semi-permanent/temporary	pond expected to be temporary (T), permanent (P), or semi-permanent (SP, in principle permanent but dries out once every several years)
Pond area	square meters (m^2)	area of the pond where the water level is the highest.
Pond depth	meters (m)	mean depth of the pond, measured along two perpendicular transects each 2 m
Turbidity	nephelometric turbidity units (NTU)	turbidity of the pond water
Total nitrogen	milligrams per litre (mg l^{-1})	total nitrogen concentration in the pond water
Total phosphorus	milligrams per litre (mg l^{-1})	total phosphorus concentration in the pond water
Chlorophyll-a concentration	milligrams per litre (mg l^{-1})	chlorophyll-a concentration in the pond water, measured in vivo, mean of three measures, proxy for phytoplankton biomass
Fish species richness	number of species	number of fish species in the pond, detected using eDNA
Emersed vegetation in the pond	percentage (%)	coverage of the pond by emersed vegetation
Emersed vegetation in the pond perimeter	percentage (%)	coverage of the perimeter around the pond by emersed vegetation.
Open water	percentage (%)	percentage of open water within the pond
Roads	presence/absence	presence of a paved or unpaved road, or its absence, at less than 100 m from the pond
Protected area	protected/unprotected	pond situated in a protected area of any kind
Arable land use at 0–100 m	percentage (%)	percentage of arable land within a 100 m radius of the pond
Urban land use at 0–100 m	percentage (%)	percentage of land use of urban origin within a 100 m radius of the pond

Molecular analyses

DNA was extracted using Qiagen's DNeasy Blood & Tissue Kit and purified with Qiagen's DNeasy PowerClean Cleanup Kit (for more details see Everts et al. 2021). The extraction process was performed in a dedicated PCR-free room for low-copy-number template extractions at INBO, with particulate air-filtered compartments, hence preventing contamination of eDNA samples. During the lysis-step in the extraction protocol, for each filter a fixed amount of internal positive control (IPC) was included. This IPC consisted of a 149 bp Dengue virus type 2 insert sequence (GenBank M29095.1) included in a plasmid (for more details see Brys et al. 2021b) and was used for both quality control of the resulting DNA extract and as an indicator of PCR inhibition or failure. The eDNA extract was purified with MagNA beads and quantified with a Quantus Fluorometer according to the manufacturer's instructions, prior to amplification. In case these quality control steps of the first filter of each pond indicated extraction failure or a too low DNA quality to further implement in the metabarcoding workflow or sequencing, the DNA from the second filter of that pond was extracted instead. Vertebrate-specific Riaz primers (12S_F1, 5′-ACTGGGATTAGATACCCC-3′;12S_R1, 5′-TAGAACAGGCTCCTCTAG-3′), were then used for DNA amplification, targeting a 142-bp fragment located on the mitochondrial 12S rRNA Gene (Riaz et al. 2011). Libraries were constructed in triplicate according to Brys et al. (2021a), followed by 150 bp paired-end sequencing on an Illumina HiSeq3000 platform (Admera Health). Libraries were constructed in triplicate according to Brys et al. (2021a). Negative PCR controls were additionally included in the workflow to assess for potential contamination in the laboratory.

Molecular data processing and cleaning

Raw sequencing data were processed following Brys et al. (2021a). Taxonomies were assigned using a custom, carefully curated reference database containing Riaz amplicons of European amphibian and fish species (Halfmaerten et al. 2020), and only sequences with a 100% match with this database were retained. The total number of reads per sample was used to standardize reads within a sample by converting raw read counts into relative read abundances (RRA). To further eliminate read noise, all species with a relative read

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Table 2. Percentage of occurrence (%) of the amphibian species found in the assessed ponds for each country: Spain (SP), Switzerland (CH), Belgium (BE), Germany (DE), Denmark (DK), Turkey (TK) and the United Kingdom (UK), and in all countries together (Global). Also, if the species is included in the Annex II and/or the Annex IV of the European Habitats directive.

Species	SP	CH	BE	DE	DK	TK	UK	Global	Annex II	Annex IV
Anura										
Alytes obstetricans	26.67							3.98		☒
Bombina bombina				17.86	10.34			3.98	☒	☒
Bufo bufo		51.72	66.67	17.86	27.59		29.63	27.86		
Bufo spinosus	36.67							5.47		
Bufotes viridis						25.00		3.48		☒
Discoglossus pictus	33.33							4.98		☒
Epidalea calamita	16.67	6.90						3.48		☒
Hyla arborea			10.00	10.71				2.99		☒
Hyla orientalis						25.00		3.48		
Hyla meridionalis	63.33							9.45		☒
Pelobates cultripes	43.33							6.47		☒
Pelobates fuscus						21.43		2.99		☒
Pelobates syriacus				64.29				8.96		☒
Pelodytes punctatus	20.00							2.99		
Pelophylax bedriagae		20.69	3.33			92.86		16.42		
Pelophylax lessonae			16.67	17.86	51.72			12.44		☒
Pelophylax perezi	46.67							6.97		
Pelophylax ridibundus		72.41	23.33					13.93		
Rana arvalis			3.33		10.34			1.99		☒
Rana dalmatina		55.17		3.57	6.90			9.45		☒
Rana temporaria	3.33	37.93	70.00		24.14		40.74	25.37		
Caudata										
Ichthyosaura alpestris		55.17	76.67	3.57				19.90		
Lissotriton helveticus	50.00	31.03	40.00	7.14				18.91		
Lissotriton vulgaris		3.45	80.00	42.86	89.66		77.78	41.79		
Ommatotriton vittatus						32.14		4.48		
Salamandra salamandra	33.33							4.98		
Triturus carnifex		37.93						5.47	☒	☒
Triturus cristatus		41.38	16.67	28.57	65.52		62.96	30.35	☒	☒
Triturus dobrogicus		3.45				53.57		7.96	☒	
Triturus marmoratus	73.33							10.95		☒

abundance of ≤ 0.001% within a sample were set to zero, and the average RRA per species across the three technical replicates was calculated.

Variable selection and analyses

To assess the effect of climatic, land use, and local abiotic and biotic variables on amphibian richness we used boosted regression trees (BRTs). Unlike conventional regression methods, BRTs combine numerous basic decision trees to enhance predictive performance. BRTs can handle complex relationships and interactions among predictors, and it is considered a robust technique that can control for outliers and nonlinearity (Elith et al. 2008, Parravicini et al. 2013). BRTs are also unaffected by multi-collinearity (Kotsiantis 2013). However, for informative reasons, collinearity among predictors for each pond was assessed by conducting a Pearson correlation matrix using the *rcorr* function of the 'Hmisc' package (Harrell 2023) in R (www.r-project.org), and can be found in the Supporting information (Fig. 1). The significance of the critical correlation coefficient was calculated by using the R function *qnorm* as $\text{qnorm}(1-\alpha)/\sqrt{(n-3)}$, where α is the significance level (0.05) and n is the number of observations (201).

BRTs were implemented using the 'gbm' package (Ridgeway 2007) in R, with additional functions outlined in Elith et al. (2008). This methodology requires four main parameters to be specified to properly calibrate the model: 1) bag fraction (bf), which denotes the proportion of input data used at each step while fitting the model to be used for testing; 2) learning rate (lr), which quantifies the contribution of each individual decision tree to the final model; 3) tree complexity (tc), which defines the number of nodes in each fitted tree, thereby determining the level of fitted statistical interactions between predictors, and 4) total number of trees (nt), the number of single decision trees on which the BRT model is built. After testing for sensitivity, we set bf to 0.75, lr to 0.001, and tc to 3 and used a Poisson error distribution. The final number of trees in the model was determined through a stepwise procedure implemented in the *gbm.step* function (Elith et al. 2008) in R, which yielded an optimal number of 4900 trees. The final model performance was assessed using the percentage of explained deviance, which was calculated as 1 – (residual deviance/total deviance) (Mouchet et al. 2015). A simplified BRT model was also calculated through the *gbm.simplify* function (Elith et al. 2008) to assess the presence of redundant

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predictors that could be degrading the performance of the model by increasing variance.

To interpret the results, the relative influence of each explanatory variable in the BRT model was assessed as the number of times a certain variable was selected for splitting a single tree, weighted by the squared improvements in error risk, and then averaged over all trees. Each relative importance was then standardized such that the sum added up to 100, so the contribution of variables to the model fit was expressed as a percentage and ranked to identify the most influential predictors explaining the highest amount of species richness variability. Subsequently, the relationship between amphibian species richness and each predictor was assessed through partial dependence plots, which show the effect of a predictor on the fitted function and fitted values of the response variable along its data range while accounting for the average effects of all other predictors in the model. Fitted functions represent the contribution of each predictor to the overall model prediction, with values on the y-axis being the contribution (or marginal effect) of that variable to the final prediction. A fitted value is the final predicted value of the dependent variable considering the combined effects of all predictors and decision trees of the model (Elith et al. 2008, Mouchet et al. 2015). We also identified the most important interactions between each pair of predictors using the *gbm. interactions* function (Elith et al. 2008), which creates a linear model for each pair of predictors, while considering the effect of all the other variables. The residual variance (interaction values) of this model indicates the strength of the interaction fitted by the BRTs, with a value of zero indicating that no interaction effects are fitted, ranging up to a value of one for total interaction.

Results

In the entire set of assessed ponds (n = 201), thirty different amphibian species were detected (Table 2). The average number of species in each pond and its SD was 3.2 ± 1.8, with Spain possessing the highest average local richness (4.7 ± 2.1) and the United Kingdom the lowest (2.1 ± 1.1). Some species were restricted to a single country, being Spain the one with the highest count of unique species (9), followed by Turkey (5), Switzerland (1) and Germany (1). The most frequent species was *Lissotriton vulgaris*, found in 41.8% of all ponds across the studied countries, followed by *Triturus cristatus* (30.4%), *Bufo bufo* (27.9%) and *Rana temporaria* (25.4%). Most of the encountered species are listed in annex IV of the European Habitats Directive (1992), and four species are included in the annex II of the same directive: *Bombina bombina*, *Triturus carnifex*, *T. cristatus* and *T. dobrogicus* (Table 2). All the observed species are categorized as Least concern by the IUCN Red List (2023), except for *Bufo spinosus* (Not assessed) and *Pelobates cultripes* (Vulnerable).

The BRT model describing the patterns of amphibian species richness explained 56.5% of model deviance. Considering the relative influence of each explanatory variable in the model, amphibian species richness was best predicted by local biotic variables (34.8%) (Fig. 2): chlorophyll-a concentration (23.2%), fish species richness (5.6%), and emersed vegetation within the pond perimeter (3.8%). The fraction of open water in the pond (1.4%) and emersed vegetation percentage inside the pond were less important (0.7%). Altogether, climatic variables had a similar importance to local biotic variables (32.2%): annual mean precipitation (14.8%), annual mean temperature (12.8%), aridity index (2.8%) and precipitation seasonality (1.7%). Local abiotic variables also played an important role (29.1%): pond area (8.4%), pond depth (7.6%), total nitrogen (6.7%) and total phosphorus (2.8%), water turbidity (3.4%), and hydroperiod (0.2%). Once local abiotic factors are accounted for, land use variables assessed in the present study had a low influence in the amphibian richness in the BRT model (3.9%): urban land use (1.8%), location of pond in a protected area (1.7%), arable land use (0.3%), and presence of nearby roads (0.1%). The simplified version of the model (i.e. without redundant predictors) had a performance of 45.3% and showed that amphibian richness was best explained by chlorophyll-a concentration (27.5%), annual mean precipitation (27.0%), annual mean temperature (19.5%), pond area (13.1%) and pond depth (12.9%), reaffirming the importance of both local pond characteristics and climatic variables on amphibian species richness.

Partial dependence plots and fitted values for the eight most influencing variables (Fig. 3) show that the highest amphibian species richness was observed in less nutrient-rich ponds (low concentrations of chlorophyll-a and nutrients) in warmer and rainier regions, as well as in relatively shallow (less than 1.5 m deep) and intermediate sized (200–2500 m^2) ponds, fishless, and with a high vegetation cover in pond shoreline areas. These ponds were located at latitudes between 41° and 43° (Spain) and longitudes between 3° and 6° (Spain, Switzerland, and Denmark). Ponds located in protected areas also show a slightly higher amphibian species richness. Data points for the fitted values of all the considered variables can be found in the Supporting information (Fig. 2). Interactions among predictors were found, the strongest of which were observed between annual mean temperature and annual mean precipitation (interaction value of 0.46), as well as pond depth and chlorophyll-a concentration (interaction value of 0.33). Interactions that present an interaction value higher than 0.10 are detailed in the Supporting information.

Discussion

We conducted a comprehensive study to evaluate the relative effect of multiple climatic, land use, and local abiotic and biotic factors on amphibian species richness in European ponds. In large scale studies, it is well-known that amphibian species richness is related to the latitudinal range, as climate predictors such as water availability and temperature are important range-limiting factors for these cold-blooded and highly moisture dependent animals (Buckley and Jetz 2007). This relationship can be observed in our results as climatic

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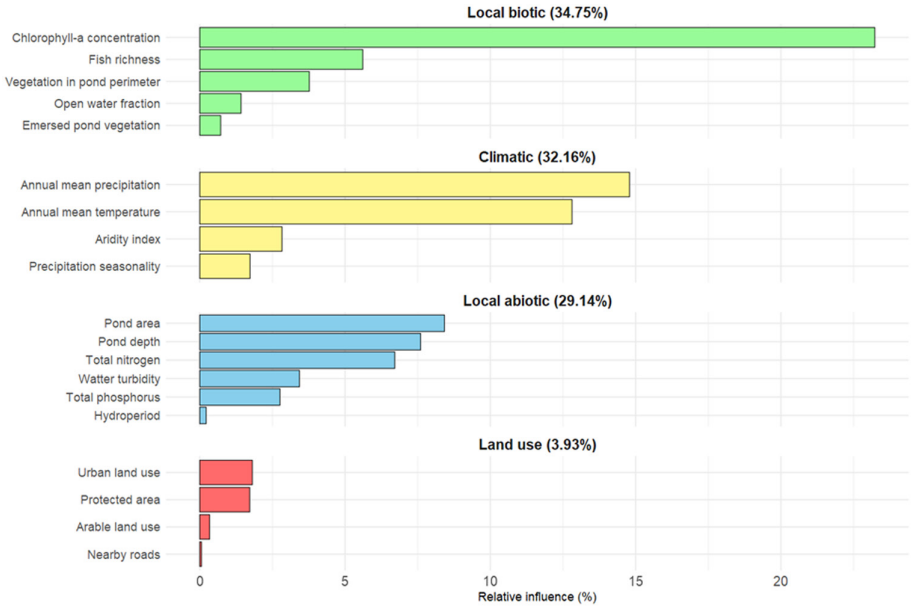


Figure 2. Relative influence of each explanatory variable in the BRT model of amphibian species richness in the 201 study ponds. All studied variables are explained in detail in Table 1.

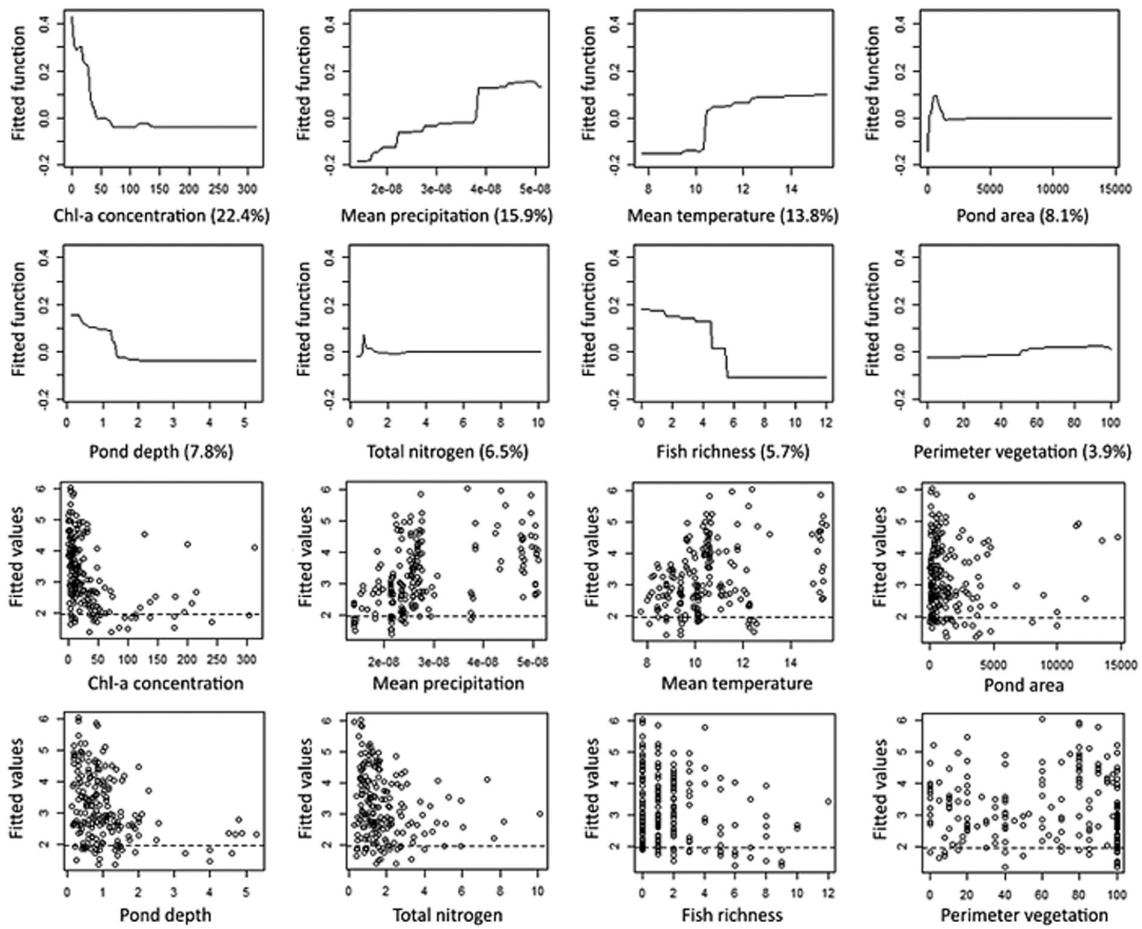


Figure 3. Partial dependence plots of the fitted function, and scatterplot of richness data for the fitted values against the eight most influential of the studied local, climatic, and land use variables considered, for the BRT model of the amphibian species richness.

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factors explain a high proportion of the variation in amphibian species richness. However, beyond this general large scale latitudinal gradient, our study provides evidence supporting that local pond characteristics, particularly chlorophyll-a concentration, but also pond area and depth, are of key importance when predicting amphibian species richness in ponds.

The observation that climatic factors affected amphibian species richness aligns with general amphibian distribution models in Europe (Araújo et al. 2006, Tsianou and Kallimanis 2019) and corroborates previous studies on ponds albeit at the within-country scale (Rosset et al. 2010, Trumbo et al. 2012). Indeed, the climatic attributes of a pond control their seasonal temperature and precipitation patterns (Eris and Ağiralioğlu 2009, Aron et al. 2023). European amphibian species richness follows a north–south gradient, shaped by historical climate stability during the Quaternary (Araújo et al. 2008) and currently influenced by contemporary rainfall and temperature drivers (Mouchet et al. 2015). These macroclimatic conditions determine water availability, a critical factor for the survival of amphibian eggs and larvae in Europe (Araújo et al. 2006, Buckley and Jetz 2007). However, despite the strong influence of climatic drivers, we observed that local abiotic and biotic pond characteristics had a key influence on amphibian species richness in our assessed ponds.

The most important predictor explaining the variation of amphibian species richness was chlorophyll-a concentration. At low concentrations, even a small increase in chlorophyll-a concentration had a strong negative impact on amphibian species richness. However, beyond 50 mg l^{-1}, further increases in chlorophyll-a had little additional effect, hinting a possible ecological threshold for European pond amphibians in this regard (Denoël and Ficetola 2007). Chlorophyll-a concentration is a key proxy for a eutrophication development, which is caused by high concentrations of nitrogen and phosphorus (Knutson et al. 2004, Johnson et al. 2007, Schmutzer et al. 2008). Pond eutrophication can decrease amphibian species richness by the reducing egg and larval survival rates (Knutson et al. 2004), diminished reproductive success (Schmutzer et al. 2008), and by increasing susceptibility to pathogenic infections (Johnson et al. 2007). Eutrophication is a common problem in ponds located in the vicinity of agricultural land, exposed to increased levels of artificial and natural fertilizers (Houlbrooke et al. 2004, Nyenje et al. 2010, Merz et al. 2023) and intense cattle disturbance (Schmutzer et al. 2008, Howell et al. 2019). Accordingly, management options aimed at increasing amphibian species richness for ponds would do well to focus on implementing measures to reduce nutrient load (Koskiaho et al. 2003, Zhou and Boyd 2015) and livestock management aimed at restricting or reducing cattle access and achieving appropriate stock levels (Schmutzer et al. 2008).

Pond area and depth were also key drivers of amphibian richness. Fitted values show that the highest richness was observed at intermediate-sized and shallow ponds. This pattern may be due to smaller and shallower ponds having a higher probability of drying, with larger and deeper ponds being more prone to fish colonization and persistence of fish populations (Semlitsch et al. 2015). These mechanisms can drive the peak of amphibian richness at intermediate values of pond size (Knutson et al. 2004, Semlitsch et al. 2015). The increase in fish species richness emerged as a significant driver of amphibian diversity decline, with our results showing a pronounced effect when three or more fish species were present in a pond, as a higher fish richness may contain generalist or even invasive species that pose larger risk to amphibians. Predatory fish are generally acknowledged as a driver of reduced amphibian species richness (Hecnar and M'Closkey 1997, Kats and Ferrer 2003, Burrow and Maerz 2022). Amphibian eggs and tadpoles, as well as adults, are notably vulnerable to predation by multiple fish taxa found in our study (i.e. *Carassius gibelio.*, *Lepomis* spp., *Esox lucius* and *Gambusia affinis*) (Hecnar and M'Closkey 1997, Kats and Ferrer 2003, Ruppert et al. 2017). Beyond direct predation, fish can also indirectly affect amphibian populations. For example, *Cyprinus carpio* can increase water turbidity and promote eutrophication by reducing zooplankton abundance and thus the grazing pressure on phytoplankton (Jeppesen et al. 2012, Kajgrová et al. 2022). Moreover, fish can compete with amphibians for vital food resources, potentially leading to competitive exclusion (Hecnar and M'Closkey 1997, Kačergytė et al. 2021), while fish introductions to ponds can serve as disease vectors, as they can carry pathogenic fungi, thus further exacerbating egg mortalities (Herczeg et al. 2021). In terms of management, ponds without naturally occurring fish should be kept fishless if the aim is to maximize amphibian abundance, richness, and diversity (Semlitsch et al. 2015). Nonetheless, it needs to be recognised that some European fish species (e.g. *Carassius carassius*) are themselves threatened and it is important to recognise this in the management decision-making process (Sayer et al. 2020).

Although pond vegetation can interact with the amphibian community via the provision of structural and functional resources, it only explained a very small amount of variation in amphibian species richness in our study. This observation contrasts with other studies that show that plant cover is a reliable predictor of amphibian richness in ponds (Santi et al. 2010, da Silva et al. 2012, Burrow and Maerz 2022). Similarly, the direct land use factors considered in our study did not exert a strong influence on amphibian species richness. Our results rather point to the importance of climatic and selected local environmental conditions (both biotic and abiotic).

We acknowledge that our study considers contemporary climatic drivers of amphibian species richness but does not include historical climate variables. Previous research has shown that paleoclimatic processes have had a major impact on current amphibian species richness gradients (Araújo et al. 2008). Thus, future studies integrating paleoclimatic data could provide a more comprehensive understanding of how historical and contemporary processes interact to shape amphibian diversity. We also acknowledge that our analysis relies only on data collected during one time period (i.e.

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sampling period) and, while we cover a broad range of potential drivers, it does not take into consideration all potential drivers of pond amphibian richness, such as macroinvertebrate densities, the effect of canopy cover, or connectivity between ponds (Semlitsch et al. 2015). Future studies should also consider evaluating fish abundance alongside fish species richness as a driver of amphibian richness. We also acknowledge the intrinsic limitations of field studies to establish clear cause and effect relationships (Mouchet et al. 2015). Yet, the use of BRTs is reliable in this context of complex data structure (Elith et al. 2008), and our results provide a clear pattern on how multiple pond characteristics play out in explaining the amphibian species richness of pond ecosystems on a large geographical scale. This study can feed directly into developing European-wide conservation initiatives (e.g. European Pond Conservation Network) and importantly provides information relevant to local-scale pond projects allowing stakeholders and decision makers to undertake better informed amphibian conservation actions (Grant et al. 2020).

In conclusion, our results emphasize the importance of local biotic and abiotic factors, especially chlorophyll-a concentration and pond size, in shaping richness patterns for European amphibians. These potential drivers of amphibian richness should be considered in the planning and implementation of amphibian conservation and management actions. Our results also highlight the value of using eDNA metabarcoding to assess amphibian richness.

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.hdr7sqvtq (Lopez et al. 2024).

Supporting information

The Supporting information associated with this article is available with the online version.

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