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INVADING NON-NATIVE POPULATIONS REPLACE NATIVE ONES OF THE ENDANGERED FRESHWATER SNAIL *THEODOXUS FLUVIATILIS* IN THE RIVER RHINE

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ABSTRACT

In the past, the freshwater snail *Theodoxus fluviatilis* was abundant in the river Rhine, but was considered to be extinct in the Upper and High Rhine in 2004. We found individuals of *T. fluviatilis* for the first time at sites in the southern part of the Upper Rhine and High Rhine in 2020, where this species went extinct 16 years ago. There are several distinct haplotypes of this species; some of them occurring in geographically separated regions of Europe. We used mitochondrial DNA (cytochrome c oxidase subunit I, COI) to trace the origin of the newly arrived *T. fluviatilis*. We examined 269 individuals collected at 29 sites and compared their sequence data with known haplotypes of *T. fluviatilis* and other species of *Theodoxus* deposited in GenBank. We also analysed a historical sample from 1995 (collected before the species went extinct), which revealed that the haplotype of the native *T. fluviatilis* in the High Rhine at that time was F28. In the recent samples, however, we found *T. fluviatilis* with the haplotypes F31 and “euxinus”, which are reported in the Ponto-Caspian region. Both haplotypes are cryptic invaders, colonizing the Rhine via the Rhine–Main–Danube canal and adversely affecting the remaining native populations of *T. fluviatilis* in Western Europe. Our findings are important for this species management by nature conservation authorities: lineages of endangered species should be preserved and supported, while the spreading of invasive, non-native lineages (or species) should be prevented.

Keywords: biological invasion; COI-sequencing; conservation; cryptic invader; non-native haplotype; Ponto-Caspian origin; tracing non-native species

Introduction

The world’s freshwater ecosystems are significantly affected by biological invasions, with non-native taxa nowadays making up a large fraction of the species, individuals and biomass (Haas et al. 2002; Ojaveer et al. 2002; Strayer 2010). However, not all invasive taxa are easily identifiable. Cryptic invasions are defined as “the occurrence of a species or genotype that was not previously recognized as alien in origin or not distinguished from other aliens” (Novak 2011). There are two basic types of cryptic invasions. Firstly, *interspecific cryptic invasion* refers to the invasion of a non-native species that goes unnoticed due to misidentification as a native or another non-indigenous species, with which it is often closely related (Morais and Reichard 2018). Secondly, *intraspecific cryptic invasion* refers to the invasion of another lineage of a species into a region where a distinct native lineage of the same species already exists. Cryptic invasions form a minor part of current research on biological invasions, despite their potential to influence native species and affect native communities to a similar extent as standard biological invasions (Morais and Reichard 2018).

Molecular markers and mitochondrial DNA sequence data have frequently been used to trace the origin and spread of cryptic invaders in freshwater bodies (Morais and Reichard 2018). Examples of intraspecific invasions are reported in several lakes and rivers in Africa by non-native genotypes of the freshwater gastropod *Melanoides tuberculata* from Asia (Van Bocxlaer et al. 2015), in Western and Central Europe by non-native races of the freshwater snail *Gyraulus parvus* from North America (Lorenková et al. 2021) and the Atlantic by distinct haplotypes of the hydrozoan *Gonionemus vertens* from the Pacific (Govindarajan and Carman 2016).

Theodoxus fluviatilis (Linnaeus, 1758) is a neritid freshwater snail with a distribution extending from Western Russia to Iberia and from southern Scandinavia to the Balkan peninsula and north Africa (Morocco; Zettler et al. 2004). Individuals attach themselves to a hard substrate, such as rocks, cobbles or other pieces of stone, in calcium-rich waters (Fretter and Graham 1962). This species is dioecious with a life cycle lasting 2–3 years (Kirkegaard 2006). Up to 100 eggs are laid in yellowish spherical capsules, which are attached to any hard substrate (Fretter and Graham 1962). Bunje (2005)

reports the geographical distribution of genetic lineages of *T. fluviatilis* in Europe. Sequence analysis of mitochondrial cytochrome c oxidase subunit I (COI) reveal that this snail has geographically nested clades displaying a distribution consistent with fragmentation and restricted dispersal. Certain regions in Europe harbour distinct native haplotypes. For example, the haplotypes F01 and F03 occur in central and northern Germany, while the haplotype F28 is only recorded at two sites in western France (Bunje 2005).

In the river Rhine and its tributaries, *T. fluviatilis* was the only abundant species of neritid until 1920 (Zettler 2008). In the following decades, its abundance decreased dramatically, most probably due to severe water pollution, resulting in the near extinction in the 1970s (Meinert and Kinzelbach 1985). During the period 1970–1986, numerous wastewater treatment plants were constructed along the river, resulting in improvements in water quality (Baur and Schmidlin 2007). Populations of *T. fluviatilis* partly recovered in the Upper and High Rhine (Kinzelbach 1987). However, another dramatic decrease occurred around the year 2000. In an attempt to preserve the native *T. fluviatilis* in Switzerland, a founder sample was translocated from the High Rhine into Lake Zurich in 1997 (Müller and Steinmann 2008). It is claimed that *T. fluviatilis* was extinct in the High Rhine and the southern part of the Upper Rhine by 2004 (Rey et al. 2005). In Switzerland, *T. fluviatilis* is included on the Red List as critically endangered (Rüetschi et al. 2012), while it is highly threatened in Germany (Jungbluth and Knorre 2011). Nature conservation authorities are obliged to preserve and support populations of species on Red Lists. In contrast, in cases of invasive, non-native species or lineages, nature conservation authorities are obliged to prevent their further spread.

In 2006, *T. fluviatilis* was refound in the Upper Rhine near the mouth of the river Main in Germany (Westermann et al. 2007). It was assumed it recolonized the Rhine via the Rhine–Main–Danube Canal, which was opened in 1992 (Westermann et al. 2007). Indeed, isolated populations of *T. fluviatilis* were first recorded in Germany in the Danube near Regensburg in 2005, which rapidly spread in succeeding years (Hirschfelder et al. 2011). Genetic analysis revealed that the haplotype in these individuals is F31; a haplotype described from Ukraine near the Black Sea and, at that time, not yet recorded in rivers in Western Europe (Bunje 2005; Hirschfelder et al. 2011). Gergs et al. (2015) confirm the presence of haplotype F31 in all individuals examined in the river Rhine between Cologne and Karlsruhe in 2010–2013 and claim that the re-established *T. fluviatilis* populations that consist exclusively of the non-native haplotype F31 are intraspecific cryptic invaders in the river Rhine. In 2020, individuals of *Theodoxus* were recorded in both the southern part of the Upper Rhine and the High Rhine at sites where the native *T. fluviatilis* went extinct 16 years ago (present study).

We aimed to determine the source of the newly established *Theodoxus* populations in the southern part of the Upper Rhine and High Rhine and discuss the consequences for the conservation of this species. We analysed the mitochondrial DNA-sequence of the cytochrome c oxidase subunit I gene in each individual and compared the molecular sequences with those of individuals sampled at one site in the High Rhine before its assumed extinction and with other haplotypes of *T. fluviatilis* deposited in the NCBI/GenBank (www.ncbi.nlm.nih.gov) and the COI sequences of other species of *Theodoxus* (*T. danubialis* (C. Pfeiffer, 1828), *T. danasteri* (Lindholm, 1908) and *T. velox* V. Anistratenko, 1999) occurring in the Ponto-Caspian region (Sands et al. 2019, 2020). We also aimed to examine the wider geographical distribution of the native haplotype of *T. fluviatilis* formerly occurring in the High Rhine, which is unknown. In particular, we addressed the following questions: (1) Which haplotypes are present in the newly established populations of *T. fluviatilis* in the High Rhine and southern part of the Upper Rhine? (2) Which haplotype was present in the original, native population (now considered extinct) in the Upper Rhine and what is the distribution of this haplotype? (3) Was it possible to preserve *T. fluviatilis* with the native haplotype from the High Rhine by translocating it into Lake Zurich? and (4) Can individuals of *T. fluviatilis* with different haplotypes be visually distinguished by differences in shell patterning?

Material and Methods

Sampling and sites

Individuals of *T. fluviatilis* were obtained from 29 sites in Switzerland, France and Germany (Table 1, Fig. 1). The High Rhine emerges from Lake Constance, flows west, mainly on the border of Germany and Switzerland (Fig. 1A). In Basel, it flows to the north (now called Upper Rhine) and forms the southern part of the border between France and Germany. Then the Rhine merges with one of its main tributaries, the river Main. Cargo shipping on the Rhine is possible from Rotterdam (North Sea) to Rheinfelden, 20 km upstream of Basel (Baur and Schmidlin 2007). The Rhine–Main–Danube Canal connects the Rhine via the Danube with the Black Sea which, in turn, is connected by canals and rivers to the Caspian Sea. The Altrhein (site 22) is a remnant of the former Upper Rhine north of Basel running parallel to the Grand Canal d'Alsace. The Altrhein has a close to natural river bed and is fed with water from the Upper Rhine with a minimum discharge of 50 m³ per second (Wirth et al. 2010). However, when the flow of the Upper Rhine exceeds 1,400 m³ per second, surplus water is released through the Altrhein, thereby causing great fluctuations in discharge and water level.

Samples were hand collected in shallow water, except at sites 8 and 29, where SCUBA-divers collected

snails at a depth of 1–4 m. Individuals were preserved in 80%-ethanol before being photographed and utilized for DNA-extraction. We determined the geographical coordinates of the sites sampled using a GPS receiver and topographical maps (scale 1 : 25,000). For each individual sampled at sites 22 and 24, we recorded on which stone it was attached. In combination with the COI-sequences, this allowed an examination of the small-scale spatial distribution of different haplotypes (whether they occur separately on different stones or co-occur on stones).

Translocation of *T. fluviatilis* into Lake Zurich

In an attempt to preserve *T. fluviatilis* in Switzerland, 100–200 individuals were translocated from the High Rhine into Lake Zurich in October 1997 (Müller and Steinmann 2008). In the first years, the translocated native snail population grew exponentially partly with the aid of further translocations, both within the lake and the rivers Limmat and Sihl, with this species extending its distribution over almost the entire Lake Zurich (Müller 2016). After 2015, however, the populations declined and *T. fluviatilis* was rare in Lake Zurich in 2020 (P. Müller, unpubl. data). To examine whether the original haplotype of *T. fluviatilis* that is assumed to have become extinct in the High Rhine is still present in Lake Zurich, we determined the haplotypes of the individuals in the translocated population collected at various sites in 2013 and 2020 (Table 1).

DNA extraction, amplification and sequencing

We isolated total genomic DNA from each individual using a slightly modified CTAB-method (Doyle and Doyle 1987). DNA quantity and quality were measured using a NanoDrop (NanoDrop Technologies Inc., Wilmington, USA) and standardized for genetic analyses. A partial sequence of the mitochondrial COI gene (600 bp) was amplified by polymerase chain reaction (PCR) using the primers F4d (5'-TACTTTTRTATATATGTTTGGT-3') and R1d (5'-TGRTAWARAATDGGRTCWCCHCCVCC-3'; Bunje 2005). PCR reactions (40 µl) consisted of 10 µl of template DNA (20–25 ng/µl), 8 µl Master Mix (5x FIREPOL Master Mix, Solis BioDyne, Estonia), 1 µl of each primer (10 µM) and 20 µl sterile water. Amplification was done in an Eppendorf Mastercycler Pro (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland) under the following conditions: initial 10 min. heat activation step at 95 °C, followed by 36 amplification cycles of denaturation at 95 °C for 50 seconds, annealing at 54 °C for 60 seconds and extension at 72 °C for 60 seconds, with a final extension step at 72 °C for 7 min. PCR products were cleaned-up using the NucleoSpin gDNA Clean-up kit (Macherey-Nagel, Oensingen, Switzerland) and sequenced by Macrogen Inc. (Amsterdam, The Netherlands) using a capillary ABI 3730 XL DNA Analyser (Applied Biosystems, Foster City, CA, USA).

The systematics and distribution of *Theodoxus* in the Ponto-Caspian region, the presumed source of the invaders, are not yet entirely resolved (Anistratenko et al. 2020). For example, recent molecular studies support the synonymy of *T. fluviatilis* and *Theodoxus euxinus* (Clessin, 1886) (Bunje and Lindberg 2007; Sands et al. 2019, 2020), while in the DNA-barcoding system *T. euxinus* is still considered as a distinct species (Barcode of Life Data System 2021). In our study, we considered the various haplotypes of *T. fluviatilis* and other *Theodoxus* species described in the literature (Bunje 2005; Bunje and Lindberg 2007; Gergs et al. 2015; Sands et al. 2019, 2020). Given the uncertain status of *T. euxinus*, we refer to its haplotype as *T. fluviatilis* haplotype “euxinus”.

We edited all sequences using CodonCode Aligner software (Codon Code Corporation, Centerville, MA, USA). Using the BLASTn tool, the molecular sequences of the individuals online were aligned to the sequences of the haplotypes of *T. fluviatilis* deposited in the NCBI/GenBank (www.ncbi.nlm.nih.gov) as well as to those of other *Theodoxus* species occurring in the Ponto-Caspian region (*T. danubialis*, *T. danasteri*, *T. velox*; Sands et al. 2019, 2020). Molecular sequences with at least 99.5% similarity to a described haplotype were considered as belonging to this haplotype. In total, we sequenced the COI from 269 individuals.

The haplotypes F28 (accession no AY765331) and TR01 (accession no KJ493817) of *T. fluviatilis* deposited in NCBI/GenBank differ in 3 base pairs (bp) from each other. Only a single COI sequence is stored from both haplotypes in GenBank. Thus, so far no information exists on the COI sequence variation in these haplotypes.

Periostracum patterning

The periostracum is the thin outer layer of the snail shell, composed of conchiolin, a type of protein. The periostracum colouration and its pattern are highly variable in *T. fluviatilis* (Zettler et al. 2004). The periostracum can be ornamented with a pattern of white drop-like spots on a dark-brown, black or redish background. In other individuals the periostracum is ornamented with zigzag stripes, while still others exhibit different combinations of white drop-like spots and zigzag stripes (Zettler et al. 2004). We analyzed the periostracum patterning in a “historical” sample of *T. fluviatilis* collected in the High Rhine near Augst (CH) in August 1997 before its presumed local extinction ($n = 50$) and in two recent samples obtained from the Altrhein (site 22, $n = 28$, collected on 15 September 2020; Table 1) and from the High Rhine (site 24, $n = 28$, collected on 14 September 2020). We photographed the shells of the snails using a digital microscope Keyence VHX-6000 (Keyence Corporation, Osaka, Japan). We then assigned each snail based on its periostracum patterning to one of five categories defined by Hirschfelder et al. (2011): D = ornamented with white drop-like spots (Fig. 3A); Z = pattern of dark zigzag

stripes (Fig. 3B, C); DZ = a combination of D and Z (Fig. 3D); HL = a combination of horizontal and longitudinal stripes; and M = monochrome, no particular ornamental pattern (this category did not occur in our samples). A few snails could not be assigned to one of these categories due to an eroded periostracum. Molecular sequencing of

the individuals made it possible to assign the periostracum patterning to the various haplotypes of *T. fluviatilis*.

Data analyses

Phylogenetic reconstruction of DNA-sequences of *T. fluviatilis* found in the river Rhine was done using the

Table 1 Overview of the sites of *Theodoxus fluviatilis* sampled, numbers of individuals examined and assigned to each haplotype.

Site sampled (waterbody, country)	Coordinates (N / E)	Altitude (m a. s. l.)	Date sampled	Number of individuals examined	Haplotype (No. of individuals assigned to haplotype) ⁵
1 Muttenz, Schweizerhalle (river Rhine, CH)	47.53265 / 7.67507	261	15.02.1995	5	F28 (5)
2 Zurich Riesbach, Höschgasse (lake Zurich, CH) ¹	47.35591 / 8.54916	405	17.05.2013	6	F28 (6)
3 Zurich Riesbach (lake Zurich, CH) ¹	47.35868 / 8.54712	405	04.09.2013	30	F28 (17), TR01 (12), F03 (1)
4 Zurich near Mythenquai (lake Zurich, CH) ²	47.35539 / 8.53617	405	17.05.2013	5	F28 (5)
5 Küsnacht near Goldbach (lake Zurich, CH) ³	47.32886 / 8.57284	405	16.05.2013	6	F28 (6)
6 Zurich, Höhe Schipfe (river Limmat, CH) ⁴	47.37257 / 8.54184	404	16.05.2013	6	F28 (6)
7 Zurich, near Gessnerbrücke (river Sihl, CH)	47.37661 / 8.53597	405	01.11.2020	25	F28 (9), TR01 (11), nd ⁶ (5)
8 Plobsheim, Ancienne gravière (quarry pond, F)	48.47831 / 7.74250	149	07.06.2015	20	F01 (13), F03 (6), F31 (1)
9 Montbozon, Haute-Saône (river Ognon, F)	47.46338 / 6.26415	241	31.05.2017	3	F28 (3)
10 Cenans, Haute-Saône (river Ognon, F)	47.42856 / 6.19654	232	30.05.2017	3	F28 (3)
11 Parcey, Doubs (river Loue, F)	47.01531 / 5.49436	196	22.06.2017	3	F28 (3)
12 Rahon, Doubs (river Orain, F)	46.98552 / 5.44935	194	22.06.2017	3	F28 (3)
13 Labeaume, Ardèche (river Beaume, F)	44.44952 / 4.30137	118	04.09.2017	3	F28 (3)
14 St. Martin-d'Ardèche (river Ardèche, F)	44.29965 / 4.56853	42	05.09.2017	3	F28 (3)
15 Méjannes-le-Clap, Gard (river Cèze, F)	44.26133 / 4.35057	95	14.09.2017	3	F28 (3)
16 Montclus, Gard (river Cèze, F)	44.26304 / 4.41988	84	05.09.2017	3	F28 (3)
17 St. André-de-Roquepertuis, Gard (river Cèze, F)	44.24816 / 4.44963	78	07.09.2017	3	F28 (3)
18 Goudargues, Gard (river Cèze, F)	44.20915 / 4.46955	69	13.09.2017	3	F28 (3)
19 Goudargues, Gard (river Source de Goudargues, F)	44.21574 / 4.46927	81	07.09.2017	3	F28 (3)
20 Sauve, Gard (river Vidourle, F)	43.94603 / 3.94701	94	06.09.2017	3	F28 (3)
21 Collias, Gard (river Gardon, F)	43.95289 / 4.48176	27	12.09.2017	3	F28 (3)
22 Istein, Altrhein (river Rhine, Ger)	47.66416 / 7.52127	230	15.09.2020	36	F31 (9), "euxinus" (27)
23 Basel, St. Alban-Rheinweg (river Rhine, CH)	47.55529 / 7.59780	248	17.09.2020	5	F31 (2), "euxinus" (3)
24 Basel, Rheinhalde (river Rhine, CH)	47.56059 / 7.62088	248	14.09.2020	32	F31 (24), "euxinus" (8)
25 Muttenz, Fuchsloch (river Rhine, CH)	47.53736 / 7.66400	261	17.09.2020	6	F31 (6)
26 Muttenz, Schweizerhalle (river Rhine, CH)	47.53370 / 7.67150	261	10.08.2020	12	F28 (1), F31 (11)
27 Pratteln, Rheinlehne (river Rhine, CH)	47.53176 / 7.68315	261	10.08.2020	10	F31 (10)
28 Pratteln, Saline (river Rhine, CH)	47.53173 / 7.68435	261	10.08.2020	7	F31 (7)
29 Stein am Rhein, Schiffflände (river Rhine, CH)	47.65982 / 8.85686	396	16.05.2020	19	F28 (18), F31 (1)

¹ 100–200 individuals collected in the river Rhine near Augst (CH; 47.53958 N, 7.71823 E) on 11.10.1997 were released in Lake Zurich (47.35591 N, 8.54924 E) on 12.10.1997

² collected in lake Zurich (CH; Zurich Riesbach, Höschgasse, 47.35591 N, 8.54916 E), released at this site on 28.05.2008

³ collected in lake Zurich (CH; Zurich Riesbach, Höschgasse, 47.35591 N, 8.54916 E), released at this site on 03.06.2008

⁴ collected in lake Zurich (CH; Zurich Riesbach, Höschgasse, 47.35591 N, 8.54916 E), released at this site on 26.06.2008

⁵ COI-sequences were assigned to haplotypes deposited in GenBank using the BLASTn tool; GenBank accession no. F28 AY765331; TR01 KJ493817; F01 AY765306; F03 AY765308; F31 AY765336; "euxinus" AY771300

⁶ could not be determined

package *phangorn* (Schliep 2011) in the statistical software R (version 3.6.3; R Core Team 2020). A maximum likelihood (ML) analysis was conducted that included all unique haplotypes with the substitution model TPM3u + I following Gergs et al. (2015). By using the *pml* function of the package *phangorn*, the likelihood of a phylogenetic tree based on given sequence alignments was computed and optimized with the function *optim.pml*. Support for reconstructed branches of the ML tree was estimated by bootstrapping with 1,000 replicates.

We used Contingency-tests to compare frequency distributions of periostracum patterns between native *T. fluviatilis* ("historical" sample) and invading *T. fluviatilis* (haplotypes F31 and "euxinus") and between the frequencies of the haplotype F31 and haplotype "euxinus". For these analyses we pooled individuals of the haplotype F31 collected at sites 22 and 24 as well as individuals of the haplotype "euxinus" from these two sites.

Results

Haplotypes recorded

We found that 97 individuals of *T. fluviatilis* belong to the haplotype F28. Analysis of the COI-sequence indicat-

ed that 78 individuals had a base-pair sequence identical to the sequence of F28 deposited in GenBank, while the sequence of 12 individuals differed in 1 bp, that of two individuals in 2 bp, that of three individuals in 3 bp and that of two individuals in 4 bp from the sequence in GenBank. Using the Alignment Megablast procedure of GenBank (www.ncbi.nlm.nih.gov), a query cover of 99% was found between the haplotypes F28 and TR01 with an identity of 99.8 ± 0.2 (\pm SE, range in bp difference: 0–5, median = 2). The query cover between TR01 and F28 was 99% (identity: 99.5 ± 0.3 , range in bp difference: 0–3, median = 1). Based on the small variation in the molecular sequence, the newly described haplotype TR01 (Gergs et al. 2015) can be considered as almost identical to the formerly described haplotype F28 (Bunje 2005) and thus as the original haplotype of *T. fluviatilis* in the High Rhine. In contrast, F01, F03 and F31 are distinct haplotypes of *T. fluviatilis*, differing by more than 5 bp from the haplotypes F28 and TR01. Furthermore, the haplotype "euxinus" collected in our study has a distinct, unique COI-sequence (accession no AY771300), which clearly differs from the other haplotypes of *T. fluviatilis* (Bunje 2005; Gergs et al. 2015; Sands et al. 2019, 2020). The BLAST tool also revealed that none of the COI-sequences of the 269 individuals examined could be assigned to the

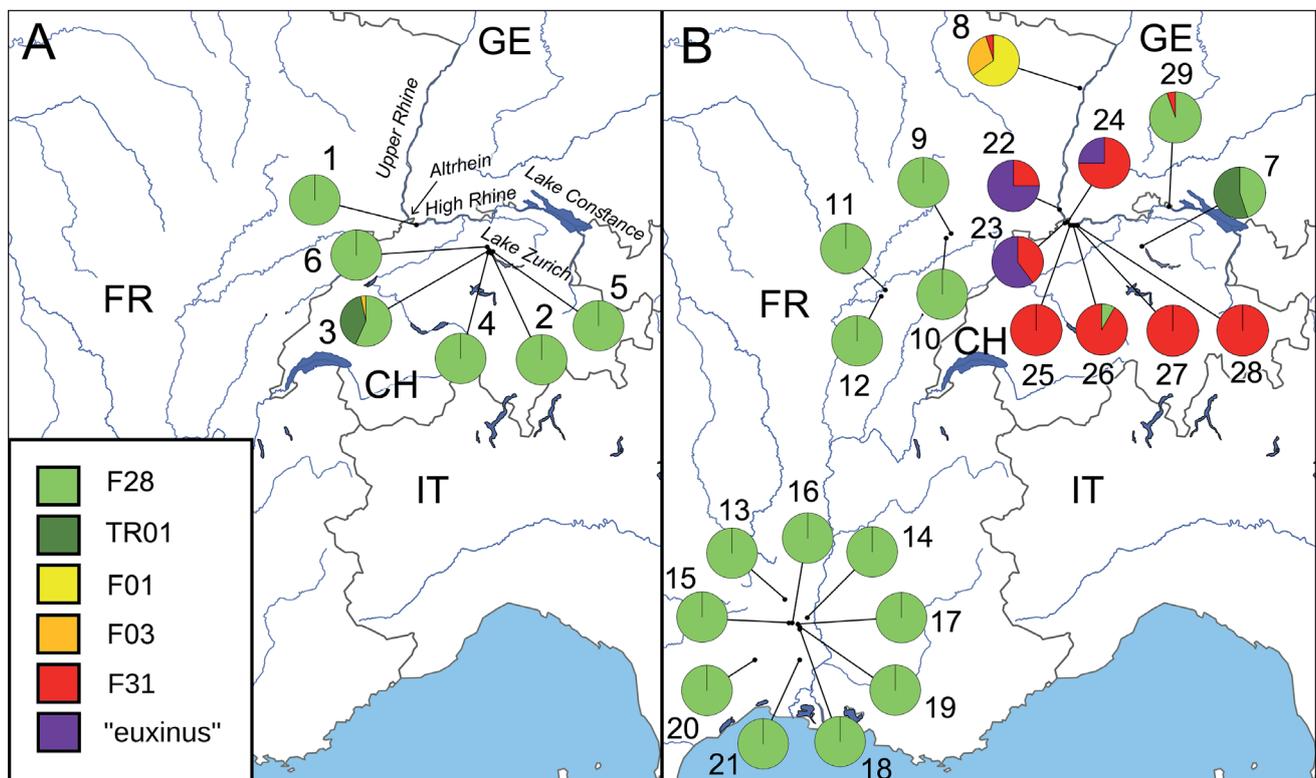


Fig. 1 Frequency distribution of *T. fluviatilis* haplotypes (in %) determined by COI-sequencing at each site sampled in this study. **A** Historical distribution of *T. fluviatilis* haplotypes at site 1 in 1995 (before the assumed extinction of the species in the High Rhine) and of populations translocated from this site into Lake Zurich in 1997 (analysed individuals were sampled in 2013). **B** Recent distribution of *T. fluviatilis* haplotypes. The originally native *T. fluviatilis* with haplotype F28 in the High Rhine was replaced by the invasive haplotypes F31 (sites 22–28) and "euxinus" (sites 22–24) in 2020. The native haplotype F28 can still be found in the High Rhine near Lake Constance (site 29), in several rivers in southwestern France (sites 9–21) and in the river Sihl near Lake Zurich (descendants of the translocated individuals). Site 8 (a quarry pond) harbours *T. fluviatilis* haplotypes found in central and northern Germany and the invasive haplotype F31. Sample sizes (number of individuals sequenced) and sampling dates are given in Table 1.

sequences of any other *Theodoxus* species deposited in GenBank.

Native haplotype in the High Rhine and its distribution

All individuals sampled in the High Rhine in 1995 before its presumed extinction 9 years later belonged to the native haplotype F28 (Table 1; site 1). Bunje (2005) found this haplotype at two sites in western France, but did not examine any specimens from the Upper Rhine or High Rhine. We extended our survey to rivers in southeastern France (e.g. to the river Ardèche and river Cèze; Table 1). All 39 individuals sequenced from sites 9–21 belonged to the haplotype F28, extending the so far known distribution of this haplotype considerably (Fig. 1). The majority of the descendents of individuals translocated from the High Rhine into Lake Zurich belonged to haplotype F28 (sites 2–6; Fig. 1). However, at site 3, the haplotypes F03 and TR01 were also recorded in Lake Zurich. The original haplotypes F28 and TR01 of *T. fluviatilis* from the High Rhine could still be detected in the river Sihl a few hundred metres from Lake Zurich (site 7) 23 years after their introduction into Lake Zurich.

Intraspecific cryptic invasion

COI-sequencing revealed that the newly established *T. fluviatilis* in the Upper and High Rhine belonged either to haplotype F31 or haplotype “euxinus”, indicating that these intraspecific invaders have now reached the upper sections of the Rhine (sites 22–29; Fig. 1). We detected the invasive haplotype F31 at low frequency also in a quarry 600 m from the Upper Rhine (site 8) and in the High Rhine where it emerges from Lake Constance (site 29; Fig. 1, Table 1). In contrast, individuals with haplotype “euxinus” were only recorded in sections of the Rhine downstreams of the dam of the hydroelectric power plant at Birsfelden near Basel (Fig. 1), indicating that individuals with this haplotype arrived later.

The ML analysis for phylogenetical reconstruction revealed that individuals of the invading haplotype F31 belong to the Danubian group with haplotypes F38 (originally found in Ukraine; Bunje 2005) and MT563453 (recorded as a newly arriving haplotype in the northern part of the Upper Rhine in 2018; Rothmeier et al. 2021) (Fig. 2). The invading haplotype “euxinus” is phylogenetically close to F30 (Fig. 2), a haplotype originally recorded in Ukraine (Bunje 2005). In contrast, F28, the native haplotype of *T. fluviatilis* in the High Rhine, is phylogenetically close to the haplotypes F22 and F23 (Fig. 2), both recorded in Northern Italy. The phylogenetical relationship of these three haplotypes can partly be explained by our new finding that the geographical range of haplotype F28 includes southeastern France (see above).

Periostracum patterning

Native *T. fluviatilis*, represented by a historical sample from 1997 at a site in the High Rhine, mainly had white drop-like spots on their shells (Figs 3A, 4), while shells of

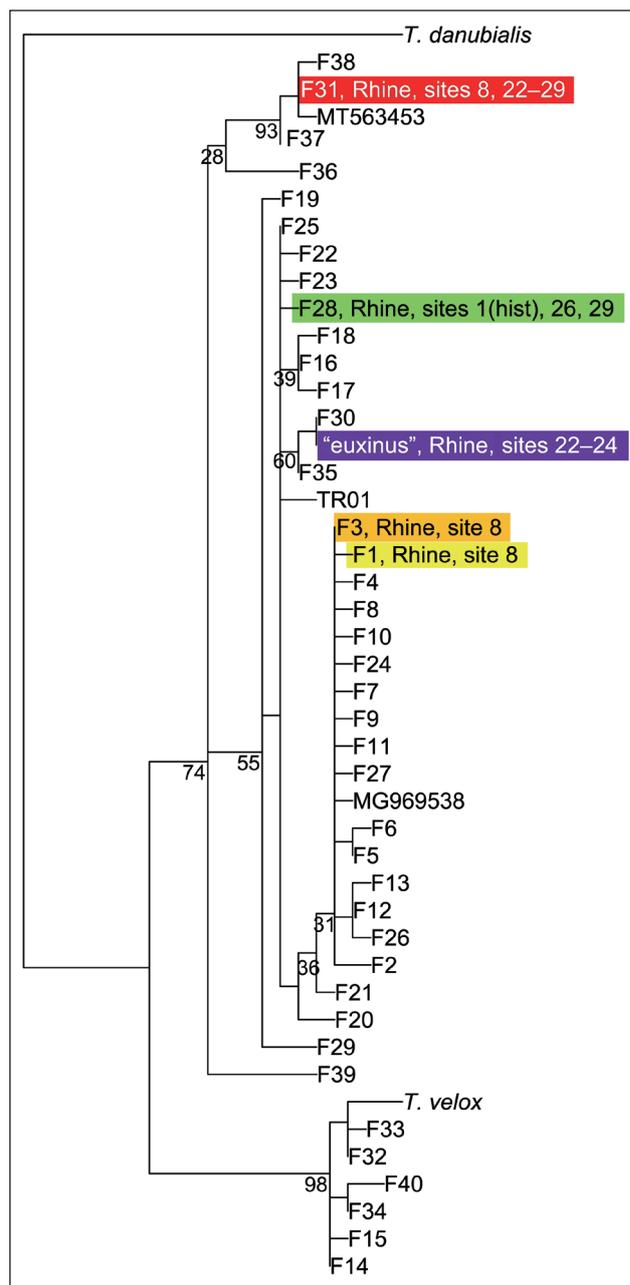


Fig. 2 Maximum likelihood tree of unique haplotypes of *T. fluviatilis* recorded in the river Rhine, rooted with the haplotype of *Theodoxus danubialis* as outgroup. Only bootstrap values ($n = 1,000$) of main branches are presented. See Table 1 for detailed information on the haplotypes found at the sites sampled. For further information on haplotypes and classification of groups see Bunje (2005), Gergs et al. (2015) and Rothmeier et al. (2021).

the invading *T. fluviatilis* (haplotypes F31 and “euxinus”) were characterized by dark zigzag stripes or a combination of zigzag stripes and drop-like spots (Figs 3B–D, 4). Individuals of both invading haplotypes differed significantly in periostracum patterning from native *T. fluviatilis* in the historical sample (Fig. 4A). However, invading individuals of the haplotypes F31 and “euxinus” did not differ in periostracum patterning (Fig. 4B).

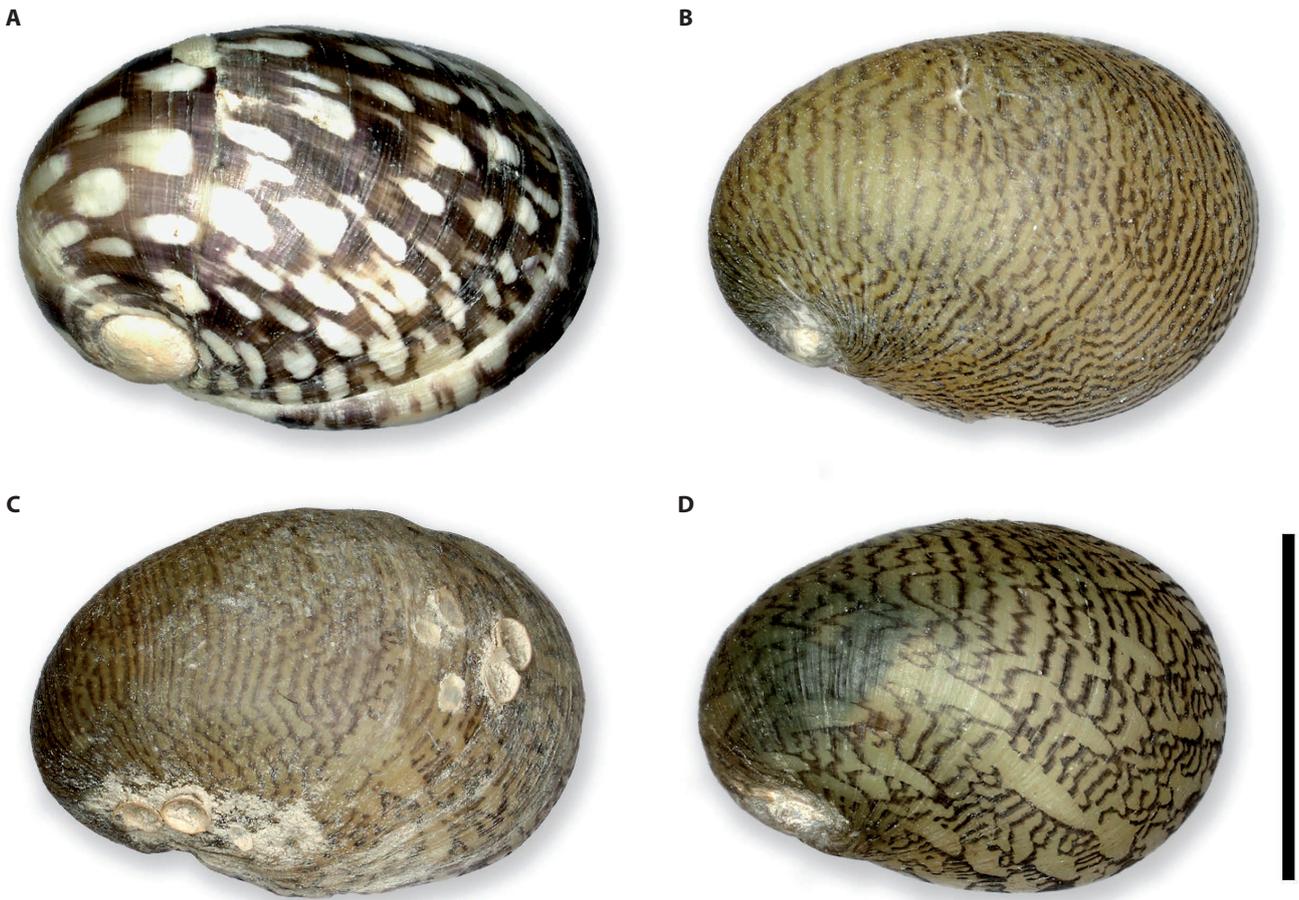


Fig. 3 Shells of *T. fluviatilis* from different sites. **A** Native *T. fluviatilis* of a historical sample from the High Rhine near Muttenz, CH (site 1; coll. P. Müller in August 1997). **B** Non-native haplotype “euxinus” from the Altrhein near Istein, GER (site 22; coll. B. Baur on 15.09.2020). **C** Non-native haplotype F31 from the High Rhine, Rheinhalde, Basel, CH (site 24; coll. B. Baur on 14.09.2020). **D** Non-native haplotype “euxinus” from the Altrhein near Istein, GER (site 22; coll. B. Baur on 15.09.2020). Scale bar indicates 5 mm.

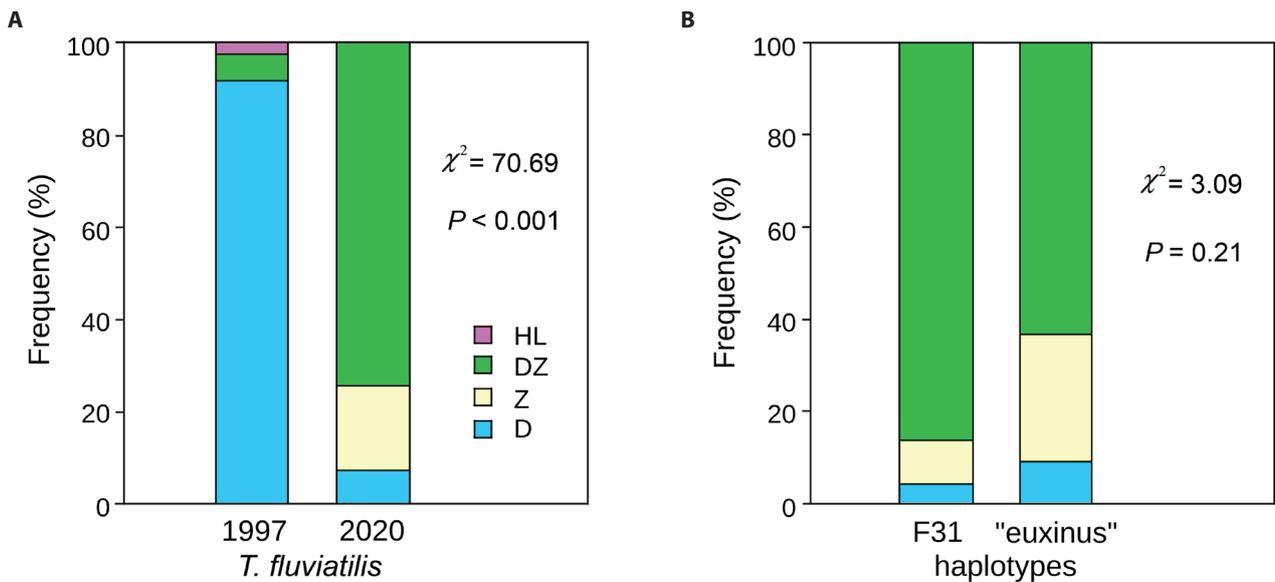


Fig. 4 Comparison of frequency distributions of periostracum patterning in *T. fluviatilis*. **A** Native *T. fluviatilis* from the High Rhine (collected in 1997 before the presumed local extinction of the species; $n = 50$) versus invading *T. fluviatilis* (haplotypes F31 and “euxinus” combined; $n = 44$) from the Upper Rhine and High Rhine in 2020. **B** Haplotype F31 (sites 22 and 24 combined; $n = 22$) versus haplotype “euxinus” (sites 22 and 24 combined; $n = 22$) from the Upper Rhine and High Rhine in 2020. D = white drop-like spots, Z = zigzag stripes, DZ = a combination of D and Z, and HL = a combination of horizontal and longitudinal stripes. The single individual with HL-type was not included in the statistical analysis.

Discussion

This study revealed that the newly established *T. fluviatilis* populations in the southern part of the Upper Rhine and High Rhine exhibit the non-native haplotypes F31 and “euxinus”. Both haplotypes can be considered as cryptic invaders (cf. Gergs et al. 2015) with a Ponto-Caspian origin. The detection of the haplotype “euxinus” at three sites is the first record of this haplotype in Western Europe (Germany and Switzerland). The correct recognition of cryptic invasions is crucial to understanding their effect on assemblages of native species and ecosystems. However, the intrinsic difficulty of identifying cryptic invaders is that they are, by definition, camouflaged and cannot be distinguished morphologically from native species already present or from known native species that may occur in other water bodies (Morais and Reichard 2018).

There is evidence from breeding experiments that periostracum patterning in *T. fluviatilis* can be influenced by environmental conditions such as water quality, type of food and mineral intake (Neumann 1959). However, the invading individuals (haplotypes F31 and “euxinus”) found in the Rhine show identical periostracum patterns to those reported in the Danube in Austria in 2001 (Schultz and Schultz 2001) and near Regensburg (Germany) in 2005 (Salewski and Hirschfelder 2006), indicating little influence of the environmental conditions of the two river systems. We showed that the periostracum patterns of the invading *T. fluviatilis* haplotypes F31 and “euxinus” did not differ from each other. Thus, visual differentiation between the two invaders is not possible. However, the periostracum patterns of both invading haplotypes differed from that of *T. fluviatilis* originally native to the High Rhine (F28), confirming the results of a similar comparison made by Hirschfelder et al. (2011) in the Danube near Regensburg (Germany). It should be pointed out that this finding relates to frequency distributions of periostracum patterning based on many shells. On the basis of its periostracum pattern an individual snail cannot be assigned to one of the two non-native haplotypes.

The diversity of haplotypes recorded in this study can hardly be compared with those reported in earlier studies (Bunje 2005, 2007; Bunje and Lindberg 2007; Gergs et al. 2015; Sands et al. 2019, 2020), because the latter focused on phylogeographical aspects and were frequently based on small numbers of individuals sampled per site; haplotype information is sometimes only available for one specimen per locality. Bunje (2005) demonstrated the presence of genetically distinct groups within *T. fluviatilis* that are separated by major geographical features (mountain chains, river systems) in Europe. However, the elimination of natural barriers between water sheds often triggers the invasion of non-native species, among them also cryptic invaders (Morais and Reichard 2018). The linear environment of rivers, streams and small

connected lakes allows non-native species to migrate along channelled fluvial paths, thereby increasing their distribution. The opening of the Rhine–Main–Danube Canal in 1992 increased the invasion rate of non-native vertebrate and invertebrate species into the Rhine drainage (Bij de Vaate et al. 2002; Baur and Schmidlin 2007; Leuven et al. 2009). It is assumed that transportation by cargo ships is responsible for the rapid spread of the invasive *T. fluviatilis* haplotype F31 from the Ponto-Caspian region to western Europe (Westermann et al. 2007; Gergs et al. 2015), as might be the case for the haplotype “euxinus”. Hull fouling on commercial ships and recreational boats and transport of gravel are considered as important vectors for the spread of non-native gastropods in aquatic habitats (Mineur et al. 2007). In the Danube, the cryptic invader *T. fluviatilis* (later determined as haplotype F31 from the Ponto-Caspian region) was first recorded in Austria in 2001 (Schultz and Schultz 2001), 2005 in Germany near Regensburg (Salewski and Hirschfelder 2006), 2006 in the northern part of the Upper Rhine near Mainz (Westermann et al. 2007), 2012 in Karlsruhe (Gergs et al. 2015) and reached the High Rhine in 2020 (present study). One may assume that the spread of this invasive snail is less rapid upstream of Basel, where cargo shipping stops, as has been shown for other non-native invertebrates (Schmidlin and Baur 2007; Schmidlin et al. 2012). However, our data demonstrate that *T. fluviatilis* (haplotype F31) has already colonized a locality close to Lake Constance (site 29). The spread of this non-native haplotype cannot exclusively be explained by transport by commercial ships. For example, we also recorded *T. fluviatilis* (haplotype F31) in a quarry pond 600 m apart from the Upper Rhine. Passive dispersal by waterbirds could also contribute to the spread of *T. fluviatilis* (Frisch et al. 2007).

The rediscovery of the native *T. fluviatilis* haplotype F28 at site 26 in the High Rhine was rather surprising. It was assumed that it went extinct in the High Rhine in 2004 (Rey et al. 2005; Westermann et al. 2007). Our results indicate that a few individuals survived in this section of the river or recolonized it later. Another surprise was the discovery of the native *T. fluviatilis* haplotype F28 at site 29, where the High Rhine emerges from Lake Constance. At this site, no neritid snail was found during 37 dives between 2010 and 2018, until the first record on 16 May 2020 (P. Steinmann, unpubl. data). Furthermore, no individuals of *T. fluviatilis* were observed during eight dives at nearby localities in Lake Constance and in the High Rhine in the previous decade (P. Steinmann, unpubl. data). Subsequent dives revealed that it had spread at site 29 over a distance of 320 m along the river bank of the High Rhine in summer 2020. We do not know how *T. fluviatilis* established this new population in the High Rhine. Most interestingly, however, one out of 29 individuals exhibited the non-native haplotype F31 from the Ponto-Caspian region. Thus, the frequency of the cryptic invader’s haplotype was still low in this population in

summer 2020. Future investigations will reveal whether the non-native haplotype F31 will become more abundant and displace the native haplotype F28 at this site.

In the northern part of the High Rhine the non-native haplotypes occupy broader ecological niches than the native haplotypes, persisting even in anthropogenically modified and disturbed habitats such as harbours (Rothmeier and Martens 2019). Non-native haplotypes of *T. fluviatilis* may also have a higher tolerance of the increase in water temperature in the river Rhine, as reported for other invading molluscs from warm regions (Müller and Baur 2011). Furthermore, *T. fluviatilis* individuals of haplotype F31 were less infested by the trematode *Plagioparus* cf. *skrjabini* (also an invader from the Caspian region) than individuals of the native haplotype (Rothmeier et al. 2021). The presence of both non-native parasites and non-native hosts (or hosts of non-native haplotypes) outside their natural range may have an effect on native biodiversity.

There is increasing evidence that introduced plant and animal taxa frequently hybridize with native taxa, leading to a growing concern that these hybridizations may compromise the genetic integrity of native taxa to the point of causing extinctions (Largiadier 2007). Hybridization events can only be demonstrated by analysing nuclear DNA. We sequenced mitochondrial DNA, which is maternally inherited. We are therefore not able to answer the question whether invading *T. fluviatilis* individuals of haplotypes F31 and “euxinus” hybridize with native individuals of haplotype F28.

Conclusions

In addition to water quality and quantity, invasive species are an important conservation challenge in freshwater habitats (Lysne et al. 2008). In the case of *T. fluviatilis* in the High Rhine, individuals with the native haplotype F28 are of conservation concern and recorded in the Red Lists of Germany and Switzerland (Jungbluth and Knorre 2011; Rüetschi et al. 2012). Nature conservation authorities are obliged to preserve and promote these populations. In contrast, individuals with the haplotypes F31 and “euxinus” are exotic invaders. In this case, nature conservation authorities are obliged to prevent the further spread of these lineages. Removing non-native species or strains from aquatic habitats is almost impossible. Re-introduction programmes with either the release of captive-propagated native snails and/or the translocation of eggs, juveniles and/or adults from viable populations aimed at restoring species or haplotypes in water bodies in their historical range, where they no longer exist, or to augment extant populations, are possible. Any re-introduction must be planned carefully because they are associated with risks such as reduction in genetic variability, introduction of disease infected individuals into the wild or the potential that habitat quality does not

match the actual needs of the introduced individuals (IUCN/SSC 2013). The endangered *Theodoxus prevostianus* (Pfeiffer, 1828) was successfully re-introduced into a spring in Hungary (Fehér et al. 2017). However, while the translocation of *T. fluviatilis* of the native haplotype F28 into Lake Zurich was very successful in the first few years, 20 years later, this translocation seems to have failed, for as yet unknown reasons. In the river Rhine, the two cryptic invaders replaced the native lineage of *T. fluviatilis* (original haplotype F28) at sites where it went extinct. Future research will show whether the invading individuals (haplotypes F31 and “euxinus”) outcompete the native lineage of *T. fluviatilis*, where it is still present.

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BREEDING MASON BEES FOR THE POLLINATION OF FRUIT ORCHARDS IN CENTRAL EUROPE

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ABSTRACT

Solitary bees pollinate wild plants and crops. In the temperate zone, they mainly pollinate fruit trees. Most species nest in the soil, but mason bees nest in wooden cavities above ground. Their presence in orchards can be augmented by provision of artificial nesting sites. Two species of mason bees frequently use these artificial nesting opportunities in Central Europe. The red mason bee (*Osmia bicornis*) is the most common species in this region, which preferentially collects pollen from oak trees. The European orchard bee (*Osmia cornuta*) prefers pollen from fruit trees, but it is more thermophilic. Its spread to higher altitudes can be expected in response to climate change. A breeding technology based on detachable wooden nests is summarized in this paper based on a literature review and experimental experience in fruit orchards in Czechia.

Keywords: artificial nesting sites; climate change; fruit orchards; mason bees; pollination

Introduction

Insect pollination supports the production of many agricultural commodities and solitary bees contribute significantly to this pollination service (Garibaldi et al. 2013; Pardo and Borges 2020). Their role is important, especially in fruit production (Mallinger and Gratton 2015; Blitzer et al. 2016). In England, the share of solitary bees in apple tree pollination is estimated at 54–58% (depending on apple variety). For honeybees, this percentage is only at 23–28% and for bumblebees 13–38% (Garratt et al. 2016). Solitary bee communities are dominated by species nesting in the ground in nests up to several tens of centimeters deep (Mallinger and Gratton 2015; Blitzer et al. 2016; Garratt et al. 2016). Less frequent, aboveground nesting bees' nest in cavities in wood or other material. Mason bees (*Osmia* sp.) and leafcutter bees (*Megachile* sp.) nest generally in cavities made by wood boring beetles. The females collect pollen on well-developed hairs on her legs (ground-nesting bees) or ventral part of abdomen (aboveground nesting bees, Macek et al. 2010).

In the USA and Japan mason bees have been used for pollinating fruit trees since the middle of the last century (Bosch and Kemp 2002; Sedivy and Dorn 2014). Alfalfa leafcutting bee (*Megachile rotundata*) pollinates lucerne in the USA and in Canada. Fruit trees (almonds, apples, cherries) in the USA are pollinated by *O. lignaria*, *O. cornuta* (imported from Europe) and *O. cornifrons* (imported from Japan). In Japan, *O. cornifrons* pollinates most apple trees. It is also used to pollinate blueberries in commercial plantations in the USA. In the southern USA,

O. ribifloris is bred for this purpose (Sampson et al. 2004; Mader et al. 2010; Sedivy and Dorn 2014). In Europe, the possibilities of using the red mason bee, *O. bicornis* (Linnaeus 1758) (syn. *rufa*), **RMB** and the European orchard bee, *Osmia cornuta* (Latreille 1805), **EOB** (Bosch and Kemp 2002; Kronic and Stanisavljevic 2006) for pollinating fruit trees has been investigated.

At the individual level, mason bees are more effective pollinators than the honeybee (*Apis mellifera*). They only need to visit a flower once for the effective pollination because they approach flowers from above and thus always come into contact with the flower's genitalia. In contrast, honeybee workers can collect nectar among the petals without coming into contact with pollen. Moreover, mason bees fly more often between individual trees and between rows of trees, which is important for cross-pollination, especially of apple and cherry trees. Mason bees can be easily bred, as they readily nest in large colonies and are willing to colonize artificial nests (Bosch and Kemp 2002; Sedivy and Dorn 2014). They are not aggressive and their stings are not as painful as those of honeybees or bumblebees are. They generally do not forage for food further than a few hundred meters from nests (Zurbuchen et al. 2010; Hofmann et al. 2020) and are thus better for targeting the pollination of a particular crop. The disadvantage is that they nest for only a few weeks each year.

The choice of the best species for fruit pollination has to take into consideration both its bionomy and habitat preferences. We review the bionomy of RMB and EOB. Their breeding in detachable wooden nests (Bosch and Kemp 2002; Kronic and Stanisavljevic 2006; Sedivy and Dorn 2014; Macivor 2017) was tested in fruit orchards

in Czechia (latitude 49–50° N) in 2018–2021. Resultant experience is summarized in the following text.

Bionomy of two species of mason bees

Both RMB and EOB are common species in Central Europe. EOB is more thermophilic and occurs mainly in lowlands in Czechia (Macek et al. 2010). Both species overwinter as adults in cocoons in their nests. In the spring, males hatch first and females a few days later. After copulation, the females establish nests in cavities in wood. Females build nests without the help of a male. They prefer holes with a diameter of 6–8 mm. They are not able to make holes in wood, so they generally use the tunnels bored by wood boring beetles or holes in wooden buildings, window frames, or walls and bricks. In cavities, they build linear nests consisting of a series of brood cells separated by partitions. The partitions are made of mud (mason bees) or pieces of leaves (leafcutter bees). One individual develops in each brood cell. The female supplies pollen mixed with nectar to each cell and lays an egg on it. The nectar is collected from a variety of sources and is combined with pollen while foraging for food. It lays an egg on the pollen mass and then closes the cell. If the weather is suitable and food is available, it takes about a day to build one cell. In order to confuse parasites the last cells are empty. There is usually 1–2 empty cells, each about 1 cm long. The nest is then closed with a massive stopper made of mud. Within one nest, females first lay female eggs, then only male eggs, which makes it easier for them to leave in spring. The female does not take care of the nests after sealing it and dies after a few weeks. The larvae turn into prepupa at the beginning of summer and remain in this stage until late summer, when they pupate. Adults hatch in October at the latest, but remain in the cocoon until spring. The cocoons of both species are different and can be distinguished. Female cocoons on average tend to be larger than male cocoons (Bosch and Kemp 2002; Krunic and Stanisavljevic 2006; Sedivy and Dorn 2014).

RMB is a polylectic species (i.e. it develops on pollen from various plant families), but usually collects pollen from oak or buttercups (Raw 1974; Persson et al. 2018; Šlachta et al. 2020). If oak is not available, it chooses blackberry (Raw 1974) or rapeseed (Holzschuh et al. 2013). It generally collects nectar from rapeseed, chestnut, maple, willow, fruit trees and *Rosaceae* plants (Jauker et al. 2012) as oak does not provide nectar. During foraging for pollen, the female first visits the sources of nectar, then sources of pollen (Raw 1974). Although it successfully develops on rapeseed pollen, this is not the preferred source of pollen (Holzschuh et al. 2013).

EOB is also a polylectic species, which focuses on collecting pollen from *Rosaceae* (Marquez et al. 1994) and is therefore being tested in southern Europe as an alternative pollinator of fruit trees (Sedivy and Dorn 2014).

It also forages for pollen from *Brassicaceae* and develops successfully on pollen of *Sinapis arvensis*, for example. It mixes pollen from a variety of sources. Some females collect pollen from up to six plant families (Eckhardt et al. 2014). In addition to fruit trees, it also collects pollen from willows and oaks, depending on their availability around nests (Kratschmer et al. 2020).

Artificial nesting sites

Mason bees are willing to nest in reed stalks. Installing reed bundles stored in plastic pipes or covered shelters as protection against water is one way of increasing the range of nesting opportunities in orchards. Such nests are spontaneously inhabited by local populations or it is possible to buy cocoons with wintering adults in cocoons via online websites. The cocoons can then be stored directly in the nests to increase the chances of their being used. The disadvantage of reed stalks is the impossibility of regulating the number of parasites in the finished nests. There is also a high probability of attacks by insectivorous birds, which can be avoided by using wire mesh to prevent their access to nests.

A more suitable solution is to use detachable wooden nests. They consist of a set of wooden boards with grooves, which when placed together create a nesting cavity. These boards can be obtained from online suppliers or can be made by a carpenter. The boards must fit tightly together and not twist when wet so that parasites cannot enter the nests through the gaps. The cocoons can be removed from the nests at the end of the season and overwintered for use next year. This reduces the incidence of parasitism, as the parasites can be sifted out. The cocoons can be kept in a small space, for example in a refrigerator, and the emergence of the bees can be delayed and synchronized with the flowering of the targeted crop. Parasitism is a major problem and cannot be completely eliminated, without the continuous control of the occurrence of parasites as they commonly occur in the wild and will always eventually find bee nests.

We experimentally verified that bees nest in cavities with diameters from 6 to 10 mm, but 8 mm is the most suitable. Although bees are able to nest in cavities with a square cross-section, a rounded cross-section is much more attractive, either circular or semi-circular (width 8 mm, length 8–10 mm). A board size of 15 cm (width) × 22 cm (length) proved to be suitable. Such long nesting cavities allow for the construction of up to 18 cells in a row, which saves bees the time spent establishing new nests. In longer cavities (over 15 cm) bees build relatively more cells with females than in shorter cavities (under 15 cm; Sedivy and Dorn 2014). The width of the cavity has a similar effect: there are more cells with females in wider cavities. With a cross-section of 8 mm, the female chambers make up about 30% of the total (Sedivy and Dorn 2014). The boards we used contained 8 nesting cavities

of semicircular shape with a width and length of 8 mm and gaps between the cavities also with a width of 8 mm. One group of 7 nesting boards thus contained 48 nesting cavities. Although such a high density of nesting holes made it difficult for bees to find a particular nest, it was not a major problem as females eventually find their nest. However, for less intensive breeding, it is recommended that there are gaps of a few centimeter between the nesting cavities for easier orientation of the nesting females.

An important factor that influences the success of occupation is the choice of a suitable place for artificial nest sites. Mason bees prefer places with unshaded free access with an entrance oriented ideally to the south-east. They should be protected from the wind (usually from the northwest) and conspicuously located so that it is easy for bees to find them. It is advisable to use the wall of a building or another significant landmark (fence or edge of a group of trees) and place the nesting site in front of it. In orchards, fencing around the orchard can usually be used, i.e. a few meters from the nearest rows of trees, where they are thus better protected against pesticide sprays and well visible to bees. Visibility is further enhanced by painting the nest sites blue and locating the nests about a meter above the ground. In orchards where there is abundance of pollen and nectar, bees fly a maximum of 50–150 meters (Sedivy and Dorn 2014).

Some of the released bees do not nest in the nest in which they were released, and look for other nesting opportunities in the area. Therefore, in order to increase their abundance, there should be many nesting sites distributed throughout an orchard. An important part of their nests are the partitions between the brood cells and the plugs closing the nests. Mason bees build them from mud, which they collect near to the nests, for example from dirt roads. The mud must be moist. Providing this material close to the nest site, or digging up the turf and occasionally pouring water on the exposed soil, will save bees time spent finding suitable material and speed up the construction of nests.

Management of the artificial nests

At the end of the season, the artificial nests can be moved from the orchard to covered areas. It is best to wait until the end of June, as they should not be moved during the first few weeks after the nests are completed so that the larvae inside brood cells do not lose contact with the pollen reserves. In June, however, they are already large enough not to be at risk when moved in nests. The best time to open the nests and remove the cocoons is October, when they will have completed their development and the temperatures are favourable for drying the cocoons and artificial nests, which are mechanically cleaned and stored in a dry place until spring. It is also possible to chemically treat them against mould. However, it is advantageous to preserve the natural scent of the

nests, because bees prefer such scented places for nesting. The smell is produced by the fatty acids contained in the cocoons. Due to the fact that the cocoons are relatively resistant to mechanical damage, they can be removed from the nests very quickly. A blunt tool with a diameter of 6 mm (screwdriver) can be used to push the contents of the nesting cavities along their entire length into a container. The mixture of cocoons, remnants of partitions and parasitized cells is then sieved through an 8 mm sieve. This separates the cocoons from impurities and parasites (parasites inside cocoons, however, escape attention). In addition, the cocoons can be cleaned of dirt using running water and then dried. However, this is not necessary.

Cocoons can be stored in large numbers during the winter in ventilated containers in which the air humidity is 65–90%. For example, paper boxes are suitable. Boxes with small holes for ventilation remain sufficiently moist for survival throughout winter. Storage is possible outdoors or in a refrigerator at a temperature of 2–4 °C. In spring, cocoons must be transferred to artificial nests at least one week in advance of the expected flowering of the fruit trees. Emergence from cocoons can be delayed by keeping them at 2–4 °C in a refrigerator. The second option is to transfer the cocoons to artificial nests at the end of February and let them hatch spontaneously. In the wild, EOB emerges from cocoons at the end of March and RMB in the second half of April. At temperatures around 20 °C, EOB emerges in a few days, especially if overwintered at a temperature higher than 4 °C, otherwise they do not emerge quickly and await more favourable temperature conditions. RMB behaves differently. It remains in the cocoons for two to three weeks, even under suitable temperature conditions. In a refrigerator (at a temperature of up to 4 °C), it is possible to keep them in cocoons until the summer.

In both species, the males hatch first and then after a few days the females. In good weather, they warm up and fly around the nest. At night, they return to their nests and look for suitable cavities. Females do not start nesting immediately as they need to acclimatize and start metabolic processes. They prefer to nest close to other nests and are attracted by the smell of cocoons. Thus, by inducing a small number of bees to nest one to two weeks before the transfer of the main population, as this will attract them to nest. A prerequisite is the availability of alternative food sources until the fruit trees bloom, e.g. willows. A large number of females will fly away to look for new nest sites, but by placing nests around an orchard, it is possible to keep them in the orchard.

Enemies

Cleptoparasites (nest parasites) are the most serious threat to mason bees in artificial nests. They feed on the food supplies of their hosts. A very common kleptoparasite is the Houdini fly (*Cacoxenus indagator*). This little

fly with distinct red eyes penetrates into unfinished nests and lays eggs in the brood chambers and their larvae eat the pollen supplies. There can be up to about 20 larvae in one chamber. In this case, the bee larva has no chance of completing its development. In addition, young larvae are able to bite through the partition into the adjacent chamber if they do not have enough food. We usually see the adults near nests under construction, where they remain at the edge of nests until the bees leave the nests to forage for food at which time they enter the nests. Invasion of chambers by this fly is easily detected when the nests are opened in autumn as they contain conspicuous larval faeces in the form of long yellow fibers. Autumn sifting of cocoons gets rid of these larvae. Due to the small size of this fly, another preventive measure is to place adhesive tapes near the nests at the time of nesting. However, this method is not very effective.

Another kleptoparasite is the mite *Chaetodactylus osmiae*. It feeds on pollen in the nests and can multiply in the brood chambers so that they completely fill them. The life cycle, which consists of the development of the egg, two nymphal stages and the adult, can be completed up to ten times a year. Subsequently, some nymphs enter a resting stage called a hypopus, which are able to survive for several years without food. This resting stage takes two forms: mobile form, with four pairs of legs with claws to attach to adult bees, which transfer them to new nests, and an immobile form, without such legs. In contrast, they wait in the nests for new bees to repopulate them. Due to the small size of these resting stages, it is very difficult to get rid of them in artificial nests. They adhere to both boards and cocoons. In cases of severe parasitism, it is better to dispose the infested artificial nests. Otherwise, chemical protection (acaricides) can be applied, even directly to cocoons. *Monodontomerus obscurus* is a parasitoid whose larvae feed directly on bee larvae. Multiple larvae usually parasitize one bee, which eventually dies. This wasp uses its long ovipositor to lay eggs in its prey by penetrating the wall of nests in reed stalks, but finds it difficult to penetrate the walls of artificial nests and rarely attack these nests. They search for their prey by smell. Wasp larvae eat the bee larva gradually and only kill it after it pupates and produces its cocoon in the host's cocoon. They can leave the nest in the same year if the temperature conditions are favourable or they overwinter in a cocoon. After leaving a cocoon, a small hole remains visible, as well as in the lid (and partitions), where they left the nest, or in the reed wall, through which they can bite directly. If wasp larvae overwinter in a cocoon, their presence can go unnoticed. In spring, however, it leaves nests in June, i.e. later than the mason bees. This makes it possible to get rid of them by removing non-hatched cocoons from the nests before June.

Another parasitoid of the mason bees is the *Anthrax anthrax*, which is a black fly, larger than the Houdini fly. The female flies into the entrance of nests and uses her abdomen to inject clay-coated eggs into the entrance.

Mobile first instars called planidia hatch from these eggs and penetrate into the depths of the nest. There, they feed first on pollen and nectar and then on the larva of the bee, but they do not weaken it too much, so that it is able to form a cocoon and eventually pupate. Only then, they kill their host and produce their cocoon in its host's cocoon. The pupa has a crest of thorns on its head, which it uses to emerge from the bee's pupa and subsequently also from the nest, damaging all the chambers along the way and killing the bee larvae in them. This can significantly damage nests. An adult fly hatches from the pupa, and pupal exuvie can be found at or near the nests.

Also cuckoo wasps (*Chrysididae*) act as parasitoids of mason bees. However, they occur relatively rarely and do not pose a great threat to mason bees in artificial nests. The nests can also contain skin beetles (*Dermestidae*), whose larvae feed on residues of organic matter and do not pose a danger to bees. We have not noticed any problems with the ants either. Mason bee larvae can be infested with a fungal pathogen of the genus *Ascospaera*, similar to honey bees. We did not have a problem with this pathogen in artificial nests, which must be thoroughly disinfected if this pathogen is detected. There is no other disease recorded for mason bees. During the wetter seasons (autumn, winter), organic residues in nests or wintered cocoons can be attacked by mould. In this case, it is advisable to treat artificial nests with an antifungal agent and dry them at a high temperature. Mould should not be present when the plates are stored disassembled, sufficiently dried and in a dry environment. To treat cocoons infested with mould, it is sufficient to dry them at high temperatures; the mould does not endanger bees inside cocoons.

Nests can be attacked by the beetle *Trichodes apiarius*. Adults feed on pollen and nectar and lay eggs on flowers or in artificial nests and their surroundings. On hatching their larvae enter bee nests and eat both pollen and bee brood. They eat the contents of one chamber after another until they destroy an entire nest. They remain in the nest and overwinter in the prepupal stage and pupate in spring. Adult larvae are strikingly pink and are up to 2 cm in size. Their cocoons cannot be overlooked during autumn sifting.

Insect-eating birds, such as great tit or spotted woodpeckers, pose a danger to nests, especially before and during winter. Not overwintering cocoons outside in artificial nests eliminates this danger. However, woodpeckers can use their beaks to significantly damage the relatively robust wooden boards of artificial nests during the nesting season. The only protection in this case is to prevent them from accessing the nests by enclosing them in wire mesh. However, this complicates the bees' access to nests as their wings rub against the mesh. Therefore, it is better to avoid this solution if possible and use it only in the case of need. Usually, nests are attacked by birds near forests or city parks, not in the open countryside and orchards.

Conclusions

RMB is less suitable for pollinating crops because it preferentially collects pollen from oaks. EOB prefers the pollen of *Rosaceae* and *Brassicaceae*, hence is more suited for pollination of fruit trees or rapeseed. EOB is more thermophilic than RMB but its spread to higher altitudes can be expected in Europe in response to climate change. The breeding technology, summarized in this paper, can be used to produce and introduce both species into fruit orchards for pollination purpose. Since EOB nests earlier than RMB, their combined breeding can be useful, since it increases the duration of the pollination service.

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MONITORING OF NATURA 2000 GRASSLAND HABITATS IN SAC ŠUMAVA

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ABSTRACT

The Bohemian Forest grasslands have emerged gradually over centuries, with political turbulence in the second half of the 20th century strongly affecting the region, including its meadows. Today, there are cultural and partly degraded meadows as well as fragments of highly valuable semi-natural mountain meadows, important biodiversity localities in the mostly wooded Bohemian Forest landscape. Their conservation value began to be taken into account in 1991, when the Šumava NP was established, and re-emphasized in 2004, when certain grasslands were recognized as Natura 2000 habitats. Maintaining favorable conditions of meadow habitats is a hard task even in strictly protected areas and the most common difficulties are listed in this paper. More attention has been paid to the management of grasslands in the region during the last decade. The Šumava NP Authority provides the necessary management of the most valued localities and has organized simple monitoring in some managed sites. This paper presents the results of the first five years of monitoring of 16 sites. Results are discussed, along with field experiences, and more effective management strategies are proposed. Appropriate and carefully executed management is recognized as a necessary tool for ensuring proper care of conservationally valuable habitats, including grasslands of European importance protected under the Natura 2000 network. Monitoring is an essential tool to observe management quality. Results of this preliminary study deliver a substantial set of experiences that can be used to improve the management and monitoring of Natura 2000 habitats, as well as other species rich meadows occurring in the Šumava NP.

Keywords: conservation; fens; management; monitoring; national park; *Molinion* meadows; Natura 2000; *Nardus* grasslands

Introduction

In the Central European landscape, grasslands are primarily semi-natural habitats, the products of local ecological conditions and long-term active management (Chytrý and Blažková 2007). The Bohemian Forest grasslands have emerged gradually over centuries, coevally with the region's colonization. For centuries, the Bohemian Forest, where Šumava Special Area of Conservation (SAC Šumava) is located, was for defensive reasons maintained as untouched, deep forest on the south-west border of the Bohemian Kingdom. Deforestation has gradually and slowly altered the region. At the highest elevations, the first gaps in untouched mountain forest were created during the second half of the 18th century or later (Záruba and Koblasa 2000). Therefore, meadows spontaneously arising in deforested areas are relatively young habitats, not more than 300 years old.

Political and social turbulence in the second half of the 20th century strongly affected the Bohemian Forest region, including its grasslands. Most of the German population was displaced after World War II and a large part of the territory was closed by the Iron Curtain. As a result, many settlements have completely disappeared, as has the traditional agricultural use of the landscape. In the “accessible” area of the region in front of the Iron Curtain fences, state farms were established, extensive drainage projects implemented and many meadows were plowed and sown with a mixture of cultivated grasses. Until 1990, so-called substitute reclamation (“improve-

ment” of less productive locations as a substitute for the occupation of agricultural land elsewhere) damaged many fen grasslands and nutrition poor mountain meadows of this region.

Today, the grasslands of the region are heterogeneous. There are cultural and partly degraded meadows as well as fragments of highly valuable semi-natural mountain meadows. Species diversity in Bohemian Forest meadows does not reach the level of species diversity of famous meadows in the White Carpathian Mountains (Hájková et al. 2011) however they are important biodiversity localities in the mostly wooded Bohemian Forest landscape. The conservation value of these meadows began to be taken into account in 1991, when the Šumava National Park (Šumava NP) was established, and emphasized again in 2004, when certain grasslands were designated as Natura 2000 habitats (i.e. habitats listed in the Annex I, the Habitats Directive 92/43/EEC) and selected for protection in SAC Šumava.

Similarly, to other parts of the Czech Republic and many European countries, currently the management of Bohemian Forest meadows is not primarily determined by the need to provide grazing and hay for cattle, a former traditional source of livelihood of the local population. Many localities, especially those species rich, are not managed for economic reasons, but for conservation purposes. Protected areas help us to fulfill our moral commitment and preserve the cultural and natural heritage for future generations. The Šumava NP Authority is the competent body of the state administration for nature

protection and is obliged to maintain the favorable condition of meadow habitats or, if necessary, to ensure the improvement of their status. This task is not easy to fulfill even in the most strictly protected area, the National Park.

Thirty years of the Šumava NP have demonstrated the most common difficulties in the management of grasslands:

(1) Other priorities in the management of this large protected area. Since the beginning of its existence, the Šumava NP has been primarily battling with the management of forests (bark beetle outbreaks, windstorms, felling, non-intervention management practices, etc.), which also provoke dynamic social and political debates; therefore, the care of grassland habitats has not received similar attention;

(2) Ensuring appropriate management in a large area of the Šumava NP is difficult, both for logistic and economic reasons;

(3) Many grasslands are held in private ownership and the influence of the Šumava NP Authority on their management is limited;

(4) The Šumava NP is an attractive recreational area and many valuable meadows have been already lost due to development activities (new pensions and apartment rental houses, touristic infrastructure, etc.).

Nevertheless, more attention has been paid to the management of grasslands in the region during the last decade. The Šumava NP Authority provides the necessary management of the most valued localities. Some sites are managed under the framework of long-term cooperation with land tenants, some are repeatedly outsourced to external contractors, and other sites are managed directly by employees of the Šumava NP Authority.

Monitoring of managed meadows is an essential tool for clearer understanding of applied measures and long-term changes of grassland habitats. Thus far, the Šumava NP Authority has organized simple monitoring in some managed sites. This paper presents the results of the first five years of monitoring. Results are discussed, along with field experiences, and recommendations for management that is more effective are proposed.

Methods

Study area

The Bohemian Forest is one of the largest forested landscapes in Central Europe, located along the Czech–Bavarian and Czech–Austrian borders. Large mountain forests together with clear mountain streams and glacial lakes, pristine wetlands, peat bogs, and mountain meadows and pastures make it a refuge for many endangered species of plants and animals. This cross-border area is home to several iconic species, such as the lynx (*Lynx lynx*), the moose (*Alces alces*), capercaillie (*Tetrao urogallus*) and the freshwater pearl mussel (*Margaritifera margaritifera*), each of which now occurs in Central Eu-

rope only in several viable populations. Typical mountain plants, such as Hungarian gentian (*Gentiana pannonica*) or mountain arnica (*Arnica montana*), flower in meadows and pastures which are remnants of traditional mountain settlements. Two national parks, the Bavarian Forest National Park (Bavarian Forest NP, Germany) and the Šumava National Park (Šumava NP, Czech Republic) were established in the Bohemian Forest in 1970 and 1991, respectively, and protect the area with the highest conservation value (Křenová and Kindlmann 2015, 2018). In their respective home countries, each National Park is among the largest terrestrial sites in the Natura 2000 network, i.e. networks of protected areas established under the Habitats Directive (92/43 / EEC) and the Birds Directive (2009/147 / EC) and designated in all 27 EU countries to protect the most valuable European habitats and species (Sundseth and Creed 2008).

The Special Area of Conservation Šumava (SAC Šumava) was designated by Czech Government Order No. 132/2005. SAC Šumava covers the entire territory of the Šumava NP and the Šumava Protected Landscape Area (Šumava PLA; Fig. 1). Twenty-one natural habitats, eight animal and three plant species (see Appendix 1 for full list of habitats and species) are subjects of protection here and are important for definition of conservation targets of the Šumava NP and Šumava PLA (Bláha et al. 2013). Grassland habitats, i.e. habitats 4032, 5130, 6230, 6410, 6430, 6510, 6520, 7110, 7120, 7140, occupy approximately 8.2% of the area of the SAC Šumava. The Šumava NP Authority, a state administration office for SAC Šumava, has selected 16 study sites for monitoring of Natura 2000 grasslands in SAC Šumava (Fig. 1; Appendix 2).

Target habitats

During the Natura 2000 habitats mapping (Härtel et al. 2009), upon the Czech Republic implementation of the Natura 2000 Directives as a part of the EU-integration process, more detailed vegetation units called biotopes (Chytrý et al. 2001, 2010) were mapped and later aggregated for habitats. Usually 1–3 biotopes form one habitat in *sensu* Annex I of Habitats Directive 92/43/EEC (Appendix 1).

Each of 16 study sites hosts one of the following biotopes:

- T2.3B – Submontane or montane *Nardus* meadows without *Juniperus communis*.
- T1.9 – Intermittently wet *Molinia* meadows.
- R2.2 – Acidic moss-rich fens.

Nardus meadows

Biotope T2.3B belongs to habitat 6230 – Species-rich *Nardus* grasslands, on siliceous substrates in mountain areas (and submountain areas, in Continental Europe). It is a priority habitat in *sensu* of Habitats Directive 92/43/EEC.

In the Bohemian Forest region, meadows of this vegetation type can be species-poor or species-rich. Different

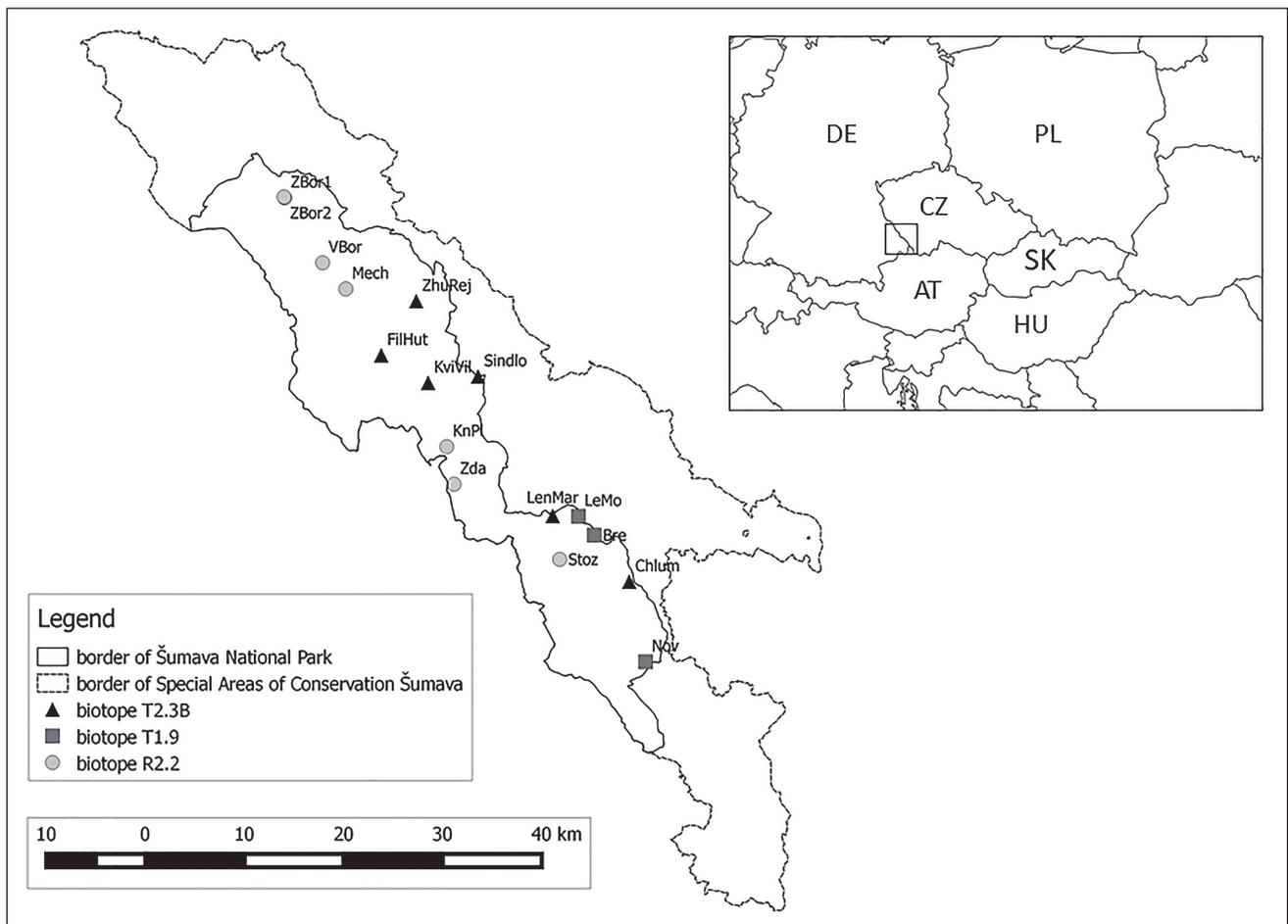


Fig. 1 The map of the study area and study sites: ZBo1 – Zadní Bor, ZBo2 – Zadní Bor 2, VBor – Velký Bor, Mech – Mechov, ZhuRej – Zhůří near Rejštěj, FilHut – Filipova Huť, KviVil – Kvilda Vilémov, Sindlo – Šindlov, KnP – Knížecí Pláně, Zdar – Žďárek, LenMar – Lenora Markovec, LenMo – Lenora Molinion, Bre – Březina, Stoz – Stožecké louky, Chlum – Chlum, Nov – Nová Pec. Different symbols are used to distinguish sites of different biotopes.

subtypes of this habitat can be found depending on local habitat conditions, depending upon the content of nutrients and soil moisture. In drier subtypes of this habitat, especially on steeper slopes and grazed areas, sparse vegetation is often formed, in which some drought tolerant species can occur (e.g. *Thesium pyrenaicum*, *Antennaria dioica*). In wetter sites (e.g. at the edges of transitional peatlands or in areas with higher precipitation), moisture-loving species are more common. *Arnica montana*, *Gentiana pannonica* (Hofhanzlová and Křenová 2007; Ekrťová 2013) and other iconic species of this habitat grow. We can also find a wide range of orchids (e.g. *Gymnadenia conopsea*, *Pseudorchis albida*) in richer *Nardus* meadows.

The degradation of stands due to abandonment or machine mowing reduce the number of species and support increasing abundance of dominants. In SAC Šumava, 2053 hectares of this habitat are mapped.

***Molinion* meadows**

Biotope T1.9 is the habitat 6410 – *Molinia* meadows on calcareous, peaty or clayey-silt laden soils (*Molinion*

caeruleae). In the past, these alternately moist *Molinia* meadows were quite abundant (for example within the montane floodplain of the Upper Vltava River; Sádlo and Bufková 2002). These meadows were primarily late, single-mown meadows. In some traditionally managed sites, their water regime was modified with shallow drainages. During the second half of the 20th century, the *Molinia* meadows have degraded and gradually transformed into other types of vegetation. Many *Molinion* meadows have been disturbed by deep drainages, fertilization or inappropriate mowing times. A bluegrass (*Molinia caerulea*), a diagnostic species, can even be missing in strongly degraded meadows where other dominants are common. Mowing twice a season as well as abandonment of these meadows cause degradation of the habitat quality and decrease of species diversity.

This vegetation type occurs on a nutrition gradient between richer *Arrhenatherum* meadows and oligotrophic pastures. On a hydrological gradient, the *Molinia* meadows are between wet *Cirsium* meadows and oligotrophic peatlands. Certain endangered species (e.g. *Dianthus superbus*, *Gentiana pneumonanthe*) can occur in these

meadows. Currently, only 36 hectares of this biotope occur in SAC Šumava.

Fen meadows

Biotope R2.2 belongs to the habitat 7140 – Transition mires and quaking bogs. These meadows, both with lower or higher species diversity, are primarily lower to medium-high grasslands with predominant sedges. Mosses are a critical aspect of this habitat and their cover is gradually decreasing with increasing soil humidity. The variability of this habitat is considerable in the Bohemian Forest region. Fen meadows are formed on acidic substrates, in nutrient-poor places with mineral substrates, during initial stages of paludification. With optimal development and conditions, fen meadows are short grasslands, with a predominance of herbs or sedges, but without a significant dominant. Change of water regime (i.e. drainages) and overgrowing with shrubs and trees are major threats to this habitat. A total of 575 hectares of this habitat are currently mapped in SAC Šumava.

Design of monitoring and data analyses

In each of sixteen study sites, paired permanent plots with analogous vegetation were set side by side. Pairs were planned as managed and unmanaged (i.e. control) plots, which in the original concept was to represent an unmown meadow and a meadow mown with an appropriate technique considered optimal in terms of the meadow phenology at the time. However, mowing has occasionally been replaced by grazing in some sites, or the plots intended to be regularly managed have remained unmown in some years. For this reason, in statistical analyses, plots were differentiated according to the planned management (treatment – MngYES / MngNO) and their actual management (mown / unmown), if necessary.

The plots are 4 × 4 m and separate by a four-meter gap between plots to avoid an edge effect. The areas are marked with wooden sticks in the field. Blue stick marked plots are designated as managed, and unmanaged (i.e. control) plots are marked with red sticks. Permanent plots were established in seven study sites (Knížecí Pláně, Mechov, Stožické louky, Šindlov, Velký Bor, Zadní Bor 1, Zadní Bor 2) in 2014 and monitoring of another nine study sites (Březina, Filipova Huť, Chlumské stráně, Kvilda Vilémov, Lenora Molinion, Lenora Markovec, Nová Pec, Zhůří near Rejštejn, and Žďárek) began in 2016. In this paper, we analyze data from 2014 and 2016–2020.

Annually, phytosociological relevés are performed in all plots, according to the combined Braun-Blanquet scale (Moravec 1994). Coverage values are recorded in percentages. Monitoring always took place at the end of June or the beginning of July, to capture approximately the same phenological phase of meadow vegetation. Recorded data (Table 1) were analyzed separately for each of the target Natura 2000 habitats.

We recorded the total number of species in the monitored plots and the diversity, calculated as a % of species out of the total number of species recorded in all localities of the given habitat. We tested differences among study sites and in the case of statistically significant differences, a subsequent comparison of study sites was performed (post-hoc comparison, Bonferroni test). We also tested differences in species abundance, diversity, total coverage (E_{total}) and moss cover (E_0) between plots with different treatment, i.e. planned management (MngYES/MngNO). If the real management differed from the planned management, we also tested differences in the numbers of species, diversity, total coverage (E_{total}) and moss cover (E_0) between plots actually managed and unmanaged (mown / unmown). Repeatedly collected data were evaluated using ANOVA, Repeated measures, General Linear Models in STATISTICA 13.3 (StatSoft, Inc. 2012).

Table 1 Parameters recorded during monitoring and used in statistical analyses.

Parameter	Description
E_{total} [%] – Total cover	Total vegetation cover in a permanent plot.
E_0 [%] – Moss cover	Moss cover in a permanent plot.
Number of species	The number of all plant species recorded in a permanent plot.
Diversity [%]	% of species from the total number of species recorded in all localities of the given habitat (i.e. for T2.3B, T1.9 and R2.2 separately)
Treatment MngYES / MngNO	A categorical variable. MngYES = a plot designated to be managed, MngNO = a plot designated as a control plot, without management.
Management MOWN / UNMOWN	A categorical variable. MOWN = planned management, mowing chiefly performed; UNMOWN = management not performed.

Results

T2.3B – Submontane or montane *Nardus* meadows without *Juniperus communis*

Monitoring of this biotope was carried out in six study sites (Fig. 1). One of them – the Šindlov study site was monitored beginning in 2014, the other sites were monitored from 2016. The highest number of plant species, 36, was recorded in the managed plot located in the Chlum study site in 2020 (Table 2). The study sites of this biotope differed in the number of recorded species and in diversity, which was expressed as % of species out of the total number of species recorded in all localities of T2.3B biotope (ANOVA, $p < 0.01$; Fig. 2).

There were no statistical differences in diversity and total coverage between managed and unmanaged (i.e. control) plots. Due to the fact that real management differed in some cases from the planned management

Table 2 The T2.3B biotope study sites. The numbers of recorded species and diversity, calculated as a % of species out of the total number of species recorded in all T3.2B biotope study sites, are shown. Mean, maximum (Max) and minimum (Min) values are presented.

Study site	Number of species			Diversity		
	Mean	Max	Min	Mean	Max	Min
Filipova Huť	21.2	29	15	55.8	76.3	39.5
Chlum	32.4	38	29	74.7	92.1	55.3
Kvilda Vilémov	28.4	35	21	81.1	100.0	60.0
Lenora za Markovcem	26.4	30	23	88.0	100.0	76.7
Šindlov	23.8	29	20	82.2	100.0	69.0
Zhůří	8.6	10	7	22.6	26.3	18.4

Table 3 The T1.9B biotope study sites. The numbers of recorded species and diversity, calculated as a % of species out of the total number of species recorded in all T1.9 biotope study sites, are shown. Mean, maximum (Max) and minimum (Min) values are presented.

Study site	Number of species			Diversity		
	Mean	Max	Min	Mean	Max	Min
Březina	33.0	41	26	80.5	100.0	63.4
Lenora Molinion	23.9	28	21	58.3	68.3	51.2
Nová Pec	24.3	31	19	59.3	75.6	46.3

method, we also tested differences between plots actually managed and unmanaged, however, no statistically significant differences were found in this case either.

The total coverage and diversity differed among study sites as well as within some study sites during our study period (Fig. 3). Year-on-year changes varied slightly including variations between managed and unmanaged plots. However, these differences were not statistically significant.

Repeated measures ANOVA analysis failed to reveal the effect of time. No statistically significant differences in diversity, total coverage or moss cover were found during our study period. This applies both to the complete analysis of all monitored plots and to the separately analyzed managed and unmanaged (i.e. control) plots.

T1.9 – Intermittently wet *Molinia* meadows

Monitoring of *Molinia* meadows was carried out in the years 2016–2020 in three study sites (Fig. 1). The highest number of plant species, 41, was recorded in 2017 in the unmanaged plot located in the Březina study site (Table 3).

There were no statistically significant differences in total coverage among the study sites, however statistically significant differences in moss cover and diversity were found (both $p < 0.01$). The highest diversity values were recorded in the Březina study site, which also had the lowest moss cover. The highest moss cover was found in the Nová Pec study site.

With regard to the fact that in 2017, 2018, 2019 and 2020 the plots determined as managed were not mowed in the Lenora Molinion study site, analyses of differences between plots with different treatments, i.e. planned management (MngYES / MngNO) were useless and comparisons of plots with different real management (mown / unmown) were performed. There were no statistically significant differences in the total coverage between mown and unmown plots. However, a comparison of mown and unmown plots revealed statistically significant differences in moss cover ($p < 0.01$; Fig. 4) and diversity ($p < 0.05$; Fig. 5). The mown plots showed significantly higher moss cover and diversity.

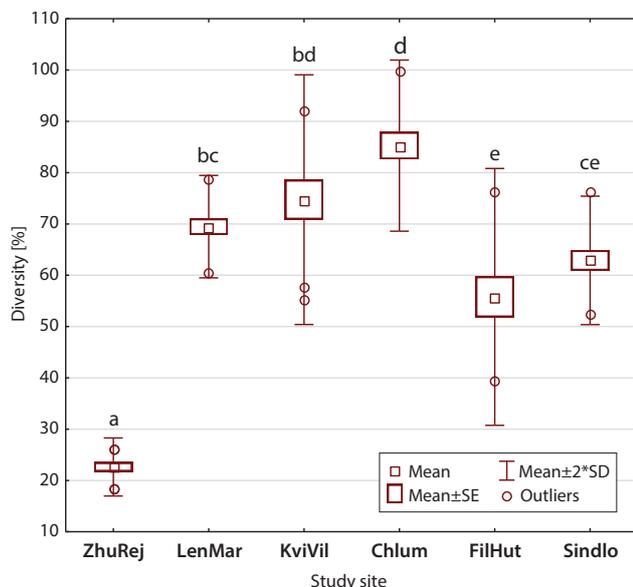
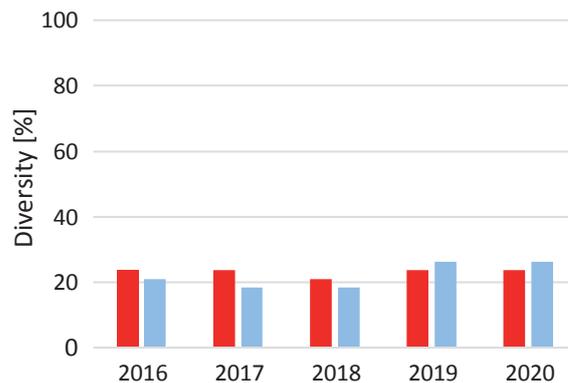
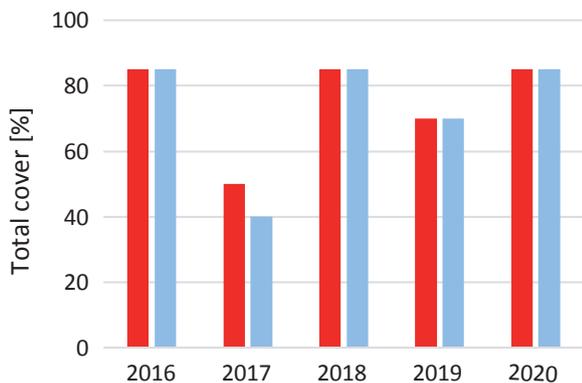
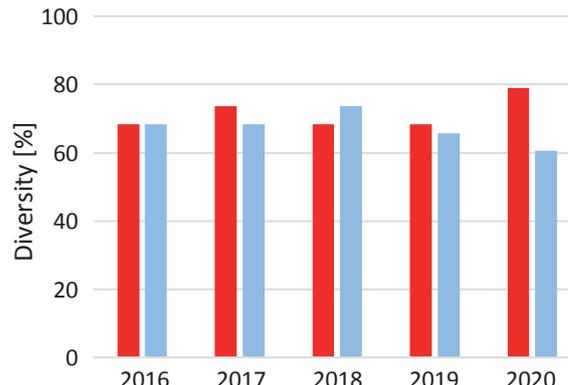
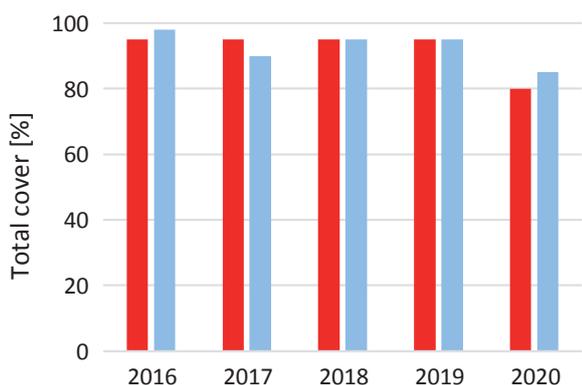


Fig. 2 One-way ANOVA. Differences among study sites in diversity calculated as a % of species out of the total number of species recorded in all T3.2B biotope study sites are shown. Mean values (points), SE (boxes) and SDs (bars) are displayed. Letters above the bars indicate results of post-hoc comparisons; i.e. different letters mark statistically different values. Study sites: ZhuRej – Zhůří near Rejstějn. LenMar – Lenora Markovec, KviVil – Kvilda Vilémov, Chlum – Chlum, FilHut – Filipova Huť, Sindlo – Šindlov.

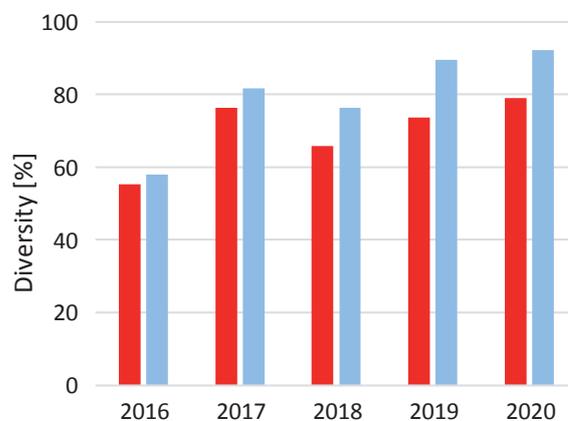
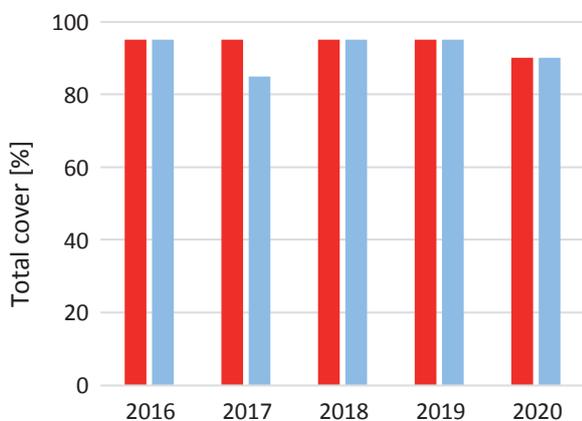
Zhůří



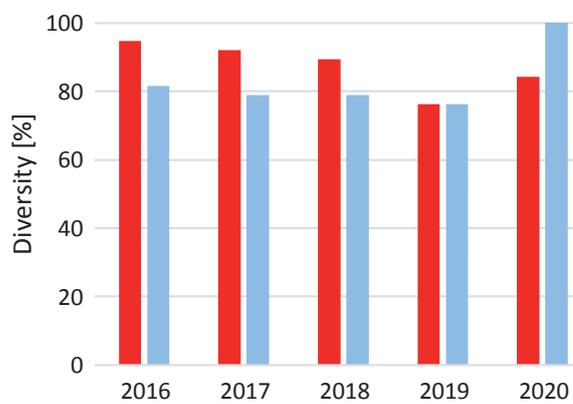
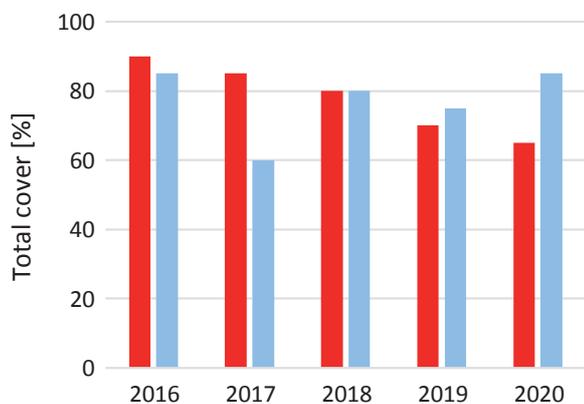
Lenora



Kvilda Vilémov



Chlum



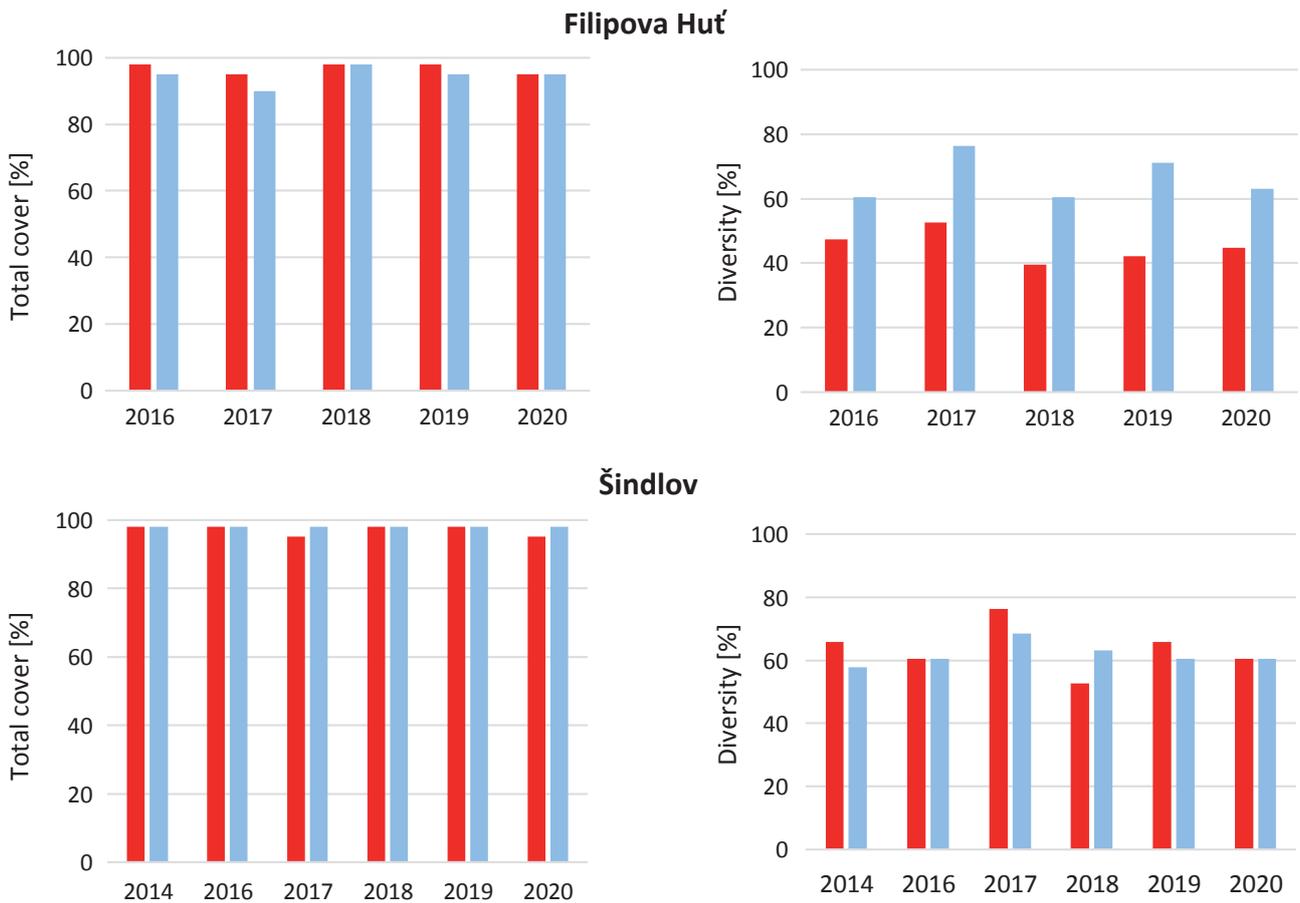


Fig. 3 Total coverage and diversity recorded in study sites in 2014 and 2016–2020. Red columns – unmanaged (i.e. control) plots, blue columns – managed plots.

Because a statistically significant effect of time was found, repeatedly measured analyses were performed. Analysis of repeated measurements of total coverage showed statistically significant differences between mown and unmown plots over time ($p < 0.001$; Fig. 6). The cov-

er both in mown and unmown areas was quite high over the years, reaching values of 95–98%. Nevertheless, it significantly increased in 2020 in unmown plots, especially in the Nová Pec study site where it was only 80% (Fig. 7).

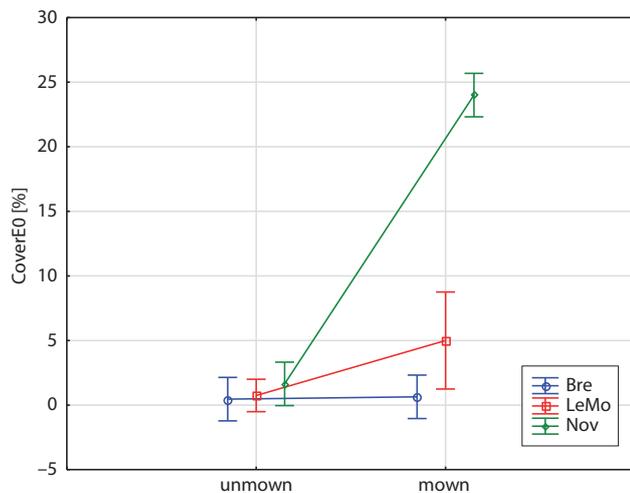


Fig. 4 Moss cover recorded in unmown and mown plots located in study sites of T1.9 biotope: Bre – Březina, LeMo – Lenora Molinion, Nov – Nová Pec. Mean (points) and 95% confidential intervals (bars) are shown.

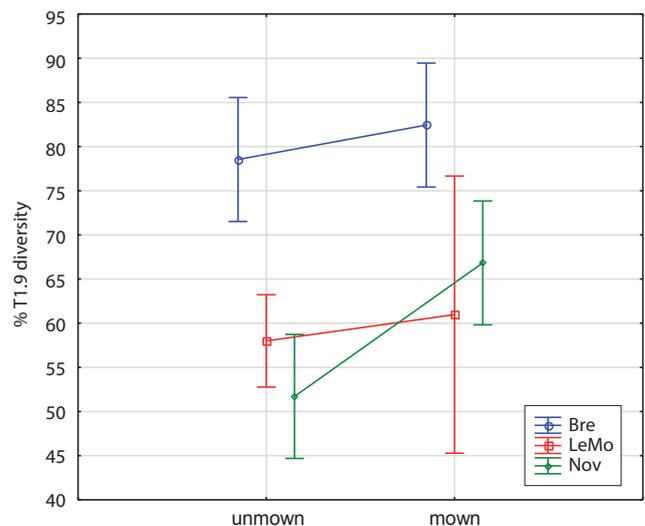


Fig. 5 Diversity recorded in unmown and mown plots located in the T1.9 biotope study sites: Bre – Březina, LeMo – Lenora Molinion, Nov – Nová Pec. Mean (points) and 95% confidential intervals (bars) are shown.

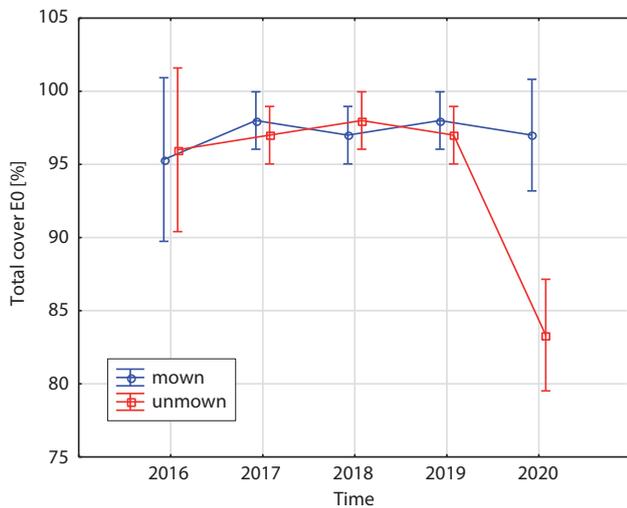


Fig. 6 Total coverage recorded in unmown and mown plots located in the T1.9 biotope study sites. Mean (points) and 95% confidential intervals (bars) are shown.

Additionally, moss cover significantly differed between mown and unmown plots during our study period ($p < 0.05$; Fig. 8). Lower moss cover was found in unmown plots in all study sites. The largest differences were recorded in the Nová Pec study, where the moss cover in the mown plot reached 20–25%, but in the unmown (control) plot decreased from 7% in 2016 to 0.1% in 2017 and slightly increased to 1% in 2020. Significant differences in the moss cover between the mown and unmown plots were recorded at Březina locality too. No significant differences in moss cover were found in the Lenora study, which was left without regular management since 2017.

No statistically significant differences in biodiversity between mown and unmown plots were found in all three-study sites (Figs. 7, 9). The smallest differences in diversity between managed and unmanaged plots were recorded in the Lenora study site, where the plot designated for regular management was mown only in 2016

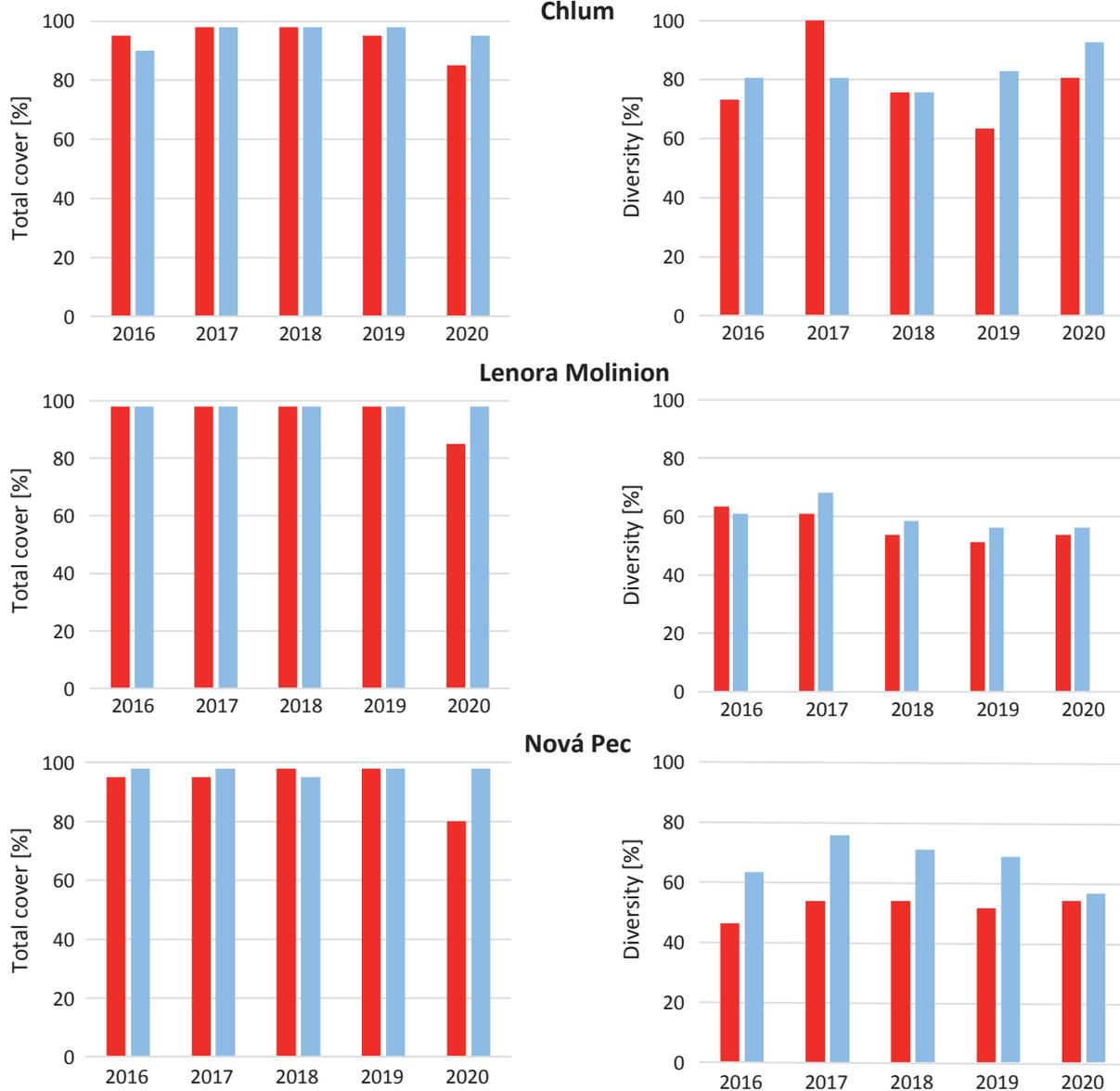


Fig. 7 Total coverage and diversity recorded in study sites in 2014 and 2016–2020. Red columns – unmanaged (i.e. control) plots, blue columns – managed plots.

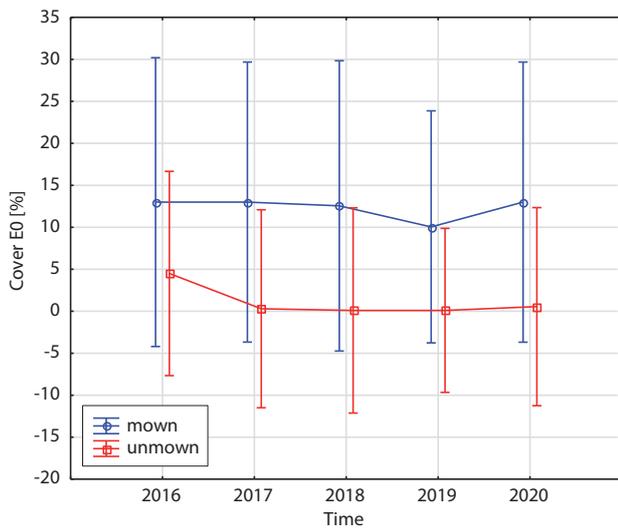


Fig. 8 Moss cover (Eo) in mown (blue) and unmown (red) plots in T1.9 biotope study sites. Mean (points) and 95% confidential intervals (bars) are shown.

and left unmanaged since that year. In the Nová Pec study site, the diversity of the mown plot slowly decreased during the study period. The largest variation in diversity was recorded in the Březina study site, both in the mown and unmown plot.

R2.2 – Acidic moss-rich fens

Seven study sites of this biotope were monitored in 2014 and 2016–2020 (Fig. 1). The highest number of plant species, 40, was recorded in 2014 in the unmanaged (control) plot in the Velký Bor study site (Table 4).

Statistically significant differences in total coverage ($p < 0.05$, Fig. 10), moss cover and diversity (both $p < 0.001$, Figs 11, 12) were found among the study sites.

Repeated measures ANOVA showed statistically significant differences in moss cover ($p < 0.05$; Fig. 13) and biodiversity ($p < 0.01$; Fig. 14) between managed and unmanaged plots during our study period. In cases of total

Table 4 The R2.2B biotope study sites. The numbers of recorded species and diversity calculated as a % of species out of the total number of species recorded in all R2.2 biotope study sites are shown. Mean, maximum (Max) and minimum (Min) values are presented.

Study site	Number of species			Diversity		
	Mean	Max	Min	Mean	Max	Min
Knížecí Pláně	32.7	37	28	32.7	37	28
Mechov	25.8	29	21	25.8	29	21
Stožecké louky	19.8	22	16	19.8	22	16
Velký Bor	33.8	40	31	33.8	40	31
Zadní Bor 1	28.0	35	21	28.0	35	21
Zadní Bor 2	25.8	30	21	25.8	30	21
Žďárek	31.5	36	28	31.5	36	28

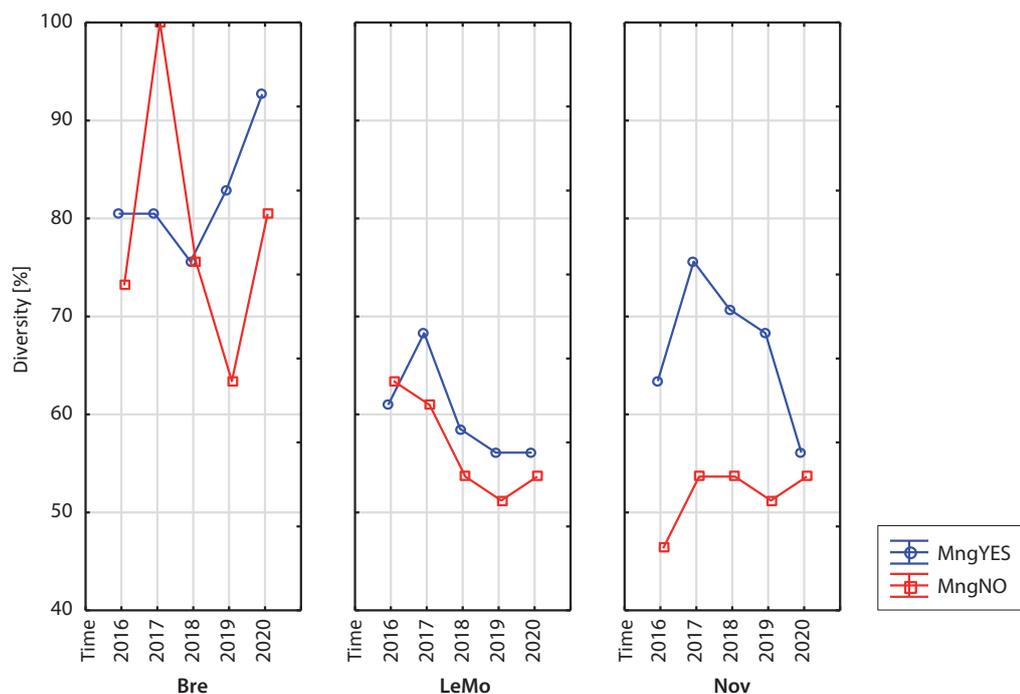


Fig. 9 Biodiversity of unmanaged (MngYES, blue) and unmanaged (MngNO, red) plots in T1.9 biotope study sites: Bre – Březina, LeMo – Lenora Molinion, Nov – Nová Pec. A plot designated as managed in the Lenora Molinion site was left unmown since 2017.

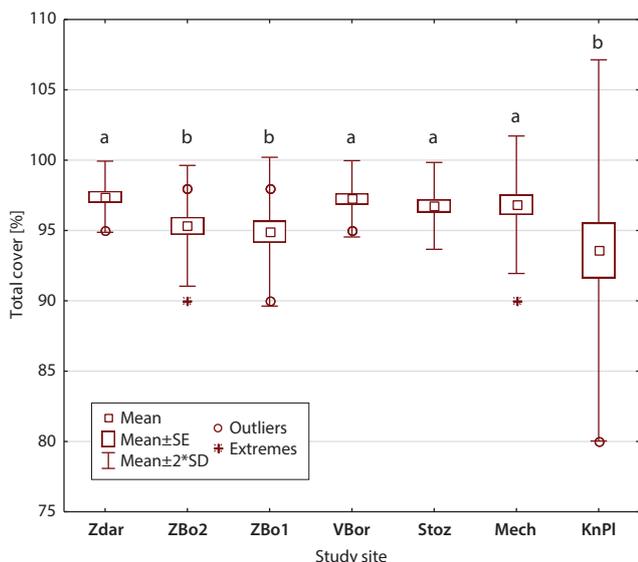


Fig. 10 One-way ANOVA. Differences among study sites in total cover are shown. Mean values (points), SE (boxes) and SDs (bars) are displayed. Letters above the bars indicate results of post-hoc comparisons; i.e. different letters mark statistically different values. Localities: Zdar – Žďárek, ZBo2 – Zadní Bor 2, ZBo1 – Zadní Bor, VBo1 – Velký Bor, Stoz – Stožecké louky, Mech – Mechov, KnPI – Knížecí Pláně.

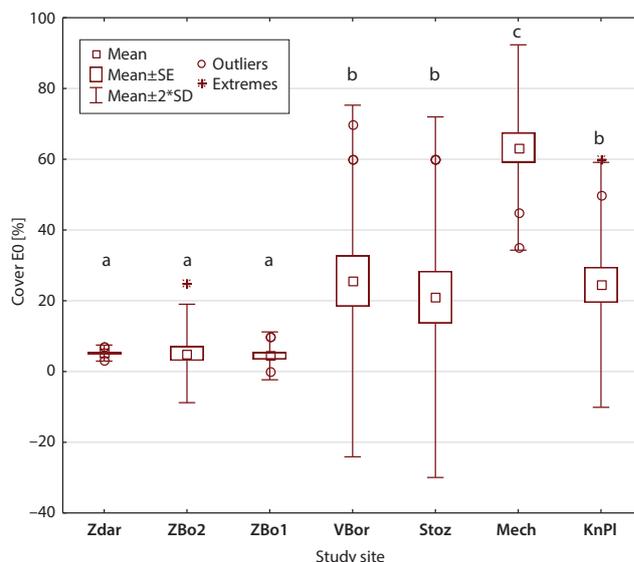


Fig. 11 One-way ANOVA. Differences among study sites in moss cover are shown. Mean values (points), SE (boxes) and SDs (bars) are displayed. Letters above the bars indicate results of post-hoc comparisons; i.e. different letters mark statistically different values. For names of localities see Fig. 10.

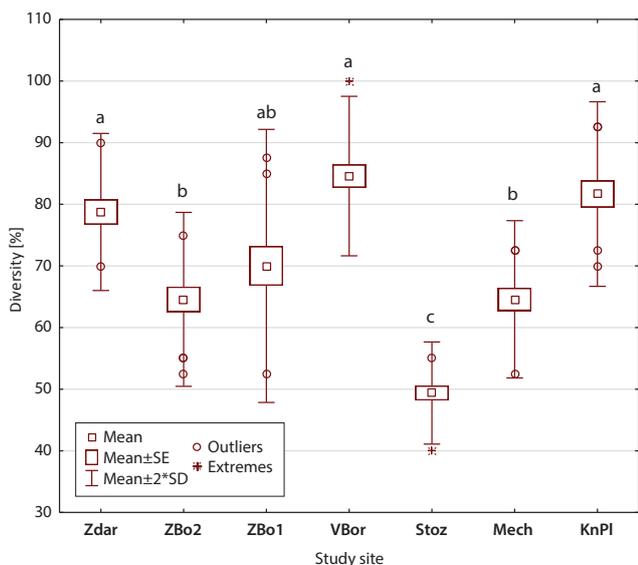


Fig. 12 One-way ANOVA. Differences among study sites in diversity calculated as a % of species out of the total number of species recorded in all R2.2 biotope study sites are shown. Mean values (points), SE (boxes) and SDs (bars) are displayed. Letters above the bars indicate results of post-hoc comparisons; i.e. different letters mark statistically different values. For names of localities see Fig. 15.

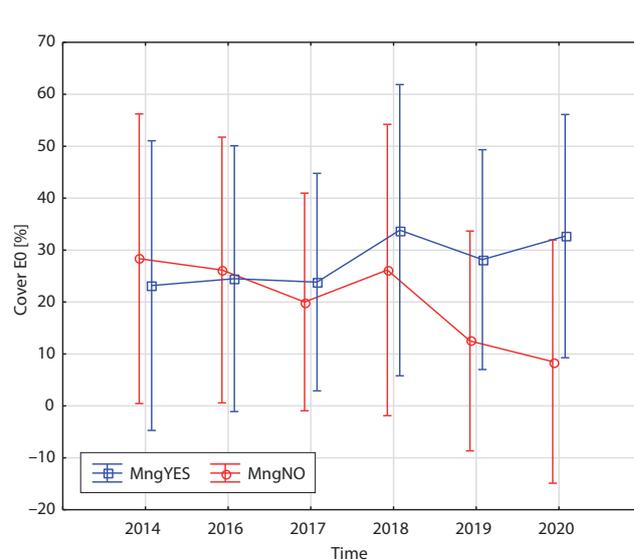


Fig. 13 Moss covers (Eo) in managed (MngYES, blue) and unmanaged (MngNO, red) plots in R2.2 biotope study sites. Mean (points) and 95% confidential intervals (bars) are shown.

coverage, no statistically significant differences between managed and unmanaged plots were found.

The moss cover differed significantly among the study sites (Figs 11, 15) as well as between managed and unmanaged plots within single study sites (Figs 13, 15). In the Zadní Bor 1 and Žďárek study sites, the moss cover did not exceed 10% during the entire monitoring period. On the contrary, the moss cover was approximately

80% in the Mechov and Velký Bor study sites. Different study sites varied in moss cover during our study period. For example, in the Knížecí Pláně study site, which was planned for mowing but remained unmown in 2019 and 2020, the moss cover increased. Similar trends were recorded in the Stožecké louky study site, where the plot, planned to be managed, was left unmown in 2019 and 2018. In this case, the 10% moss cover recorded in 2014

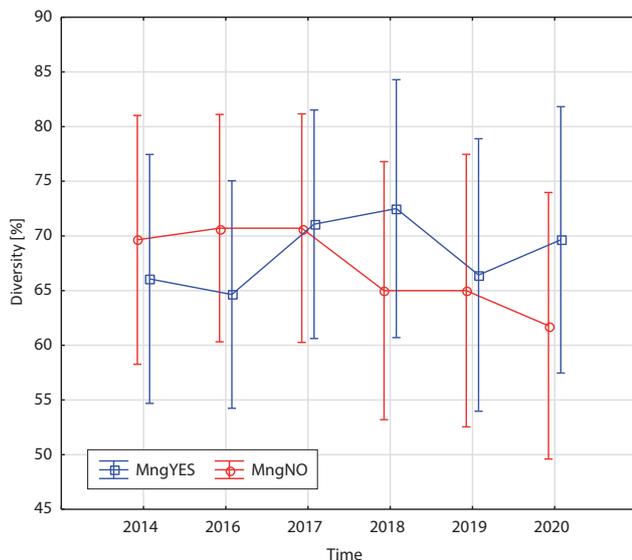


Fig. 14 Diversity in managed (MngYES, blue) and unmanaged (MngNO, red) plots in R2.2 biotope study sites. Mean (points) and 95% confidential intervals (bars) are shown.

increased to 60% in 2018–2020. At the same locality, the moss cover in the control, unmanaged plot, fluctuated between only 1–3% throughout the monitoring period. Significantly higher moss cover in a control plot, i.e. unmanaged, was recorded in the Velký Bor study site, where 70% of moss cover recorded in 2014 decreased to only 10% in 2020. Moss cover in the managed plot of this study site fluctuated between 5–15% during the entire study period.

Study sites differed in their diversity calculated as a % of species recorded in all R2.2 biotope study sites (Figs 12, 15). The highest diversity was found in the Velký Bor study site, where 40 plant species were found in the unmanaged plot in 2014 when monitoring began. In 2020, only 33 species were recorded in the same plot. Its paired managed plot hosted 31–34 plant species during the entire study period. With a few exceptions, there were no significant differences in the diversity of managed and unmanaged plots within single study sites. The Zadní Bor 1 and Žďárek study sites showed slightly higher diversity in the managed plots. Diversity slightly increased in managed plots in the Knížecí Pláně and Stožecké louky study sites, however, these plots were unmown in 2019 and 2020.

Discussion

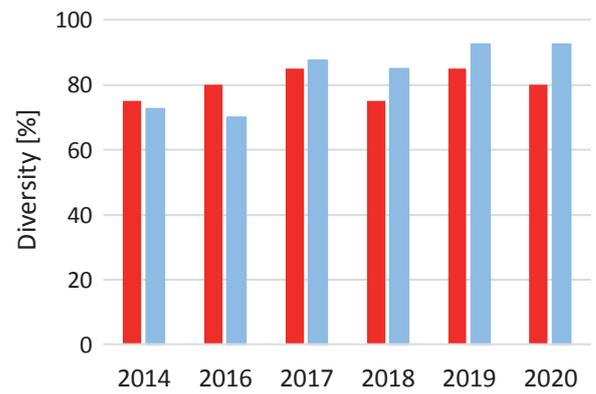
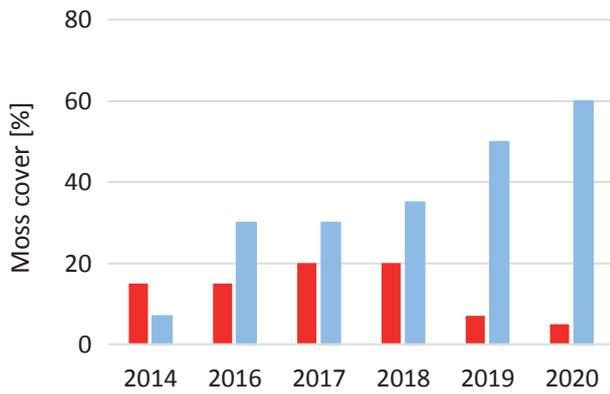
Several gaps and changes in regular management of meadows, where permanent plots were located, partly affected the results of this study (Table 5). There were various reasons for missing seasonal mowing, usually logistical obstacles faced by the owners or tenants of these meadows. These consequences are discussed in detail for individual types of meadow habitats.

Logistical obstacles and lack of capacity partly limited the design of our monitoring too. To avoid the effect of subjective evaluation, a team of only two highly experienced botanists collected data from all study sites during the entire study period. Because of this time-consuming fieldwork, no replications of monitored pair plots could be set in each study site. We believe that despite these shortcomings in monitoring design, the results of this preliminary study elucidate numerous relevant findings and experiences.

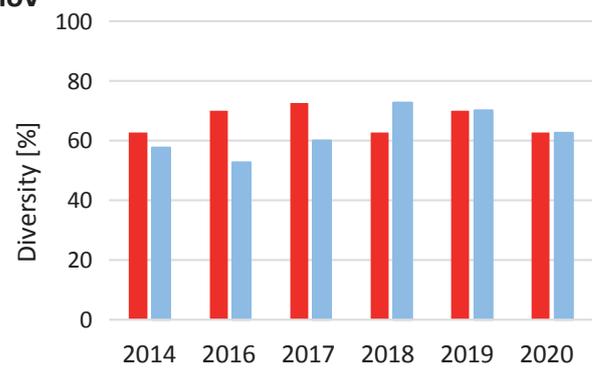
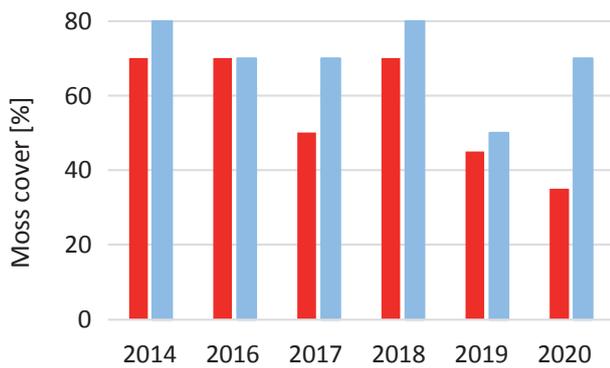
Analyzes of data from the **T2.3B biotope** study sites confirmed our field observations and experiences. There are significant differences among the monitored study sites, which are caused both by geographical differences among the study sites (discrete altitudes, hydrological and soil conditions etc.) and by their differing management histories. However, our results from a majority of study sites show that thorough management is necessary for successful conservation of this biotope. Mowing, a combination of grazing and mowing, grazing, or grazing with mowing of the remainder are considered suitable management practices for the *Nardus* grasslands (Háková et al. 2000; Korzeniak 2016). Machine mowing is insufficient to improve the condition of the habitat and increase species diversity, as it leaves grass insufficiently cropped, with grass litter remaining onsite. Careful digging and removal of biomass is crucial. During machine hay raking, the moss layer is usually not disturbed sufficiently and thus no gaps occur, a requirement for regeneration of many plant species. Therefore, some authors suggest using rotators to support the creation of gaps in dense *Nardus* meadows (Kurtogullari et al. 2019).

There was an increase of biodiversity, particularly in the managed plot recorded in the Kvilda Vilémov study site, which was mowed in 2016 and 2019 and grazed in 2017, 2018 and 2020, each year at a different time. This result could indicate that a combination of mowing and grazing can support biodiversity of the T2.3B biotope. The Filipova Huť and Chlum study sites were mown only in 2016 and 2017. They have been unmanaged since 2018 (Table 5). However, the changes in these two sites were ambiguous. In the Filipova Huť study site, (planned but unmown since 2018), plot diversity fluctuated slightly year to year. However, this plot's diversity was slightly higher than in the unmanaged (control) plot from the beginning until the end of the monitoring period. A different situation was recorded in the Chlum study sites, unmanaged since 2018. In this study site, total coverage increased with the end of management, with 38 species recorded in 2019. It was the highest number of species recorded in monitored plots of the T2.3B biotope. Species from the surrounding unmanaged areas of the study site (e.g. *Calluna vulgaris*, *Lembotropis nigricans*) have invaded the previously mown area. There were also problems with appropriate management in the Lenora and Šindlov study sites, which were mown according to plan in 2016–2018, but in 2019 and 2020 only the monitored plots were

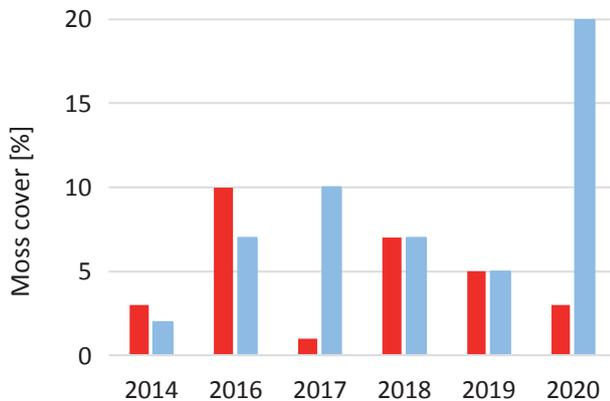
Knížecí Pláně



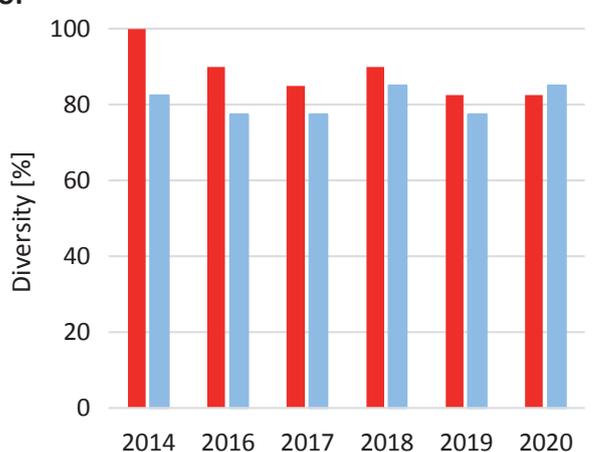
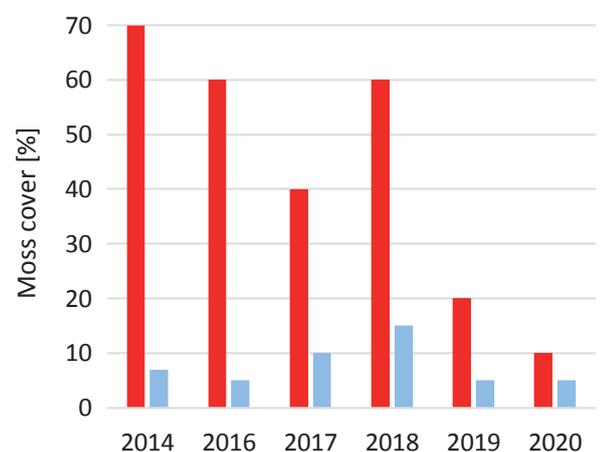
Mechov



Šindlov



Velký Bor



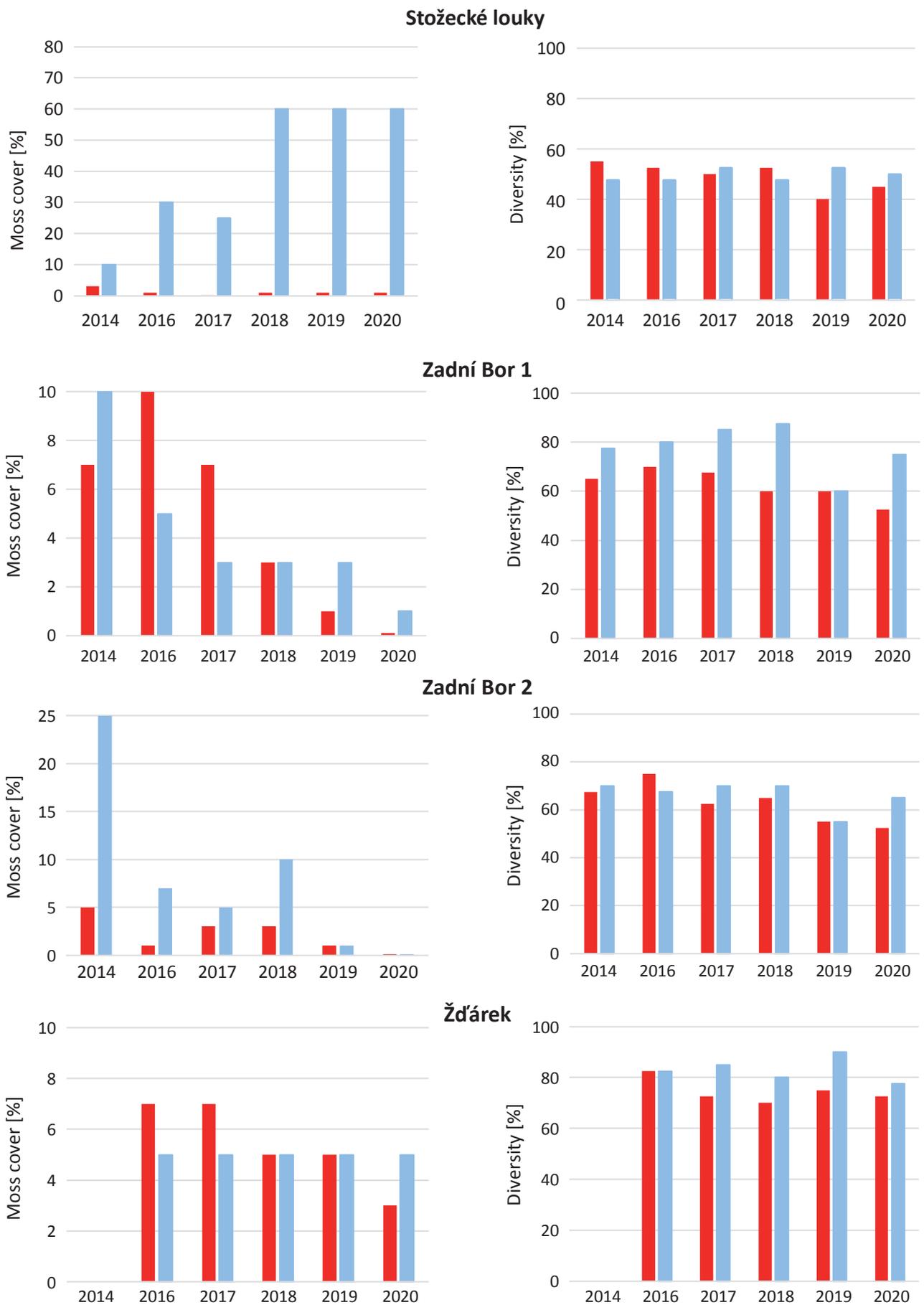


Fig. 15 Moss cover and diversity recorded in study sites in 2014 and 2016–2020. Red columns – unmanaged (i.e. control) plots, blue columns – managed plots. Different scales were used for y-axes to improve visibility of moss cover.

Table 5 Real management measures applied in plots with planned mowing in 2014, 2016 – 2020. M – mown, G – grazed, 0 – without management.

	Study site	2014	2016	2017	2018	2019	2020	Notes
T2.3B	Filipova Huť		M	M	0	0	0	
	Chlum		M	G	0	0	0	unmown only grazed in May 2017
	Kvilda Vilémov		M	G	G	M	G	unmown only grazed in Aug 2017, Oct 2018, Sep 2020
	Lenora Markovec		M	M	M	M	M	
	Šindlov	M	M	M	M	M	M	
	Zhůří near Rejštejn		M	M	M	M	G	only machine mowing in 2019
T1.9	Březina		M	M	M	M	M	
	Lenora Molinion		M	0	0	0	0	
	Nová Pec		M	M	M	M	M	
R2.2	Knížecí Pláně	M	M	M	M	0	0	
	Mechov	M	M	M	M	0	M	
	Stožecké louky	M	M	M	M	0	0	
	Velký Bor	M	M	M	M	M	0	
	Zadní Bor 1	M	M	M	0	M	0	
	Zadní Bor 2	M	M	M	0	M	0	
	Ždárek		M	M	M	M	M	

hand mown (Table 5). The Zhůří site is the most disparate from this group of study sites. This is a former fallow, which was managed irregularly after the end of plowing in the 1990's. The site was mown by hand in 2016–2018, machine mown in 2019 and not mowed but grazed by sheep in September 2020. The increase of diversity here has been very gradual – from 21% in 2016 to 26.3% in 2020. One of the central problems is the lack of diaspores of the target species, chiefly due to the large area of this site and very slow spread of seeds (e.g. through ants).

We can summarize that regular mowing, raking and a mix of mowing and grazing are the best measures for *Nardus* meadows. In the Kvilda and Lenora study sites, we note that good management can improve the condition of grasslands. The Šindlov and Filipova Huť study sites host more or less stable plant communities and with management no significant changes will likely occur. However, without maintenance management, negative changes – particularly a decline in species diversity – could occur in ten or more years.

In the **T1.9 biotope**, it is obvious that the three monitored study sites each show marked differences. The Březina study site represents a species-rich drier type of *Molinion* meadow, the Lenora Molinion study site is a floodplain type and the Nová Pec study site is a poor acidic type. Unfortunately, management of these *Molinion* meadows has been poor, without the regular mowing and high-quality biomass excavation considered crucial for this type of habitat (Kulik 2014). The Lenora Molinion study site was mown only in 2016 and left unmanaged since 2017. This is why the results on the unmown plot and the plot planned to be mown differ little. This locality has to be excluded from some analyses.

In the Nová Pec study, higher diversity was recorded in the managed plot than in the control plot, however there has been a partial decrease in the managed plot since 2018. A possible cause is a change in management methods, noted in the quality of litter raking.

Diversity slightly increased in the mown plot located in the Březina study site, which was the only regularly mown T1.9 biotope during the entire study period. This study site shows lower cover of mosses since the beginning of monitoring. However, we found a statistically significant difference in moss cover and diversity between mown and unmown plots during the study period. In this study site, the mown plot showed higher moss cover and higher diversity. Differences of moss cover between mown and unmown plots were not as significant as found in the Nová Pec study site. Diversity recorded in the mown plot located in the Březina study site slowly increased during our study period – from 80.5% in 2016 to 93% in 2020.

Our study results and field experience show that current management, recently carried out in the monitored *Molinion* meadows (T1.9 biotope), does not effect species diversity significantly. We surmise that inconsistent and poor quality management explain this situation. Experience from other areas, for example SAC Boletice (Vydrová and Grulich 2018), shows that regular mowing positively affects species diversity. With well-executed management, differences in the species richness of *Molinion* meadows should be visible and recordable after several (three-five) years.

The largest group of study sites, seven, were assigned to the **R2.2 biotope** – fen meadows. Monitoring of fen meadows began in 2014, in 2015 the monitoring was suspended and from 2016 to 2020 the areas were monitored

annually. In the case of fen meadows, the planned management was imperfectly performed in all study sites in each year. Some plots, which were planned to be managed, were not mown one or more times during the entire study period (Table 5).

We found differences in the total coverage, the moss cover and diversity among study sites. They differ in their abiotic conditions (principally hydrology), history of management and human intervention (drainages etc.).

The Knížecí Pláně, Velký Bor and Žďárek study sites are in relatively good condition, with only slight degradation. These species-rich sites are also more stable, so changes are likely to be slow. The Mechov study site represents a different vegetation type (transitional mire) with a significantly higher moss cover. In the long term, it will be appropriate to monitor the spread of *Carex brizoides* in this site. The Stožecká louka study site is of another vegetation type with the strong effect of wetting probably a cause of the lowest species diversity.

The Zadní Bor 1 and 2 study sites are significantly affected by successive changes because their water regime was fundamentally damaged in the past. Their vegetation is unstable and both study sites show a similar trend in the decrease of moss cover during the study period. The results thus far suggest that mowing will probably not have a very large and rapid effect on improving the quality of the habitat in these previously drained study sites. However, the observed changes to date may also be influenced by the fact that in 2018 and 2020 the managed plot was left unmown.

Water regime quality is a crucial parameter for fen meadows (R2.2 biotope), however their restoration is possible (Isselstein et al. 2002; Billeter et al. 2007). Regular and well-executed management must be ensured. Even in sites with only slightly changed water regimes, long-term management is necessary and significant transformation is likely to take several years (five or more). In sites with strong wetting (the Stožecké louky study site), again, high quality management measures are crucial. The results strongly demonstrate the failures of proper management, both in terms of fluctuations in dominants and changes in the moss cover.

Conclusions

We can conclude that appropriate and carefully executed management is a necessary tool for ensuring proper care of conservationally valuable habitats, including grasslands of European importance protected under the Natura 2000 network. Monitoring is an essential tool to observe management quality and improve care. Results of this preliminary study deliver a substantial set of experiences that can be used to improve the management and monitoring of Natura 2000 habitats and other species rich meadows occurring in the Šumava NP.

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Appendix 1: Habitats that are subject to protection in SAC Šumava. Priority habitats are marked by *. Grassland habitats are in bold.

Code	Habitats of Annex I of Habitats Directive	Biotope units for mapping (see Chytrý et al. 2001, 2010)
3130	Oligotrophic to mesotrophic standing waters with vegetation of the <i>Littorelletea uniflorae</i> and/or of the <i>Isoëto-Nanojuncetea</i>	M2.2 – Annual vegetation on wet sands M3 – Vegetation of perennial amphibious herbs V6 – <i>Isöetes</i> vegetation
3150	Natural eutrophic lakes with <i>Magnopotamion</i> or <i>Hydrocharition</i> – type vegetation	V1 – Macrophyte vegetation of naturally eutrophic and mesotrophic still waters
3160	Natural dystrophic lakes and ponds	V3 – Macrophyte vegetation of oligo lakes and ponds
3260	Water courses of plain to montane levels with the <i>Ranunculion fluitantis</i> and <i>Callitricho-Batrachion</i> vegetation	V4A – Macrophyte vegetation of water streams with currently present aquatic macrophytes
4030	European dry heaths	T8.2B – Secondary submontane and montane heaths without <i>Juniperus communis</i>
5130	<i>Juniperus communis</i> formations on heaths or calcareous grasslands	T8.2A – Secondary submontane and montane heaths with <i>Juniperus communis</i>
6230*	Species-rich <i>Nardus</i> grasslands, on siliceous substrates in mountain areas (and submountain areas, in Continental Europe)	T2.1 – Subalpine <i>Nardus</i> meadows T2.3B – Submontane or montane <i>Nardus</i> meadows without <i>Juniperus communis</i>
6410	<i>Molinia</i> meadows on calcareous, peaty or clayey-siltladen soils (<i>Molinion caeruleae</i>)	T1.9 – Intermittently wet <i>Molinia</i> meadows
6430	Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels	A4.2 – Subalpine tall-forb vegetation A4.3 – Subalpine tall-fern vegetation T1.6 – Wet <i>Filipendula</i> grasslands
6510	Lowland hay meadows (<i>Alopecurus pratensis</i>, <i>Sanguisorba officinalis</i>)	T1.1 – Mesic <i>Arrhenatherum</i> meadows
6520	Mountain hay meadows	T1.2 – Montane <i>Trisetum</i> meadows
7110*	Active raised bogs	R3.1 – Open raised bogs R3.3 – Bog hollows
7120	Degraded raised bogs still capable of natural regeneration	R3.4 – Degraded raised bog
7140	Transition mires and quaking bogs	R2.2 – Acidic moss-rich fens R2.3 – Transition mires
8220	Siliceous rocky slopes with chasmophytic vegetation	S1.2 – Chasmophytic vegetation of siliceous cliffs and boulder screes A6B – Acidophilous vegetation of alpine cliffs
9110	<i>Luzulo-Fagetum</i> beech forests	L5.4 – Acidophilous beech forests
9130	<i>Asperulo-Fagetum</i> beech forests	L5.1 – Herb-rich beech forests
9180*	<i>Tilio-Acerion</i> forests of slopes, screes and ravines	L4 – Ravine forests

Appendix 2: Study sites. A description of the study sites, their brief histories, management and phytosociological classification following Moravec (1994) are presented together with positions of permanent plots, their codes, GPS coordinates and elevations.

Name	Březina	Code	Bre	Biotope	T1.9
GPS coordinates	48°53'58.78"N, 13°50'55.54"E			Elevation [m a.s.l.]	754
The study plots, located on a gentle SE slope above the Vltava river floodplain, were established in 2016. Vegetation type corresponds to the <i>Junco effusi-Molinietum caeruleae</i> ass., Tüxen 1954. The area is without significant wetting, grasses dominate here. The canopy is tall, uniform, without visible gaps nor open spots. In the past, this meadow was likely mowed regularly. Currently, machine mowing is applied. No significant negative effects were recorded here.					
Name	Filipova Huť	Code	FilHut	Biotope	T2.3B
GPS coordinates	49°01'47.62"N, 13°31'16.4"E			Elevation [m a.s.l.]	1105
The study plots, established in 2016, are located on a gentle slope with a south exposition between a main road and the Filipohuťský creek. Local vegetation is close to the <i>Festuca capillatae-Nardetum strictae</i> ass., Klika et Šmarda 1944. In the past, drainages likely disturbed the water regime of this site. More intensive management measures were applied before the Šumava NP was established. The area is without visible wetting, the canopy is lower with predominate grasses. Currently, machines are used for mowing. The eutrophication effects coming from the nearby road were detected in the area, however, not yet within our study plot. Some larger grass species, e.g. <i>Dactylis glomerata</i> , have expanded from the road ditches.					
Name	Chlumské stráně	Code	Chlum	Biotope	T2.3B
GPS coordinates	48°51'46.51"N, 13°54'18.36"E			Elevation [m a.s.l.]	764
The study plots, established in 2016, are located on a gentle SW slope above the Vltava river floodplain. It is in the vicinity of a biotope T8.2B – <i>Secondary submontane and montane heaths</i> . Local vegetation is classified as a transition between the <i>Campanulo rotundifoliae—Dianthetum deltoidis</i> ass., Balátová-Tuláčková 1980 and <i>Vaccinio-Callunetum vulgaris</i> ass., Búker 1942. The vegetation is not homogeneous. In the past, the site was likely grazed. The site was damaged by drought in 2019 and strongly marked by game in 2020. The NP Administration has failed to ensure regular management of this site since 2018.					
Name	Knížecí Pláně	Code	KnPI	Biotope	R2.2
GPS coordinates	48°57'27.8"N, 13°37'44.6"E			Elevation [m a.s.l.]	990
The study plots, established in 2014, are located on a NNE gentle slope above the Vltavský creek. Vegetation corresponds to the <i>Caricetum nigrae</i> ass., Braun 1915. There are visible remnants of old drainages (created before 1945), apparently this site was grazed or harvested for litter. The canopy is homogeneous, dense, with predominant sedges and grasses. Currently, machines are used for mowing of the site. However, the remains of unharvested grass and litter remain.					
Name	Kvilda – Vilémov	Code	KviVil	Biotope	T2.3B
GPS coordinates	49°0'42.7"N, 13°35'18.27"E			Elevation [m a.s.l.]	1139
The study plots, established in 2016, are located on a steep south slope above a local road from Kvilda to Borová Lada, in the Vltava river valley. The vegetation corresponds to the <i>Campanulo rotundifoliae — Dianthetum deltoidis</i> ass., Balátová-Tuláčková 1980. This historically grazed grassland was mown with machines in 2016 and 2019 and sheep-grazed in 2017, 2018, and 2020. The canopy is a quite tall and dense. Grasses predominate and members from the <i>Apiaceae</i> family are abundant too. The current expansion of <i>Arrhenatherum elatius</i> indicates eutrophication, probably a result of air pollution.					
Name	Lenora – Molinion	Code	LeMo	Biotope	T1.9
GPS coordinates	48°54'49.69"N, 13°49'23.42"E			Elevation [m a.s.l.]	751
The study plots, established in 2016, are located in the Vltava floodplain and the Olšinka creek flows nearby. Vegetation is approaching the <i>Junco effusi-Molinietum caeruleae</i> ass., Tüxen, 1954. In the past, the site was most likely mowed. The canopy is tall, very dense; patches of grasses, sedges and herbaceous plants alternate. The site was flooded in 2019. Machines are used for mowing of this site, however some unmown segments together with decomposed litter are very common here.					
Name	Lenora – za Markovcem	Code	LenMar	Biotope	T2.3B
GPS coordinates	48°54'37.37"N, 13°47'14.02"E			Elevation [m a.s.l.]	769
The study plots, established in 2016, are located on a gentle NW slope above the Řasnice river floodplain. The vegetation is classified as the <i>Festuca capillatae-Nardetum strictae</i> ass., Klika and Šmarda 1944. Historically, the site was probably grazed and some intensification of management were tried before 1990. The canopy is dense and lower grass species predominate here. Currently, machine mowing is applied. Collecting of hay is imperfect and litter accumulates on the site.					
Name	Mechov	Code	Mech	Biotope	R2.2
GPS coordinates	49°05'04.0"N, 13°27'37.5"E			Elevation [m a.s.l.]	848
The study plots, established in 2014, are located on a gentle W slope between the Vchynice-Tetov floating channel and the Plavební creek. Vegetation was classified as the <i>Caricetum nigrae</i> ass., Braun 1915. In the past, this site was probably mowed for litter. Historical interventions in the water regime are evident. <i>Carex brizoides</i> spreads from the edges of this site. Currently, machine mowing is used, nevertheless collecting of hay and removing of litter is imperfect.					

Name	Nová Pec – Molinion	Code	Nov	Biotope	T1.9
GPS coordinates	48°47'38.72"N, 13°56'34.46"E			Elevation [m a.s.l.]	730
The study plots, established in 2016, are located on a flat terrace above the Jezerní creek floodplain. Vegetation is classified as the <i>Junco effusi-Molinietum caeruleae</i> ass., Tüxen 1954. It is a drier and species-poorer type of this association. In the past, the site was probably regularly mown, then abandoned, and a succession of woody species began. <i>Carex brizoides</i> invades this meadow too. Currently, the site is mown with machines; hay and litter are not carefully removed.					
Name	Stožecké louky	Code	Stoz	Biotope	R2.2
GPS coordinates	48°52'24.9"N, 13°48'21.9"E			Elevation [m a.s.l.]	805
The study plots, established in 2014, are located in a flat spring area near the Mlýnský creek. Vegetation is classified as the transition between the <i>Caricetum nigrae</i> ass., Braun 1915 and <i>Sphagno recurvi-Caricetum rostratae</i> ass., Steffen 1931. In the past, the site was likely mowed for litter and the water regime modified. A succession of tall vegetation (e.g. <i>Filipendula ulmaria</i> , <i>Scirpus sylvaticus</i> and <i>Carex brizoides</i>) has begun since the site was abandoned. The canopy is tall, medium dense, species of the <i>Cyperaceae</i> family are common. Machine mowing of this site commenced in 2014.					
Name	Šindlov	Code	Sindlo	Biotope	T2.3B
GPS coordinates	49°01'28.9"N, 13°39'28.9"E			Elevation [m a.s.l.]	936
The study plots, established in 2014, are located on a terrace between the Vydří and Studený creeks. The vegetation is close to the <i>Festuco capillatae-Nardetum strictae</i> ass., Klika and Šmarda 1944. It is a species-poorer type of this association. The canopy is homogeneous over a large area, medium-high, medium-dense. The site is mown with machines.					
Name	Velký Bor	Code	VBor	Biotope	R2.2
GPS coordinates	49°06'15.2"N, 13°25'24.2"E			Elevation [m a.s.l.]	838
The study plots, established in 2014, are located on a gentle SE slope above the Křemelná river. Vegetation was classified as the <i>Caricetum nigrae</i> ass., Braun 1915. This is a species-richer type of the association with a dense canopy. In the past, the site was likely mowed for litter. Old drainages are still visible and partially functional. Currently, machines are used for mowing of this site.					
Name	Zadní Bor 1	Code	ZBor1	Biotope	R2.2
GPS coordinates	49°09'24.2"N, 13°21'28.6"E			Elevation [m a.s.l.]	857
The study plots, established in 2014, are located on a gentle SE slope above the nameless right-hand tributary of the Křemelná river. The vegetation is classified as a degraded type of the <i>Caricetum nigrae</i> ass., Braun 1915. In the past, this large area was drained and managed as a litter meadow. The flow of the nameless stream was artificially deepened and many drainages are connected to it. Machines are used for mowing of this site, nevertheless quality of management is poor. Repeatedly, large unmown segments and much unremoved litter were observed on this site.					
Name	Zadní Bor 2	Code	ZBor2	Biotope	R2.2
GPS coordinates	49°09'26.5"N, 13°21'26.3"E			Elevation [m a.s.l.]	859
The study plots, established in 2014, are located on a gentle SE slope above the nameless right-hand tributary of the Křemelná river, close to the plot ZBor1. The vegetation is classified as the <i>Caricetum nigrae</i> ass., Braun 1915, a degraded type. In the past, this large area was drained and mown for litter. The flow of the unnamed stream was artificially deepened and many drainages were connected to it. Machines are used for mowing of the site, nevertheless management is poor. Repeatedly, large unmown segments and much unremoved litter were observed on this site.					
Name	Zhůří u Rejštejna	Code	ZhuRej	Biotope	T2.3B
GPS coordinates	49°04'56.4"N, 13°33'30.2"E			Elevation [m a.s.l.]	1146
The study plots, established in 2016, are located on a gentle W slope above the road Horská Kvilda – Rejštejn. The vegetation can be classified as an uncertain type of the <i>Violion caninae</i> ass., Schwickerath in 1944. Species diversity is low, grasses dominate and dicotyledonous plants are rare. The area was plowed up until about 1990 and then abandoned for 30 years. Machine mowing was applied in 2016–2018. In 2019, the whole site (including a control plot) was grazed by sheep. Sheep grazing took place again in 2020; the control plot remained untouched.					
Name	Žďárek	Code	Zda	Biotope	R2.2
GPS coordinates	48°55'33.89"N, 13°38'50.9"E			Elevation [m a.s.l.]	1051
The study plots, established in 2016, are located on a gentle SSE slope in a spring area of the Židovský creek valley. The vegetation is classified as the <i>Caricetum nigrae</i> ass., Braun 1915. It is a species rich community. The canopy is dense, without gaps. In the past, the site was probably mown for litter. Several old drainages exist at the edges of this site. At present, machines are used for mowing of this site.					

EFFECT OF CLIMATE CHANGE ON FIR FOREST COMMUNITIES IN THE MOUNTAINS OF SOUTH-CENTRAL GREECE

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ABSTRACT

The endemic oro-Mediterranean fir (*Abies* spp.) forests in Greece are valuable ecosystems with high ecological and economic importance. In the mountains in south-central Greece, the availability of moisture plays a crucial role in the floristic composition, structure and distribution of fir forest communities. Because of the predicted adverse climate changes for the Mediterranean zone, suitability of many habitats for the growth of fir forests will change. This study aims to quantify the degree to which these forests will be affected by climate change. Current and future climatic conditions in the area studied were estimated for two periods of time (2041 – 60, 2061 – 80) and two climate change scenarios (RCP 4.5, RCP 8.5). Vegetation relevés were classified and ordinated. Recursive partitioning was used to reveal the most important factor for discriminating the main plant communities and determining the ecological threshold between them. Current and future suitability of bioclimatic space for fir forest communities was identified. The effect of climate change was assessed based on predicted changes in the potential distribution, in terms of size and location, of fir forest communities. Two plant communities that reflect the differentiation of fir forests into xerophytic and mesophytic forest types are described. Among several climatic variables, spring drought was the best factor discriminating these two forest communities. The quantification of the threshold for drought in these two plant communities was used to predict their potential distribution in the area studied. Potential distribution of fir forests in south-central Greece will change in the future, due to a reduction in suitable bioclimatic space and shift to higher altitudes. The effect is expected to be greater for xerophytic forest communities, especially in the worst-case scenario (RCP 8.5) and in 2061–80.

Keywords: *Abies cephalonica*; *Abies x borisii-regis*; exposure; MaxEnt; sensitivity; Sterea Ellas

Introduction

Fir (*Abies* spp.) forests are a widespread landscape feature in the mountains of southern and central Greece, with high ecological and economic importance. Three closely related fir taxa occur in Greece, *Abies cephalonica* Loudon (Greek fir), *A. alba* Mill. (silver fir) and their natural hybrid *A. x borisii-regis* Mattf. (Mitsopoulos and Panetsos 1987; Christensen 1997). The endemic Greek fir is the dominant forest species in the mountains of Peloponnese (southern Greece) and Sterea Ellas (south-central Greece). In the northern mainland part of Greece, Greek fir is replaced by *A. x borisii-regis*. The silver fir is only found in the northernmost parts of the mainland (Christensen 1997).

Although fir forests in Greece receive abundant precipitation during autumn and winter, they may suffer from drought in summer (Aussenac 2002). Drought plays a crucial role in the floristic composition, structure and distribution of coniferous forest communities in southern and south-central Greece (Bergmeier 2002; Samaras et al. 2015). In south-central Greece, there are two types of fir forest vegetation: mesophytic and xerophytic (Samaras et al. 2015). Drought is the main abiotic factor that affects and weakens fir trees, making them vulnerable to bark beetle attacks (Tsopeles et al. 2004). Periods of extreme drought can cause extensive dieback as occurred

in 1988–89 throughout Greece, and more recently in 2000–02 and 2009 on many mountains in southern and central Greece (Markalas 1992; Brofas and Economidou 1994; Raftoyannis et al. 2008).

Climate change projections for the Mediterranean zone indicate that extremely dry years will occur more frequently and drought periods will be much longer in the future (Lindner et al. 2014). Because of these environmental changes, dieback and mortality of fir forests may increase in the future. Climate change will also affect the suitability of many habitats for the growth of fir forests, which are expected to result in a change in their distribution associated with the new conditions. Vegetation dynamic models indicate a possible shift of fir forests to higher altitudes (Fyllas and Troumbis 2009; Fyllas et al. 2017). Species distribution models are another useful tool, which are extensively used to analyse the effect of climate change on different species (Elith and Leathwick 2009; Navarro-Cerrillo et al. 2018).

For the protection, conservation and rational utilization of the valuable fir forest ecosystems in Greece, it is necessary to take appropriate management measures, which will help to address the effect of climate change (Chrysopolitou and Dafis 2014). However, in order to adapt forest management to climate change, the most affected areas must be identified and the degree to which fir forests might be affected by climate change determined.

We hypothesize that:

1. potential distribution of fir forests in Greece will shift to higher altitudes;
2. climate change will mostly affect xerophytic fir forest communities

To test these hypotheses, we chose a representative area in south-central Greece covered by extensive fir forests, to analyse and quantify the degree to which these forests will be affected by climate change. Our *objectives* were:

- to estimate current and future climatic conditions in the area studied;
- to study the floristic and ecological differentiation of fir forests in the area studied;
- to identify current and future suitability of bioclimatic space for fir forests;
- to assess the effect of climate change on fir forest plant communities in the area studied.

Material and methods

Study area

The area studied is located in the central part of south-central Greece (Sterea Ellas). The area consists of three mountains (Goulinas, Vardousia and Iti), rising to 2495 m a.s.l. (summit of Korakas) and lies between 38°32'30" and 38°56'42" N and 21°57'24" and 22°29'30" E (Fig. 1).

The dominant type of forest in this region is the endemic oro-Mediterranean *Abies cephalonica* or mixed *A. cephalonica* and *A. × borisii-regis* forest, which covers 29% of the area studied. *A. × borisii-regis* is considered a natural hybrid between *A. alba* and *A. cephalonica* and is morphologically intermediate between these two species (Christensen 1997). Recent studies on the genetic variation of fir populations in Greece have confirmed that *A. × borisii-regis* cannot be distinguished from *A. cephalonica* based on isozyme or molecular markers (Fady and Conkle 1993; Scaltsoyiannes et al. 1999; Drouzas 2000) and it is often difficult to distinguish them based on morphological traits. For the purposes of this study all fir trees in the area studied are considered to belong to the species *A. cephalonica* s.l.

The dominant bedrock in the area studied is flysch, while Jurassic limestone and scree slopes (or terraces) occur to a small extent (Kallergis et al. 1970). The climate in the fir forest zone is typically Mediterranean with wet, cool winters and dry summers. Nevertheless, there are large local differences due to the complexity and variety of the topography.

Climatic analysis

The knowledge of current and future climatic/bioclimatic conditions is an important element in any assessment of the effect of climate change. We, therefore, estimated the *current local climatic conditions* in the area studied and generated high-resolution climatic maps

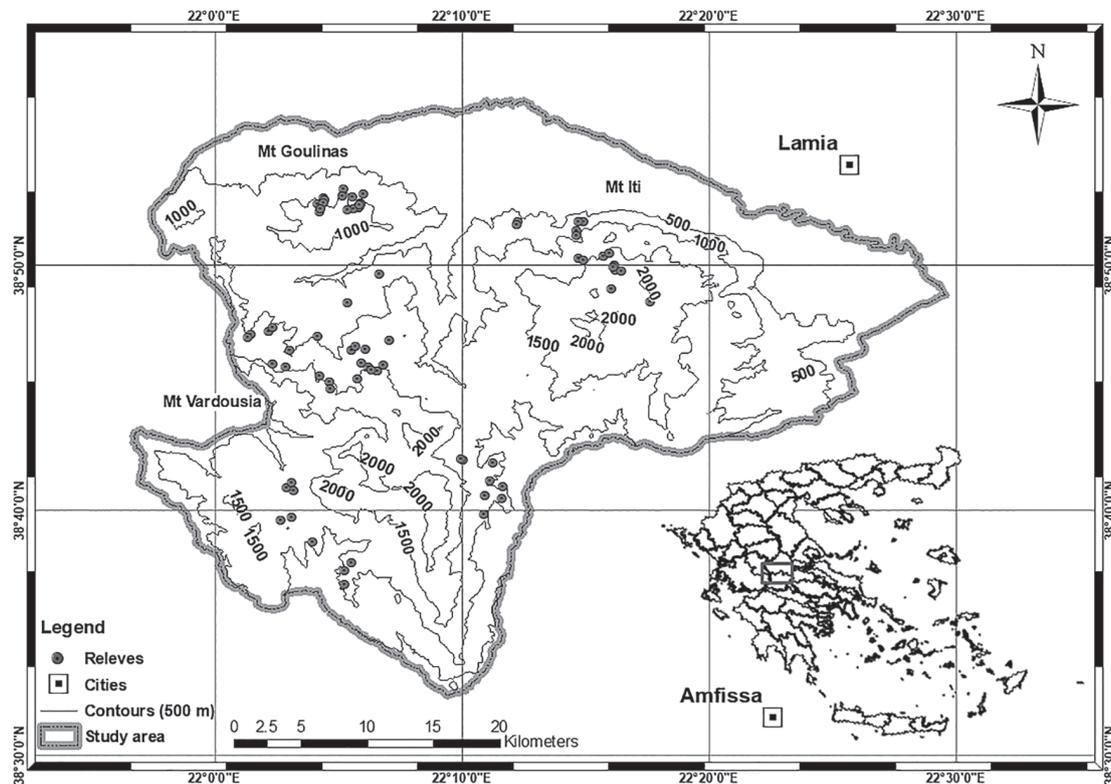


Fig. 1 Map of the are studied with the location of the 67 plots indicated by black dots. Coordinate reference system: WGS 84.

(horizontal resolution of 3 arc seconds ~ 90 m). The raster maps were generated for different time periods such as monthly periods, the driest period (4 driest months, June–September), vegetation period (April to October), annual period and four seasons.

For the spatial estimations of *air temperature* (T) and *precipitation* (P), we used ordinary kriging, linear regression and regression-kriging models. In the estimate, we accounted for altitude and the spatial correlation of the climatic data from 20 weather stations over the period 1975–2010 (Samaras et al. 2017). The computational steps follow the general framework for geostatistical mapping of environmental variables in Hengl (2009). To calculate the monthly mean of daily *global irradiation* (Rs), the r.sun model (Hofierka and Šúri 2002; Šúri and Hofierka 2004) was used in the GRASS-GIS software version 7.2 (GRASS Development Team 2017). The model considers the spatial variation of fine-scale physiographic features like altitude, surface inclination, exposure and topographic shadowing effects. For the analytical computational steps see Samaras et al. (2017). The *reference potential evapotranspiration* (PETref) was calculated by using the empirical equation of Abtew (1996), for which maximum air temperature (Tmax) and Rs values, calibrated for the local conditions, are required (Samaras et al. 2014). For the quantification of drought, we used a modified version of the Transeau's *Humidity Index* (HI – Tuhkanen 1980), which combines the two main factors related to drought. The HI was calculated as the ratio of P to PETref ($HI = P/PETref$). Nineteen bioclimatic variables were created from monthly data of P, Tmax and Tmin, using “dismo” R package (Hijmans et al. 2021).

For the estimation of the *future local climatic conditions*, we used the CHELSA-[CMIP5] climatic dataset for the years 2041–2060 and 2061–2080 (<https://chelsea-climate.org/future/>). CHELSA-[CMIP5] climatic data are based on the 1979–2013 reference CHELSA climatic dataset (Karger et al. 2017) and consist of temperature and precipitation estimates at a horizontal resolution of 30 arc seconds (1 km) in the form of raster (GeoTIFF) files. In order to take into account the uncertainty of future climates, due to different future anthropogenic greenhouse gas emissions, we used 2 different scenarios (RCP 4.5 and RCP 8.5), proposed by the IPCCs fifth assessment report (IPCC 2013). The RCP 4.5 scenario is nearly equivalent to Paris accord emission reduction, while the RCP 8.5 represent current rates of emissions (Schwalm et al. 2020). From the 36 Earth system models of the CMIP5 ensemble, we chose 5 models (CESM1-CAM5, FIO-ESM, GISS-E2-H, IPSL-CM5A-MR, MIROC5) that show the lowest amount of interdependence, according to Sanderson et al. (2015). By averaging the projected climatic conditions of the 5 models, we obtained estimates of the future temperature and precipitation for the area studied (future CHELSA climatic data). In order to further downscale the future CHELSA climatic data at a higher horizontal resolution (3 arc seconds),

we calculated temperature and precipitation changes as percentage of reference CHELSA climatic data. Then we added or extracted these changes from the raster maps of the current climatic conditions, generating the future temperature and precipitation estimates for the area studied at a horizontal resolution of 3 arc seconds (future climatic data). To simplify the calculation, we assumed that there will be no changes in the monthly mean daily global irradiation in the future. The future climatic data for temperature (means, max, min) and precipitation were finally used to estimate future possible changes in PETref and HI for different time periods and 19 more bioclimatic variables.

Vegetation analysis

The aim of the vegetation analysis was to study the floristic and ecological differences of the mesophytic and xerophytic fir forest plant communities, to delineate their distributions and, finally, predict their current distribution in the area studied.

For the vegetation analysis, 50 personal, unpublished relevés from the current distribution range of fir forests in the area studied were selected. At each location, a rectangular plot of 200 m² was sampled. In each plot, the species composition was recorded. Structural information was obtained by assessing the covers of three layers of vegetation (herb, shrub and tree layer). For all species and layers, the cover-abundance was estimated using the extended (9-point) Braun-Blanquet scale (Van der Maarel 2006). The nomenclature of vascular plants follows Dimopoulos et al. (2013, 2016). Topographic information was recorded for each plot including altitude, inclination and exposure. Exposure (measured in degrees) is considered to be a poor variable for quantitative analysis (McCune and Keon 2002) and therefore has to be transformed prior to data analysis. Two new variables were created, “exposure to the north” and “exposure to the east”, with the use of trigonometric functions (Leyer and Wesche 2007). Additional vegetation data from 17 relevés were obtained from Samaras et al. (2008, 2015).

Vegetation relevés were classified based on the floristic composition and species cover values. A hierarchical agglomerative *cluster analysis* based on Bray-Curtis distance (Bray and Curtis 1957) was performed. For the determination of diagnostic taxa for the vegetation units, fidelity values for the species were calculated using a modification of the Φ index (Tichý and Chytrý 2006). To reveal expected gradients in vegetation, *Non-Metric Multidimensional Scaling* (NMDS) was used (Kruskal 1964a,b), with Bray-Curtis distance. *Recursive partitioning* was used to derive classification trees for the response variables (plant communities) (Breiman et al. 1984), in order to reveal the most important factor for the discrimination of the main plant communities and to determine the threshold between them. All the analyses were performed in R software, version 4.0.3 (R Core Team 2020), with the use of various R packages. The computational

Table 1 Sensitivity effect categories based on the threshold values of overlap and ratio metrics.

	Overlap and Ratio Sensitivity thresholds (%)							
	< 30	30–50	50–70	70–100	100–130	130–150	150–170	> 170
Overlap effect category	Very High	High	Moderate	Low				
Ratio effect category	Very High	High	Moderate	Low	Low	Moderate	High	Very High
	Negative Effect				Positive Effect			

steps follow the general framework of the data analysis in Samaras et al. (2015).

Assessment of the effect of climate change

Effect is a function of *exposure* and *sensitivity* to climate change, without considering the adaptive capacity of a species. *Exposure* is the nature, magnitude and rate of climatic change experienced by a species (external factor) (IPCC 2007). *Sensitivity* is the degree to which a species is affected by climate change (intrinsic factor) (IPCC 2007, 2013).

The methodology used to assess the effect of climate change on fir forest plant communities is based on a modification of the analytical framework of Sajwaj et al. (2009) and Harley et al. (2010). As a measure of exposure to climate change, we used the *projected future climatic conditions* for the area studied. Sensitivity assessment was based on changes in the *potential distribution* (in terms of size and location) of *Abies cephalonica* s.l. in terms of two metrics:

1. *Overlap* – the percentage of *current potential distribution* (current suitable bioclimatic space) that is covered by the *projected future potential distribution* (future suitable bioclimatic space).
2. *Ratio* – the relative change in total potential distribution range (suitable bioclimatic space).

Overlap and ratio metrics were used to define *effect categories* based on the threshold values of Table 1 (from Sajwaj et al. 2009). Overlap indicates the need for shifting species range in order to maintain the total area of its current distribution. Ratio indicates a reduction or expansion of total suitable bioclimatic space.

To estimate the *future potential distribution* of *Abies cephalonica* s.l., we used a species distribution modeling (SDM) analysis (MaxEnt). MaxEnt (Phillips et al. 2006; Pearson et al. 2007; Phillips and Dudík 2008) is a machine learning niche model that describes the correlation between a focal species current distribution (using presence-only data) and a set of environmental variables. The SDM analysis was done using the MaxEnt software version 3.4.1 (Phillips et al. 2021). The geospatial analysis was implemented in QGIS (QGIS Development Team 2021).

The *current actual distribution* of fir forests was obtained from vegetation maps of the area studied. The maps were digitized at the same resolution with climatic data (3 arc seconds). From the current actual distribution of fir forests, we randomly selected 20% of all grid cells,

which corresponds to 11,648 occurrences of *Abies cephalonica* s.l. We repeated this procedure 10 times, creating 10 distribution datasets. Initially, 29 environmental variables were selected as predictors. Among them, PETref and HI for the 4 driest months, vegetation period, spring, summer and autumn and 19 more bioclimatic variables. In order to avoid multicollinearity among the continuous climatic variables, we calculated Pearson correlation coefficients between all possible combinations of variables. All variables with high correlations ($r > |0.75|$) were excluded from the analysis.

For each fir distribution dataset, we ran 10 models with MaxEnt using the default settings and the auto-features mode, as suggested by Phillips and Dudík (2008). Seventy five percent (75%) of fir species' occurrences were used to train MaxEnt models while the remaining 25% was used to test the accuracy of each model. The output of the model (a continuous suitability map) was converted into presence/absence data by using the Equal Training Specificity and Sensitivity threshold. The effect assessment procedure was carried out for both fir species and their plant communities.

Results

Current and future climatic conditions

Several thematic maps were created showing the current and future climatic conditions in the area studied

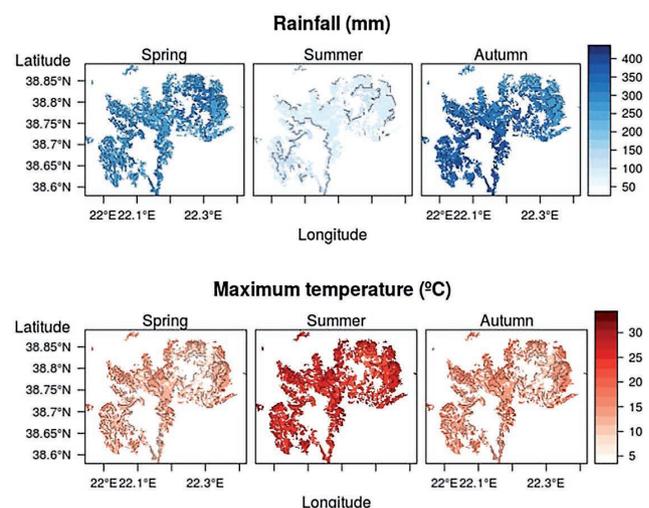


Fig. 2 Mean seasonal values of rainfall and average maximum temperature in the current distribution of fir forests in the area studied.

Table 2 Range of the predicted seasonal values for Humidity Index (HI) for two periods of time (2041–60, 2061–80) and two climate change scenarios (RCP 4.5, RCP 8.5). The HI values for the reference period (1979 to 2013) are in bold and the largest changes are underlined.

Time	Scenario	Spring	Summer	Autumn	Vegetation period
1979–2013		0.42–4.27	0.07–0.38	0.66–6.39	0.17–1.19
2041–2060	RCP 4.5	<u>0.37–3.36</u>	0.07–0.35	0.60–5.59	0.15–1.04
2041–2060	RCP 8.5	0.33–2.96	0.06–0.31	<u>0.53–5.14</u>	0.12–0.91
2061–2080	RCP 4.5	<u>0.34–3.13</u>	0.06–0.32	0.60–5.62	0.14–0.99
2061–2080	RCP 8.5	0.30–2.59	0.05–0.28	<u>0.47–4.51</u>	0.11–0.81

(Fig. 2). In the current distribution area of fir forests, mean seasonal rainfall varies between 145 to 355 mm during spring, 50 to 130 mm during summer and between 162 to 414 mm in autumn (Fig. 2). The average maximum temperature ranges between 5.3 and 21.1 °C in spring, 18.1 and 32.5 °C in summer and between 7.2 and 23.0 °C in autumn (Fig. 2). Global irradiation during vegetation period ranges from 7.6 to 22.7 MJ/m². PETref during vegetation period varies between 341 and 1119 mm and HI, for the same period, ranges from 0.17 to 1.19 (Fig. 3).

Based on both RCP greenhouse gas emissions scenarios (RCP 4.5 and RCP 8.5), HI index is predicted to gradually decline up to 2080. The largest decrease in HI is expected during spring and autumn, while smaller changes are expected during summer (Table 2).

Plant communities

The classification of the vegetation plots revealed two plant communities (Table 3), clearly distinguishable by their floristic and ecological characteristics. The two plant communities are:

Plant community A (Xerophytic forest) (Table 3, A): this community is made up of pure fir forest stands and mixed stands of *Abies cephalonica* s.l. with *Quercus petraea* subsp. *polycarpa* and occasionally *Q. frainetto* or *Q. pubescens*. The shrub layer consists mainly of *Abies cephalonica* s.l., *Juniperus oxycedrus* subsp. *oxycedrus* and *Quercus coccifera*. Many other woody species such as *Quercus frainetto*, *Fraxinus ornus*, *Ilex aquifolium* and *Phillyrea latifolia* occur at a lower frequencies in the shrub layer. It is characterized by a group of thermophilous plants indicative of dry conditions (i.e. *Quercus coccifera*, *Sedum cepaea*, *Fraxinus ornus*, *Cistus creticus*, *Asplenium obovatum*).

Plant community B (Mesophytic forest) (Table 3, B): this community is made up of pure fir forest stands and mixed stands of *Abies cephalonica* s.l. with *Fagus sylvatica* subsp. *sylvatica*. The shrub layer consists mainly of *Abies cephalonica* s.l., and *Juniperus oxycedrus* subsp. *oxycedrus*. A few other woody species (i.e. *Juniperus communis* subsp. *nana* and *Sorbus aria*) occur at low frequencies in the shrub layer. It is differentiated from the xerophytic community by a group of species indicating moderately moist conditions (i.e. *Geum urbanum*, *Arabis alpina*, *Brumus benekenii*).

Multivariate analysis of floristic data identified the main vegetation gradient, depicted by the horizontal axis (NMDS1) of the ordination diagram (Fig. 4). The two plant communities were well differentiated along the gradient, with the xerophytic community occupying the left part of the ordination diagram and the mesophytic community the right part (Fig. 4). By fitting different environmental variables onto the ordination scores, the underlying ecological gradient that explains the floristic differentiation of the fir forests was revealed. The direction and strength of the ecological gradients is represented by the direction and length of the vectors respectively (Fig. 4). The horizontal axis of the ordination diagram reflects a seasonal drought gradient that was quantified using the HI index. The left part of the ordination diagram represents areas of low altitude and humidity, while the right part more humid areas of high-altitude fir forests.

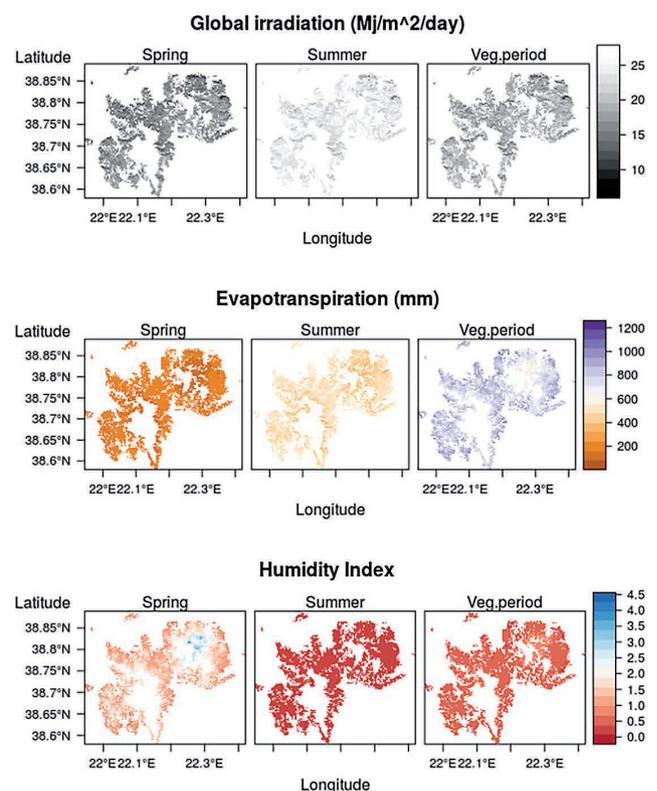


Fig. 3 Global irradiation, potential evapotranspiration and Humidity Index in spring, summer and vegetation period (April to October) in the current distribution of fir forests in the area studied.

Table 3 Synoptic table of the fir forest vegetation units (plant communities) in the area studied based on cluster analysis (Flexible beta with $\beta = -0.25$, Bray-Curtis distance). Only the diagnostic taxa are presented along with their frequency (Freq), fidelity (Φ) and indicator (IndVal) values.

Communities	A (n = 44)	B (n = 23)			
Number of relevés (n = 67)	Freq (%)	Freq (%)	Φ	IndVal A	IndVal B
Community A (Xerophytic forest)					
<i>Luzula forsteri</i> subsp. <i>rhizomata</i>	89	17	0.714	0.869	0.886
<i>Quercus coccifera</i>	66	4	0.645	0.982	0.659
<i>Crepis fraasii</i> subsp. <i>fraasii</i>	64	4	0.626	0.919	0.636
<i>Sedum cepaea</i>	50	0	0.577	1.000	0.500
<i>Carex distachya</i>	45	0	0.542	1.000	0.455
<i>Fraxinus ornus</i>	43	0	0.525	1.000	0.432
<i>Cistus creticus</i>	34	0	0.453	1.000	0.341
<i>Torilis arvensis</i>	34	0	0.453	1.000	0.341
<i>Pilosella bauhini</i> subsp. <i>magyarica</i>	52	13	0.418	0.819	0.523
<i>Trifolium grandiflorum</i>	39	4	0.417	0.932	0.386
<i>Quercus petraea</i> subsp. <i>polycarpa</i>	30	0	0.416	1.000	0.296
<i>Thymus longicaulis</i>	36	4	0.398	0.954	0.364
<i>Asplenium adiantum-nigrum</i>	27	0	0.397	1.000	0.273
<i>Asplenium onopteris</i>	25	0	0.378	1.000	0.250
<i>Hedera helix</i> subsp. <i>helix</i>	25	0	0.378	1.000	0.250
<i>Digitalis laevigata</i> subsp. <i>graeca</i>	23	0	0.358	1.000	0.227
<i>Prunella vulgaris</i>	23	0	0.358	1.000	0.227
<i>Ruscus aculeatus</i>	23	0	0.358	1.000	0.227
Community B (Mesophytic forest)					
<i>Lapsana communis</i> subsp. <i>adenophora</i>	20	96	0.762	0.833	0.957
<i>Pilosella cymosa</i> subsp. <i>sabina</i>	5	70	0.673	0.949	0.696
<i>Lamium garganicum</i> subsp. <i>garganicum</i>	5	65	0.637	0.943	0.652
<i>Stellaria media</i>	16	78	0.625	0.884	0.783
<i>Sedum hispanicum</i>	9	65	0.581	0.907	0.652
<i>Geum urbanum</i>	11	61	0.515	0.897	0.609
<i>Silene multicaulis</i> subsp. <i>multicaulis</i>	9	57	0.505	0.940	0.565
<i>Arabis alpina</i>	7	52	0.497	0.950	0.522
<i>Digitalis ferruginea</i> subsp. <i>ferruginea</i>	7	52	0.497	0.909	0.522
<i>Dactylis glomerata</i>	45	91	0.493	0.733	0.913
<i>Geocaryum capillifolium</i>	32	78	0.467	0.796	0.783
<i>Festuca rubra</i> subsp. <i>juncea</i>	0	35	0.459	1.000	0.348
<i>Verbascum epixanthinum</i>	0	35	0.459	1.000	0.348
<i>Myosotis sylvatica</i> subsp. <i>cyanea</i>	66	100	0.453	–	–
<i>Arrhenatherum elatius</i>	25	70	0.446	0.822	0.696
<i>Carduus tmoleus</i> subsp. <i>cronius</i>	0	30	0.424	1.000	0.304
<i>Ranunculus sartorianus</i>	0	30	0.424	1.000	0.304
<i>Pimpinella tragioides</i> subsp. <i>polyclada</i>	2	35	0.418	0.976	0.348
<i>Galium aparine</i>	11	48	0.399	0.902	0.478
<i>Aremonia agrimonoides</i>	73	100	0.397	–	–
<i>Daphne oleoides</i> subsp. <i>oleoides</i>	0	26	0.387	1.000	0.2609
<i>Bromus benekenii</i>	2	30	0.381	0.976	0.3043
<i>Cardamine hirsuta</i>	20	57	0.371	0.761	0.5652
<i>Acinos alpinus</i>	11	43	0.360	0.779	0.4348
<i>Silene vulgaris</i>	11	43	0.360	0.877	0.4348

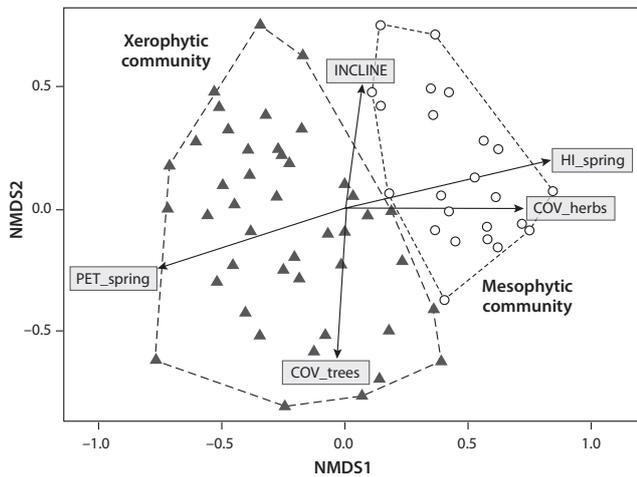


Fig. 4 Distribution of the vegetation units along the two axes (NMDS1, NMDS2) of the ordination (NMDS). The different symbols indicate vegetation units identified by the cluster analysis. The envelopes include the two main plant communities. The stress for the solution with two axes is equal to 0.18. Three environmental variables are projected as vectors on the ordination. The direction and strength of the gradients is represented, respectively, by the direction and length of the vectors.

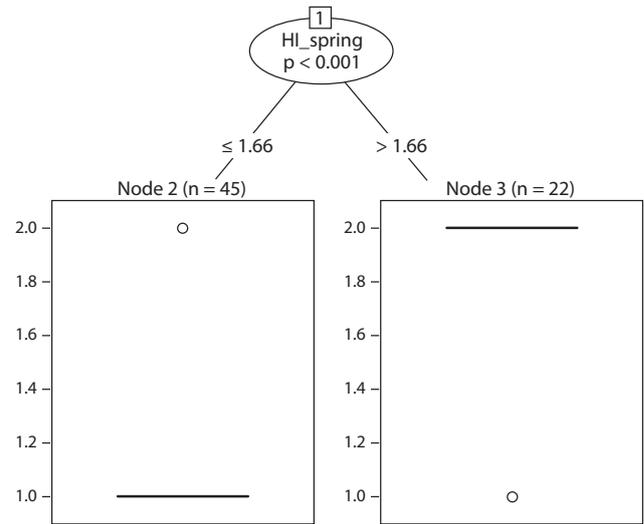


Fig. 5 Classification tree for the two fir forest communities (Node 2=Xerophytic community; Node 3=Mesophytic community) based on environmental variables. The drought threshold is indicated by HI_spring (Humidity Index during spring). The level of significance chosen was $\alpha = 0.05$.

Recursive partitioning revealed HI during spring (HI_spring) as the best discriminating variable among all the factors analysed (Fig. 5). Most of the plots from the xerophytic forest community were included in the left panel of the classification tree (Fig. 5, Node 2), while the majority of the plots from the mesophytic forest community were included in the right panel (Fig. 5, Node 3). A

xerophytic forest community is present when the value of HI in spring is less or equal to 1.66. In contrast, the occurrence of the mesophytic forest community is associated with HI_spring values of more than 1.66. No other plot subgrouping was detected using any of the remaining environmental variables analysed in this study. The above ecological threshold value was used to predict the

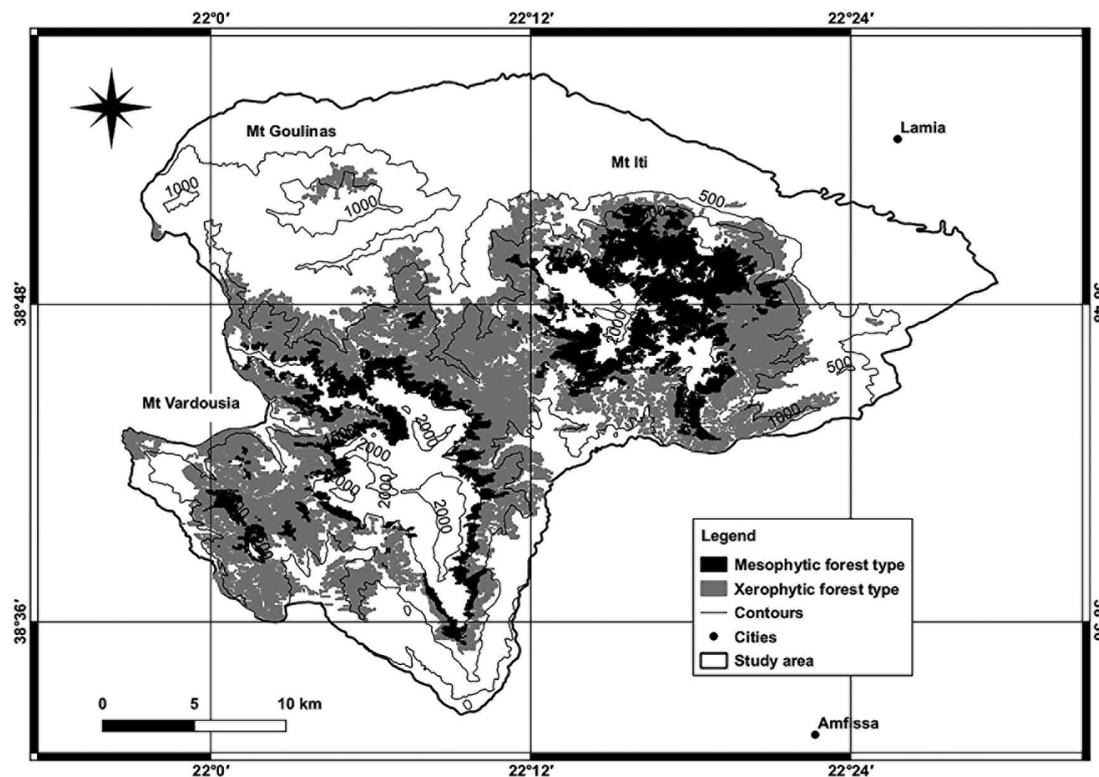


Fig. 6 Map of the area studied with the current actual distribution of Xerophytic (grey colour) and Mesophytic (black colour) fir forest stands. The distribution threshold of the two fir forest communities is based on their ecological threshold of HI_spring (Humidity Index during spring).

Table 4 Heuristic estimate of relative contributions of the variables (%) included in the analysis (bio1: annual mean temperature, bio8: mean temperature in wettest quarter, bio12: annual precipitation, HI_spring: Humidity Index in spring, PET_dry: Potential Evapotranspiration in the 4 driest months – June to September).

MaxEnt model	bio1	bio8	bio12	HI_spring	PET_dry
Model 1	3.2	3.0	1.7	83.5	8.6
Model 2	2.9	3.0	1.7	86.1	6.4
Model 3	3.5	2.9	1.6	83.3	8.7
Model 4	2.9	2.9	2.0	83.8	8.4
Model 5	3.2	3.0	1.7	83.8	8.3
Model 6	3.1	2.6	1.9	83.8	8.6
Model 7	2.4	2.8	2.3	86.5	6.0
Model 8	2.9	2.8	1.7	86.4	6.3
Model 9	3.0	2.7	1.7	86.1	6.6
Model 10	3.4	2.3	1.8	83.7	8.7
Average	3.1	2.8	1.8	84.7	7.7

current actual distribution of the two plant communities in the area studied (Fig. 6).

Xerophytic communities occur on Mt. Vardousia, Mt. Goulinas and Mt. Iti, in the meso-Mediterranean and the lower part of the supra-Mediterranean zones, between 660 and 1360 m a.s.l. Mesophytic communities occur on Mt. Iti and Mt. Vardousia at higher altitudes (1230–1840 m a.s.l.) than the previous community and is totally absent from Mt. Goulinas. It ranges from the

lower part of the supra-Mediterranean zone up to the timberline (Fig. 6).

Suitability of bioclimatic space for fir forests

Of the 29 environmental variables that were selected as predictors, only 5 (HI_spring, PET_dry, bio1, bio8, bio12) had low intercorrelations ($r < |0.75|$) and therefore were included in the MaxEnt model. The most important variable, defined by the MaxEnt model, was by far

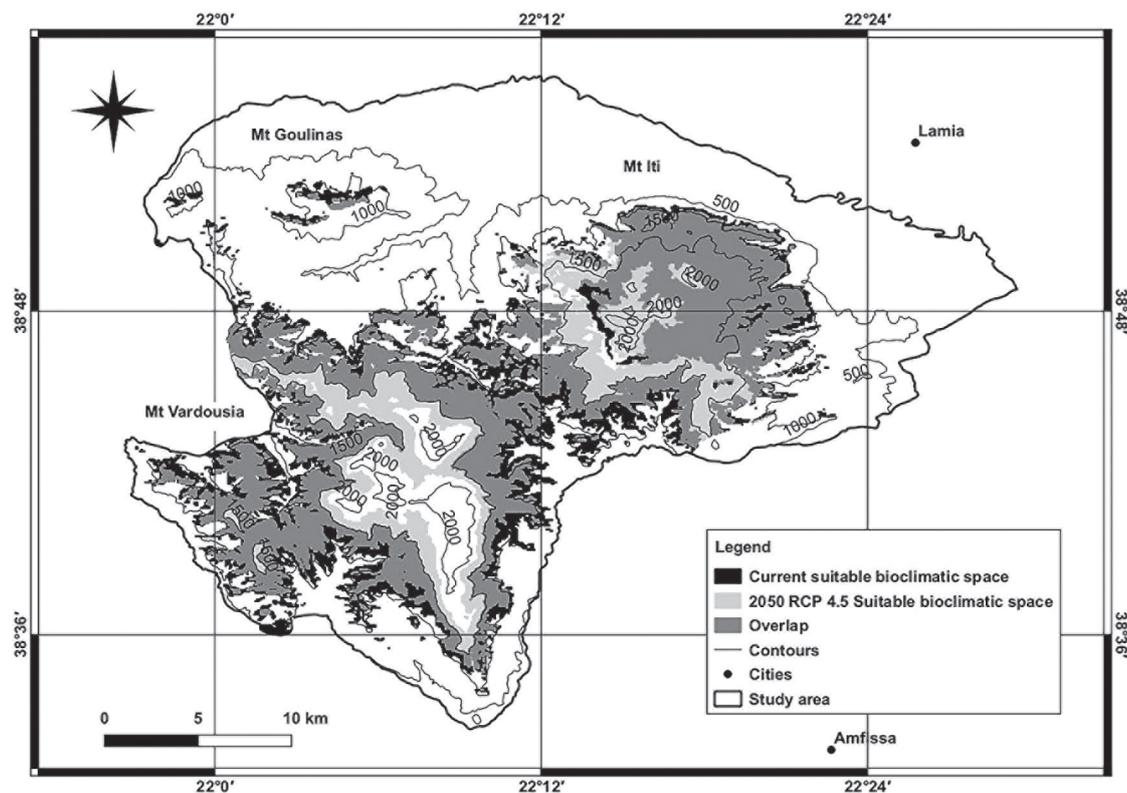


Fig. 7 Map of the area studied with the current suitable bioclimatic space (black colour), the predicted suitable bioclimatic space in 2050 for the RCP 4.5 scenario (light grey colour) and their overlap (dark grey colour).

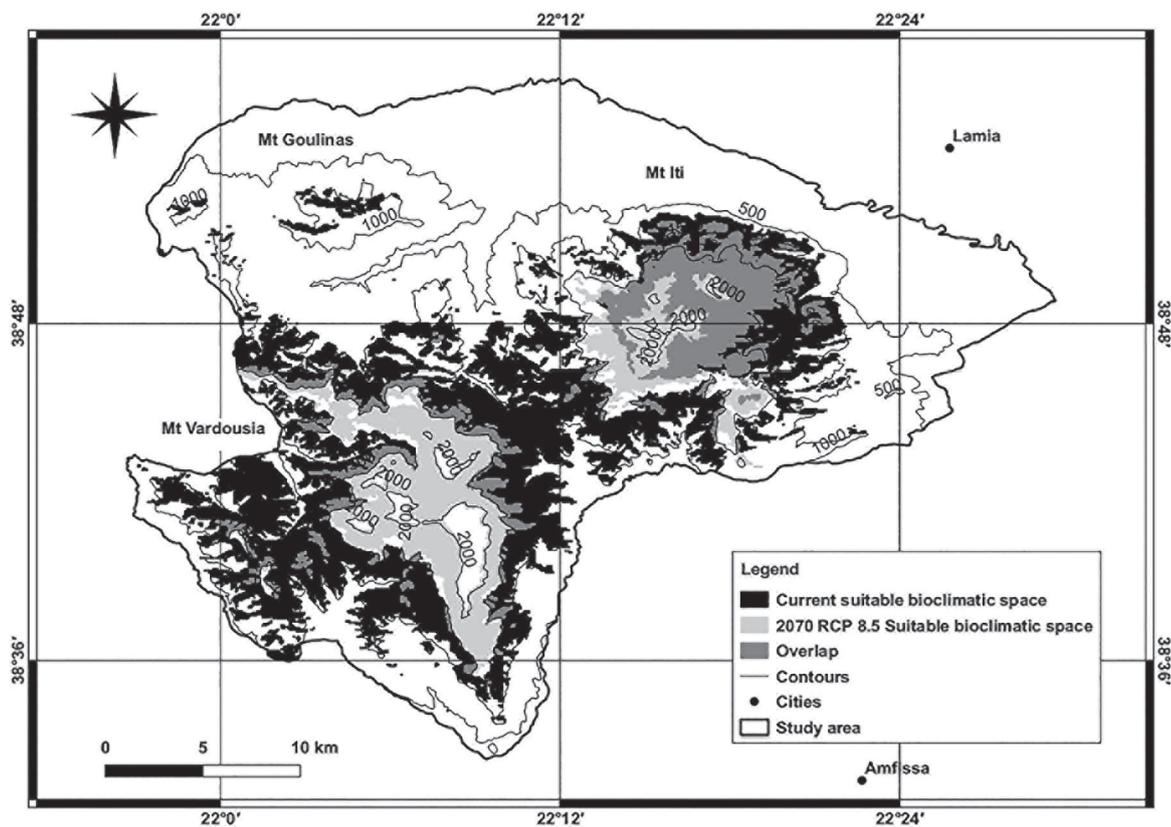


Fig. 8 Map of the area studied with the current suitable bioclimatic space (black colour), the predicted suitable bioclimatic space in 2070 for the RCP 8.5 scenario (light grey colour) and their overlap (dark grey colour).

HI_spring (84.7%) followed by PET_dry (7.7%), while the least important variable was bio12 (1.8%) (Table 4).

The current suitable bioclimatic space for *Abies cephalonica* s.l., predicted by the MaxEnt models, covers an area of 31,515.9 ha. Based on the RCP 4.5 scenario, the future suitable bioclimatic space is predicted to decline to 30,256.3 ha by 2050 (Fig. 7) and to 21,990 by 2070. The suitable bioclimatic space for fir forests is predicted to be even smaller following the RCP 8.5 scenario (22,555.9 ha by 2050 and 16,554.1 by 2070) (Fig. 8).

Effect of climate change

The effect of climate change on fir forests varies and depends on the projected period and the emissions scenario. Considering the whole distribution of fir forests, a moderate to very high effect of overlap and a low to moderate ratio is expected (Table 5).

If we consider the RCP 4.5 scenario, the effect of climate change on fir forests due to the reduction of suitable bioclimatic space (ratio) is expected to be low in 2050 (96%) and moderate in 2070 (69.8%), see Table 5. The negative effect of ratio is expected to be higher for the xerophytic plant community, with a value of 93.5% in 2050 and 66% in 2070 (Table 6). In contrast, the mesophytic community is expected to have a positive effect of ratio in 2050 with an expansion of its suitable bioclimatic space (102.6%) and a low negative ratio in 2070 (79.8%). Based on the RCP 8.5 scenario, although the reduction of suitable bioclimatic space is expected to be higher, compared to the RCP 4.5 scenario, the ratio effect on fir forests is projected to be low in 2050 (71.6%) and moderate in 2070 (52.5%), see Table 5. In 2050 the negative ratio effect is expected to be almost the same for both xerophytic and mesophytic communities, but in 2070 a higher neg-

Table 5 The overlap and ratio metrics for the assessment of the effect of climate change on fir forests in two periods of time (2041 – 60, 2061 – 80) and for two climate change scenarios (RCP 4.5, RCP 8.5).

Time	Scenario	Overlap	Overlap effect	Ratio	Ratio effect
2050	RCP 4.5	65.2%	Moderate	96.0%	Low
2050	RCP 8.5	39.8%	High	71.6%	Low
2070	RCP 4.5	41.7%	High	69.8%	Moderate
2070	RCP 8.5	21.1%	Very high	52.5%	Moderate

Table 6 The overlap and ratio metrics for the assessment of the effect of climate change on fir forest communities in two periods of time (2041 – 60, 2061 – 80) and for two climate change scenarios (RCP 4.5, RCP 8.5).

Time	Scenario	Xerophytic community				Mesophytic community			
		Overlap	Overlap effect	Ratio	Ratio effect	Overlap	Overlap effect	Ratio	Ratio effect
2050	RCP 4.5	53.6%	Moderate	93.5%	Low	42.0%	High	102.6%	+ Low
2050	RCP 8.5	18.7%	Very high	71.5%	Low	27.8%	Very high	71.7%	– Low
2070	RCP 4.5	24.9%	Very high	66.0%	Moderate	30.6%	High	79.8%	– Low
2070	RCP 8.5	1.6%	Very high	48.9%	High	10.6%	Very high	62.2%	– Moderate

ative ratio effect is predicted for xerophytic communities (48.9%), see Table 6.

Considering the RCP 4.5 scenario, the overlap effect on fir forests is expected to be moderate in 2050 (65.2%) and high in 2070 (41.7%), see Table 5. The negative overlap effect is expected to be higher for the xerophytic plant community, with a value of 53.6% (moderate) in 2050 and 24.9% (very high) in 2070 (Table 6). The mesophytic community is expected to have a high negative overlap effect in both 2050 (42%) and 2070 (30.6%). Based on the RCP 8.5 scenario, the overlap effect on fir forests is predicted to be high in 2050 (39.8%) and very high in 2070 (21.1%). Very high overlap effect is predicted for both projection periods for the xerophytic and mesophytic communities, although the overlap values for the xerophytic community are predicted to be 10% more than for the mesophytic community (Table 6).

Discussion

Floristic and ecological differentiation between the fir forests

A difference in the fir forest vegetation in xerophytic and mesophytic plant communities was recorded in the area studied. These results confirm those of previous studies on fir forest vegetation in southern and south-central Greece. Bergmeier (2002) reports the same pattern and suggests that water supply is the crucial factor driving the floristic variation of coniferous forests and woodlands of *Abies cephalonica*, *Pinus nigra* and *Juniperus drupacea* on Mt. Parnon (southern Greece). Similarly, Samaras et al. (2015) report that the floristic variation within the fir forest vegetation on the Oxia-North Vardousia mountain system (south-central Greece) reflects the differences between mesophytic and xerophytic forest communities, associated with the seasonal drought gradient from low to high altitudes.

The xerophytic plant community has many floristic elements in common, including two “character species” (*Trifolium grandiflorum* and *Luzula forsteri*), with the association *Trifolio grandiflori-Abietetum borisii-regis* Barbéro and Quézel 1976, described by Barbéro and Quézel (1976) from the mountains of Oxia and Timfristos and the southern part of Mt. Vardousia. It also resembles the *Crepis fraasii-Abies cephalonica* community, described by

Samaras et al. (2015) from Mt. Oxia and the northern part of Mt. Vardousia. The mesophytic plant community has many floristic elements in common with the association *Lilio chalconicae-Abietetum cephalonicae* Barbéro and Quézel 1976, described by Barbéro and Quézel (1976) from different mountains in Peloponnisos (southern Greece). It also resembles *Abies cephalonica* community described by Karetos (2002) from Mt. Iti and *Sanicula europaea-Abies cephalonica* community described by Samaras et al. (2015) from Mt. Oxia and the northern part of Mt. Vardousia. A syntaxonomic review of fir forest vegetation in Greece is needed, for a better understanding of their floristic and ecological differentiation. For a rigorous syntaxonomic discussion of Greek fir forests see Samaras et al. (2015).

Possible changes in the distributions of fir forests

Based on the current climatic conditions in the area studied, fir forests are subject to intense water stress during the summer period (Table 2). According to both climatic scenarios, drought intensity is expected to increase significantly during spring and autumn, while in summer the changes are expected to be less severe. Our results are similar to those of previous studies on Mt. Iti (Samaras et al. 2017), confirming the importance of climatic changes (related to drought) in the reduction of suitable bioclimatic space for fir forests.

Both ratio and overlap sensitivity metrics are important for the assessment of the effect of climate change. A low ratio indicates a reduction in the size of a species’ potential distribution. On the other hand, a projected low overlap between current and future potential distributions indicates that the species has to shift its range in order to maintain the total area of its current distribution. Although recent studies indicate that such a shift is possible and has already occurred in some tree species (Boisvert-Marsh et al. 2014; Monleon and Lintz 2015), other studies show that range shift may be limited for many species of trees (Zhu et al. 2012). Obstacles (physiographic barriers, dispersal abilities, low levels of breeding productivity, lack of suitable habitat) other than climate can lead a species not shifting its range (Sajwaj et al. 2009). Low levels of overlap, therefore, may result in a higher negative effect, even if the ratio metric is high.

Our results confirm previous studies on the dynamics of vegetation in fir forests in Greece, that show a possible shift in their range to higher altitudes (Fyllas and Troumbis 2009; Fyllas et al. 2017). The shift is predicted to be stronger in the distant future (2061–80). The overlap between current and predicted suitable bioclimatic space in 2070, based on the worst-case RCP 8.5 scenario, is predicted to differ on the three mountains in the area studied (Fig. 8). The effect of climate change will be very high on Mt. Goulinas, where fir forests are expected to disappear by 2070, due to lack of suitable bioclimatic space.

Conclusions

There are two types of fir forests in south-central Greece: mesophytic and xerophytic. The most suitable variable for quantifying the distribution of these two types of forest is the Humidity Index during spring. The potential distribution of fir forests in south-central Greece is expected to change in the future, due to a reduction in suitable bioclimatic space. Furthermore, their potential distribution will shift to higher altitudes, but only in the highest mountains, such as Mt. Iti and Mt. Vardousia. Climate change will mostly affect xerophytic fir forests, which are predicted to lose more than half of their current potential distribution by 2061–80 and in the worst-case RCP 8.5 scenario.

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THE INFLUENCE OF RICE-DUCK FARM SOIL ON THE NITROGEN CONTENT OF DIFFERENT VARIETIES OF JAPONICA RICE

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ABSTRACT

Nitrogen fertilizers are widely applied to increase rice yields, but excessive fertilization poses an environmental risk. It has been shown that rice-duck farming can be more efficient in terms of N use by improving rice growth. Several pathways have been proposed for how ducks may improve rice growth in paddy fields. The aim of this study was to investigate the isolated effect of rice-duck farm soil on rice, namely the N content in different plant organs and whether it differs among rice varieties. In a 116-day greenhouse pot experiment, six different Japonica rice varieties (JinU99, Jinyuan98, Jindao18, Jinyuan89, Jinhei1 and Jindao201) were grown in fertilized duck and no-duck soil after which the N content in their organs and the numbers of surviving and grain-producing plants were compared. The straw and leaf N concentrations were positively influenced by rice-duck farm soil while in the roots, this effect was recorded in only two rice varieties. Grain N content differed among varieties, but was not significantly influenced by soil type. Overall, N concentrations in straw and leaf, and roots of some rice varieties, but not in grain grown on duck soil were higher than that in those grown in soils not influenced by rice-duck farming. This study for the first time demonstrates that rice-duck farm soil alone can influence rice growth, namely an increase in the N content of certain rice plant organs.

Keywords: co-culture; nitrogen; rice plant; soil

Introduction

Rice is the most important cereal crop for more than 3 billion people in the world and for about 60% of the Chinese population (Xiong et al. 2013). China is the largest producer of rice (Frolking et al. 2002). Between 1977 to 2005, the total annual grain production in China increased by 71% to 484 million tons (Ju et al. 2009). While the human population is increasing in China and across the world, cultivable land resources are limited. Addressing food security requires novel strategies to increase crop production, including sustainable fertilizer use strategies.

Nitrogen (N) fertilizer is used to enhance rice production (Zhang et al. 2012). However, nitrogen use efficiency is relatively low in rice fields because of N losses via ammonia volatilization, denitrification, surface runoff and leaching of the soil in floodwater systems (Vlek and Byrnes 1986). These losses represent a substantial environmental threat because of the emissions of ammonia and greenhouse gases and groundwater pollution (Bijay-Sing and Craswell 2021). Avoiding these losses is an important goal for the development of environmentally focused rice cropping strategies.

Previous studies have shown that rice-duck farming can lead to lower N losses and higher N use efficiency and improved rice growth than conventional rice farming (Yu et al. 2009; Gao et al. 2019). The rice-duck co-culture has more than a 400-year-old history in China and has

been widely adopted in many other Asian countries such as Japan, South Korea, Malaysia and Philippines because of its economic, environmental and ecological benefits (Zheng et al. 2016). The presence of ducks in paddy fields promotes rice production and quality (Suh 2014; Teng et al. 2016; Li et al. 2017). Ducks control weeds and pests (Liu et al. 2004; Quan et al. 2005) and promote soil fertility via their droppings (Teng et al. 2016). Moreover, ducks provide mechanical effects including plowing, muddying and mechanical stimulation of rice by engaging in their activities including walking, swimming, eating, grooming, paddling and rubbing (Luo and Gliessman 2016). Duck activities not only stimulate rice growth, but can also increase its lodging resistance (Zhang et al. 2013). Understanding the mechanisms by which ducks influence rice plants in paddy fields is necessary to continue improving rice-duck farming systems towards higher N use efficiency. For example, rice-duck farming can induce higher N content in certain rice organs (Ebissa et al. 2018). Several direct and indirect pathways are proposed for why ducks influence rice growth, based on field observations of rice-duck farming and conventional rice cultivation. In such conditions, the different pathways may act together and their isolated effects cannot be tested. No study has to our knowledge focused on isolating the effect of rice-duck farming soil on rice growth.

The main objective of the current study was to investigate the effect of rice-duck farm soil on rice seedling survival and rice N content and whether it differs among dif-

Table 1 Times and amounts of fertilizer applied at the Ninghe experimental farm.

	Basic fertilizer	Transplanting	Tiller fertilizer		
Date	25-April	10-May	17-May	26-May	17-June
Fertilizer	(NH ₄) ₂ HPO ₄ :	Rice seedlings	(NH ₄) ₂ SO ₄ :	(NH ₄) ₂ SO ₄ :	(NH ₂) ₂ CO:
Rate (kg ha ⁻¹)	112.44		149.25	5.97	1.12
Rate (kg N ha ⁻¹)	23.84		31.63	1.27	5.22

(NH₄)₂HPO₄: ammonium diphosphate; (NH₄)₂SO₄: ammonium sulphate; (NH₂)₂CO: urea

ferent Japonica rice varieties. We hypothesized that rice plants grown in duck soil will have a higher N content.

Materials and Methods

Study site and experimental design

The study was carried out using soil and rice seedlings collected at the Ninghe experimental farm (39°18′–39°50′ N, 117°08′–117°56′ E), located in North China. At the experimental farm, two areas were studied: duck and no-duck fields (each 3 × 7 m). The amounts of fertilizer applied at the farm are shown in Table 1. In the duck field, twenty ducks were released at the vegetative stage in 2017. Soil samples for soil characterization were collected from each field using an auger in October 2017 as described in Ebissa et al. (2018). For the purpose of the present study, only the 0–20 cm layer was sampled, yielding three samples from each of four plots in both the duck and no-duck fields (n = 12 per treatment). In April 2018, seeds of six Japonica rice varieties (JinU99, Jinyuan98, Jindao18, Jinyuan89, Jinhei1 and Jindao201) were sown at the farm in an area not influenced by ducks close to the two fields. In June 2018, rice seedlings were collected together with soil from the surface layers of each of the two fields.

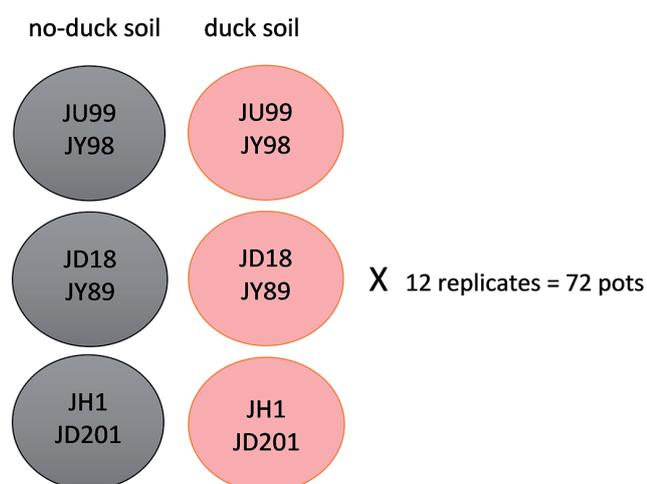


Fig. 1 Experimental design of the greenhouse pot experiment. Pots with either duck soil or no-duck soil were each planted with two seedlings of 6 varieties using the following pattern. JU99: Jin U99, JY98: Jinyuan 98, JD18: Jindao 18, JY89: Jinyuan 89, JH1: Jinhei no. 1, JD201: Jindao 201.

The rice seedlings and the duck and no-duck soil were then brought to a greenhouse for an indoor pot experiment. Treatments were arranged in a total of 72 pots and included 2 types of soil (duck vs. no-duck) and 3 pairs of rice varieties (JU99+JY98, JD18+JY89, JH1+JD201) in 12 replicates (Fig. 1). Each pot (Ø 22 cm, height 22 cm) contained approximately 5 kg of soil and two seedlings, one of each variety. Fertilizers were applied to each pot: 0.75 g (urea), 0.25 g (calcium superphosphate) and 0.5 g (potassium sulfate) on June 29, 2018 (day 1 of the experiment). The same amounts of fertilizer were applied on day 34, amounting to a total N application of 321 N kg ha⁻¹. The rice plants were watered 3 to 5 days a week until maturity and harvested on October 22, 2018 (day 116 of the experiment).

The harvested rice plants were sorted into grain, straw, leaf and root. The roots were washed to avoid any contamination with soil. The rice plant samples were oven-dried for three days at 75 °C and ground to fine powder. The soil samples were air-dried, ground and sieved to pass through a 0.15-mm mesh. The following soil properties were evaluated: pH, total N, NO₃⁻, NH₄⁺, soil organic matter, total P and particle size distribution, as described in Ebissa et al. (2018) (Table 2).

Data analysis

All data were analyzed using R software. A two-way ANOVA was conducted to examine the interaction between soil types and rice variety on N concentrations in each plant. A paired t-test was used to compare survival

Table 2 Physicochemical properties (±SD, n = 12) of soil at 0–20 cm depth in the experimental fields (2017). SOM – soil organic matter.

Property	Unit	Duck soil	No-duck soil	t-test
pH		7.42 ± 0.09	7.48 ± 0.04	ns
Total N	(g kg ⁻¹)	1.04 ± 0.19	0.93 ± 0.26	ns
NH ₄ ⁺ -N	(mg kg ⁻¹)	3.92 ± 4.46	2.23 ± 0.94	ns
NO ₃ ⁻ -N	(mg kg ⁻¹)	25.98 ± 15.48	35.66 ± 14.96	ns
Total P	(g kg ⁻¹)	0.89 ± 0.16	0.91 ± 0.11	ns
SOM	(g kg ⁻¹)	22.75	19.60	
Clay	(%)	32	35	
Silt	(%)	63	56	
Sand	(%)	6	9	

ns: non-significant

and grain production of varieties. An unpaired t-test was used to compare the soil properties of the duck and no-duck fields.

Results and Discussion

The main objectives of this study were to investigate the effect of rice-duck farm soil on rice seedling survival and rice N content and whether it differs for different rice varieties.

Number of surviving and grain-producing plants

Out of the 12 rice seedlings of each variety at the beginning of the experiment, between 9 and 12 survived to the end of the experiment (Fig. 2). Highest survival was recorded for varieties JD18, JY89 and JU99 and the lowest for JY98 and JD201, without any major effect of soil. Not all the seedlings produced grain at the end of experiment, with the number ranging between 4 and 12 for different varieties and soil types (Fig. 2). The number of grain-producing plants was higher in duck soil than no-duck soil for half of the varieties (JY89, JH1, JD201) while the opposite was true for the other half (JU99, JY98, and JD18). However, neither survival nor grain production of the varieties were significantly influenced by soil type (paired t-test, $p > 0.05$). JD18 produced the highest number of grain-producing plants in duck soil while in no-duck soil this was also true for JU99. To our knowledge, there is no study that compares the suitability of different rice varieties for rice-duck co-culture and our preliminary data indicate that some varieties might be more suitable than others. Future studies should however focus also on the potential differences in biomass and grain yield of the varieties and include also monocropping treatments to exclude the potential effects of competition.

N concentrations in rice plants

In line with our hypothesis, the N concentrations in rice plants were influenced either by soil type, the variety

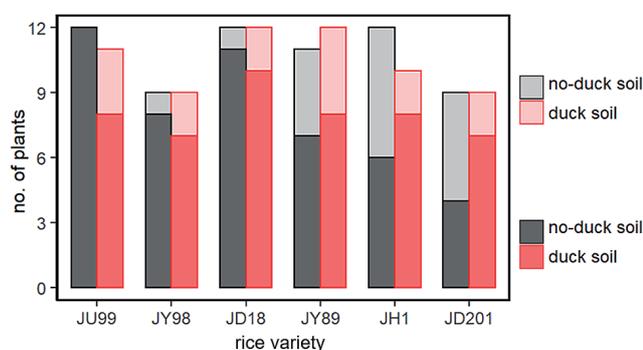


Fig. 2 Number of plants of each variety that survived (light-coloured) and produced grain (dark-coloured) at the end of the 116-day pot experiment. Twelve seedlings were planted at the beginning of the experiment.

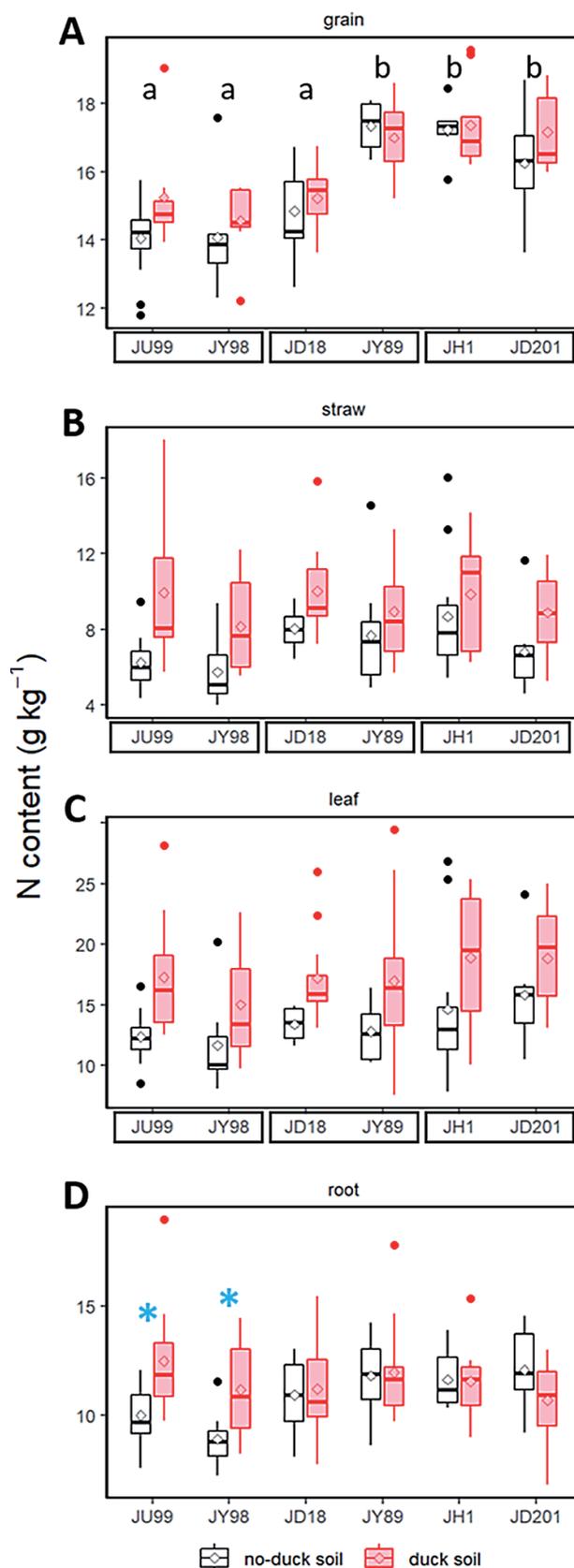


Fig. 3 Nitrogen content of grain (A), straw (B), leaves (C) and roots (D) of six varieties of Japonica rice grown in soil from a rice-duck field (duck soil) and a field without ducks (no-duck soil). Only significant main or interaction effects of two-way ANOVA are listed, see Table 1 for details. *, ** and *** denote significance at 0.05, 0.01 and 0.001 probability levels, respectively. Different letters and asterisks indicate significant differences (Tukey post-hoc test, $p < 0.05$).

Table 3 The effects of types of soil and variety of rice and their interaction on the N contents of grain, straw, leaves and roots as tested by two-way ANOVA.

Variable	df	N content (g kg ⁻¹)							
		Grain		Leaves		Straw		Roots	
		F	P	F	P	F	P	F	P
Soil types (ST)	1	3.206	0.0770	25.555	<0.0001	21.876	<0.0001	3.615	0.0598
Rice variety (RV)	5	18.293	<0.0001	1.938	0.0940	2.154	0.0640	1.958	0.0902
ST × RV	5	0.739	0.5960	0.127	0.9860	0.735	0.5980	2.963	0.0150

*, **, and *** denote significance at 0.05, 0.01, and 0.001 probability levels, respectively. Different letters and asterisks indicate significant differences (Tukey post-hoc test, $p < 0.05$).

or their interaction depending on the part of the plant (Fig. 3, Table 3). Concentrations in grain did not differ between duck soil and no-duck soil, but were different for the varieties being higher in Jinyuan 89, Jinhei 1 and Jindao 201 than in Jin U99, Jinyuan 98 and Jindao 18 (Fig. 3A). In contrast, straw and leaf N concentrations were higher for plants grown in duck soil (Fig. 3B, C), which is in accordance with our hypothesis. Neither straw nor leaf N concentrations were associated with rice variety. Finally, also the concentrations in roots were higher for plants grown in duck soil, but only in varieties JY99 and JY98 (Fig. 3D). Similarly, to straw and leaf, variety did not affect N concentrations in roots.

The variability in N content was higher in leaf and straw than in grain and root. Not all plants produced grain at the end of the experiment, which might be explained by the plants being in different phases of development. It is reported that the N content of leaves can vary at different stages of the development of rice, ranging between 4.9% to 50 g kg⁻¹, with lowest values at booting and highest at the vegetative stage (Wang et al. 2014; Gholisadeh et al. 2017).

That duck soil can increase the N content of rice plants is in accordance with the results of the preceding field experiment of Ebissa et al. (2018). To our knowledge, no other study has so far compared the N content of rice plants produced by rice-duck and conventional farming, but other related parameters have been studied. Nitrogen is an important component of proteins and N content of leaves is strongly correlated with chlorophyll content (Singh et al. 2002; Wang et al. 2014; Yang et al. 2014), which in turn is related to grain yield (Gholisadeh et al. 2017). In line with our findings, Teng et al. (2016) report higher contents of soluble protein and chlorophyll in duck treatments than in no-duck treatments at certain times during the growing season. Also, Li et al. (2019) report increases in leaf soluble protein content. This corresponds with many other previous studies that have shown the positive effect of rice-duck farming on rice growth and production compared to conventional fields (Liu et al. 2004; Saleh and Seyyed 2015). These effects include increased grain yield (Hossain et al. 2004; Saleh and Seyyed 2015), plant height (Hossain et al. 2004;

Saleh and Seyyed 2015), grain number per panicle (Ahmad et al. 2004; Saleh and Seyyed 2015), one thousand grain weight (Karbalaie 2004), number of tillers per hill (Mohammadi et al. 2013) and harvest index (Saleh and Seyyed 2015).

Since we found increased N content in certain rice organs on duck soil, future studies should aim to also quantify N uptake to explore if this corresponds also to higher N use efficiency. In general, the N application rate in paddy fields ranges between 50 and 500 kg ha⁻¹ (Che et al. 2015). In our experiment, 321 kg N ha⁻¹ was applied to the pots and 62 kg N ha⁻¹ was applied in the field before collecting the seedlings (total 383 kg N ha⁻¹) to simulate conventional fertilization rates. Further studies should also explore the potential for fertilizer reduction on rice-duck farm soil as a next step towards environmentally focused rice cropping.

Soil in the duck and no-duck fields

In order to understand the mechanisms resulting in the higher N content of rice grown in duck soil, soils collected from duck and no-duck fields were analyzed. We expected the duck soil to have higher content of organic matter and nitrogen and thus act as soil enriched with organic fertilizer. However, we found no significant differences ($p > 0.05$) between the duck and no-duck field in any of the investigated soil properties related to soil fertility, namely soil pH, total N, NH₄⁺ and NO₃⁻, and total P (Table 2, note that difference in SOM could not be tested). This contrasts with the findings of Yang et al. (2004) who report that soil from rice-duck co-culture was higher in soil organic matter, total N, available N as well as available P and K. Teng et al. (2016) report the effect of rice-duck farming on the availability of nutrients in the soil (namely NH₄⁺, alkali hydrolysable N and available P) throughout the growing season and conclude that differences only occur in September and are not present in October (when the soil was sampled in present study). This could explain the discrepancy at least in the highly dynamic available N pools.

We expected to find higher total and available N pools in the duck soil since it is estimated that the total excreted faeces per duck can reach 10 kg, which contains 47 g

N, 70 g P and 31 g K (Xiong and Zhu 2003). On a daily basis, fresh droppings of a duck weigh an average 0.14 kg and contain 7.1 g N kg⁻¹ (Long et al. 2013). However, a study in which duck faeces alone or in combination with mineral fertilizer were added to soil showed no positive effects on rice growth, yield or protein content (Isobe et al. 2005), suggesting that the nutrient content alone may not be the most important factor determining the fertility of rice-duck farm soil. Increase in soil enzyme activity or soil physical characteristics such as lower bulk density and higher aggregation in duck-influenced soil (Yang et al. 2004) may be equally or more important, but were not measured in the present study. Overall, we can conclude that in our study we did not find any differences in the soil parameters of duck and no-duck fields. Future studies should consider wide range of soil properties (including different soil organic matter fractions, enzyme activity or soil physical characteristics) and a targeted analysis of the soil at the start and at the end of the pot experiment.

Direct and indirect effects of ducks on rice growth

This study is the first to confirm that rice-duck farming soil alone can positively affect rice growth, namely the nitrogen content of the rice plants. Previous studies were conducted only in the field where the ducks could have influenced rice growth by various direct and indirect pathways. For example, water and soil disturbance caused by ducks walking in rice fields may inhibit weed germination. This can result in up to 99% weed reduction after four years of rice-duck cultivation. Ducks are also effective at reducing pests (Quan et al. 2005) while at the same time are not harmful or even beneficial in terms of arthropod diversity (Qin et al. 2011) or nematode abundance (Teng et al. 2016). Stimulating effects of duck activities on rice plants can cause some changes in shape, height, stalk thickness, effective tillering and other growth characteristics of rice plants (Takao 2001; Shen 2003; Zhang et al. 2007, 2011; Wang et al. 2008; Huang et al. 2012; Zhang et al. 2012). Last but not least, ducks improve soil properties (Yang et al. 2004). Apart from the effect of duck droppings on soil chemistry, the movement and feeding activity of ducks in rice plots causes variations in soil distribution, thus resulting in improving soil physical properties, which subsequently improve the root systems of rice plants (Furuno 1996).

Differences in the responses of the rice varieties

This study is also the first to compare the responses of different varieties of Japonica rice to rice-duck farming soil; however, the differences were small. The compared Japonica rice varieties did differ in grain N content but this was irrespective of soil type. Similarly, some differences were found also in the survival of rice plants, again irrespective of the soil. Only the root N content response to duck soil differed among the varieties, but a monocropping study would be needed to assess if these differences affect yield, biomass production and N uptake.

Conclusion

Rice-duck farming can improve rice growth via several pathways. The objective of this study was to investigate the effect of rice-duck farm soil on rice N content and its variability among rice varieties. We found that N concentrations in certain organs of the rice varieties grown in duck soil were higher compared to those grown in no-duck soil. Namely, N concentrations in straw, leaves and roots of some rice varieties were higher when grown in duck soil. Neither survival or grain production of the varieties were significantly influenced by soil type. Our study is the first to confirm that duck-influenced soil can promote rice growth, namely its nitrogen content. We give several recommendations on further research towards the application of rice-duck farming for sustainable rice production. Further experiments should investigate grain yield, biomass, and wider range of soil properties in response to different fertilization rates to further assess the effect of duck soil on rice growth and nitrogen use efficiency.

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PERSPECTIVE OF SOLID WASTE GENERATION IN ALBANIA BASED ON A COMBINATION OF CORRELATION AND INTERPOLATION

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ABSTRACT

Waste management is a serious problem in developing countries. Solid waste (SW) statistics provide an indication of compliance with the targets of the Waste Framework Directives (WFD). This paper includes an analysis of specific variables that directly influence trends in the production of SW. Albania is divided into 12 first-level administrative units, counties. The objective of this paper is to concentrate on the geographical areas that provide the most striking results. The analyses use linear regression and Pearson correlation in which the dependent variable is waste generation and the independent variables, geographic, demographic and economic, are among those that are of greatest importance in the production of SW. The specific relationships are highly significant ($r > 0.98$) and are the input for the IDW interpolation maps. The disaggregated panorama is used to develop policy recommendations for waste management in the affected areas. Prediction of urban waste through a combination of mathematical equations and geographical tools is part of the results of this study.

Keywords: correlation; GIS; geographic dispersion; interpolate; spatial map

Introduction

Despite some advances in enhancing environmental protection, rapid urbanization and inequality in the developing world makes the collection of municipal solid waste (MSW) even more difficult (Vieira and Matheus 2018). Waste management is ranked among the basic challenges faced by developed countries worldwide, especially those municipalities that carry the burden of the largest share of the issue. SW is one of the main by-products of urban life (Pan et al. 2019). According to the European Environment Agency, Albania has very few recycling/reusing systems for waste and few engineered landfills for the disposal of waste. Understanding trends in waste production in terms of several variables is likely to improve waste management. Waste separation is almost non-existent and recycling is very low, also most waste continues to be disposed of unsafely, which is a serious cause for concern (European Commission 2014). The trend in the rural-urban process in Tirana is not unusual in the European Western Balkans (EWB). A famous quote from Peter Drucker says: “You can’t manage what you can’t measure”. Hence, the goal of the current analyses is to identify the most important variables in waste generation followed by a spatial perspective of the affected areas. According to Smrekar et al. (2019), the increase in unfavourable residential conditions in some urban areas due to uncontrolled waste management can result in increased social degradation and differentiation and jeopardize the implementation of sustainable urban development. Production of solid waste is closely connected to population growth, general consumption and economic activity. Based on Rybova et al. (2018), we try to find the same significant correlations between

socio-demographic aspects and SW in order to account for the much higher share of inter-municipal variability using geographically weighted regression, because this method is able to consider changing strength and even direction of the relationships in different spatial units.

Waste management must be strictly controlled, as it directly affects human health and the environment. The substances involved include volatile organic compounds (VOCs), polychlorinated biphenyls (PCBs), heavy metals, radioactive materials and pharmaceuticals (Pecci 2017). Waste management is closely related to geographical dispersion. To identify important variables and their spatial correlation it is crucial to develop an operational strategy. Through GIS technology, we manage environmental measures based on the combination and analysis of multiple layers. Statistical data based on mathematical formulas, such as linear regression or correlation are important for defining trends. The outcomes elaborated through geospatial tools improve the policy on waste disposal, which cannot be restrained, but controlled. This paper aims to benefit from GIS utilities overlapping multiple layers reflecting different themes to highlight specific issues (Hysenaj 2016). As is the case with maps in general, overlays are more helpful than indicators to accommodate reflexive scrutiny and plural perspectives (Rafols et al. 2010). To identify the most important variables that affect waste generation brings the goal of a prediction model much closer.

Evaluation Strategy

To develop relationships between variables and waste generation, many researchers use correlation and regres-

sion analysis. To better understand the spatial extent of the issue and the symbiotic behaviour between applied variables, interpolation techniques are used to quantify the situation and define a future perspective. Overlay maps provide significant advantages in the readability and contextualization of disciplinary data and in the interpretation of cognitive diversity (Rafols et al. 2010). We combine multiple layers in order to produce heuristic interpretations of the internal dynamics of a research field. According to waste prediction models, many different variables are important, such as number of residents, income, household size, residency type, age groups, employment, consumer price index (CPI), gross domestic product (GDP), level of education, culture, geography and climate (Ordonez-Ponce et al. 2004). We analysed a subset of these variables for the area studied.

Correlations between waste generation *per capita* and demographic and economic parameters

The data from each of the 12 administrative units in Albania was analysed using linear regression analyses in Microsoft Excel 2016 software and the Pearson correlation formula between waste generation per year (dependent variable) and the chosen list of demographic and economic factors (independent variables). Because of the

recent great increase in population in Albania, this study mainly focused on demographic variables and their effect on urban waste management. The first set of scatter plots define the relationships between independent variables and urban waste per capita per year. Fig. 1 indicates a low degree correlation with population size. The R-value is positive. There are stronger relationships with population density and urban population (Figs 2 and 3). These scatter diagrams reveal that urbanization and population density have greater effects on waste per capita generation than population size.

Waste production per capita is also positively correlated with the independent variable *GDP per capita* (Fig. 4) and economic performance should also be considered.

Correlation between generation of waste per county and economic parameters

The second set of scatter plots reveal the relationships between generation of urban waste per county per year and independent variables (Figs 5–8). All four cases show strong positive increases ($R > 0.70$). The correlation coefficients and their significance (Table 1) indicate that the predictions at the county level are likely to be accurate. As reported previously (Troschinetz 2005; Senzige et al. 2014; Challcharoenwattana and Pharion 2018), the re-

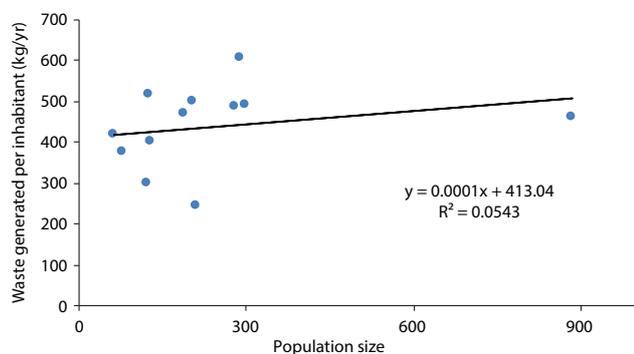


Fig. 1 Waste generated per inhabitant (kg/yr) against population size.

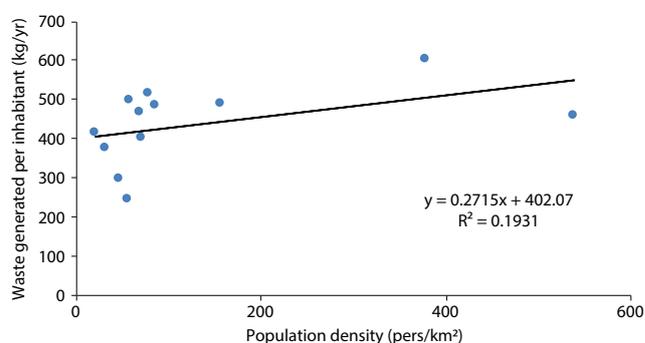


Fig. 2 Waste generated per inhabitant (kg/yr) against population density (pers/km²).

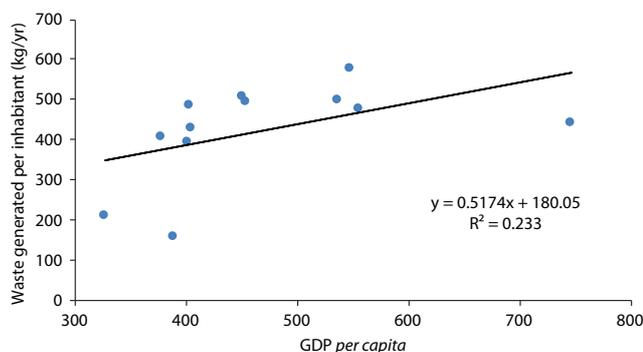


Fig. 3 Waste generated per inhabitant (kg/yr) against GDP *per capita*.

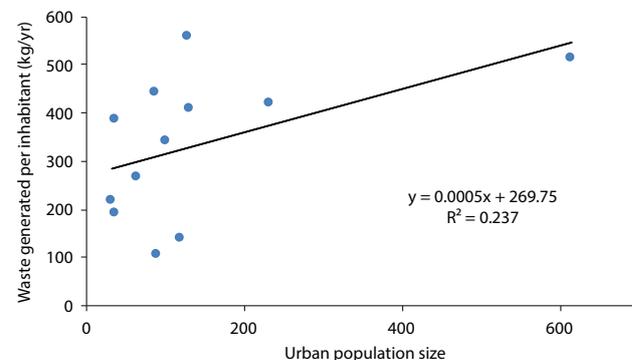


Fig. 4 Waste generated per inhabitant (kg/yr) against urban population size.

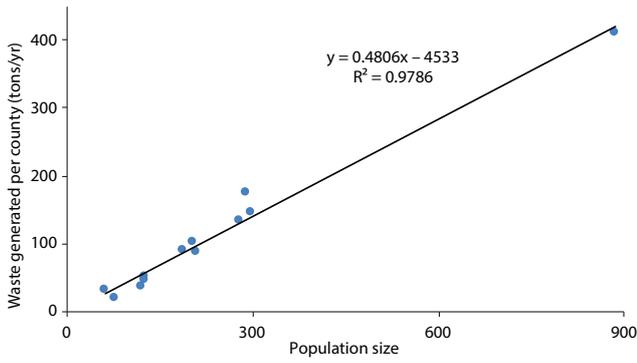


Fig. 5 Waste generated per county (tons/yr) against population size.

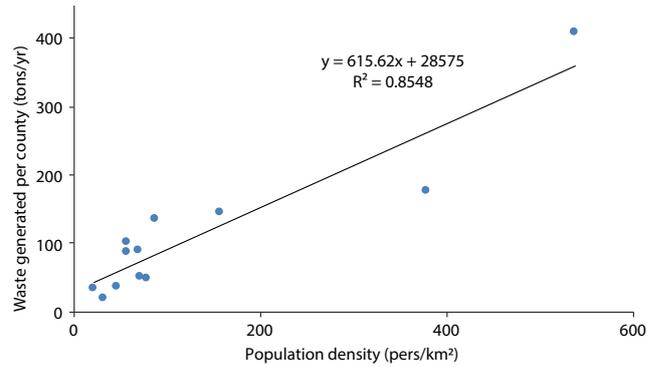


Fig. 6 Waste generated per county (tons/yr) against population density (pers/km²).

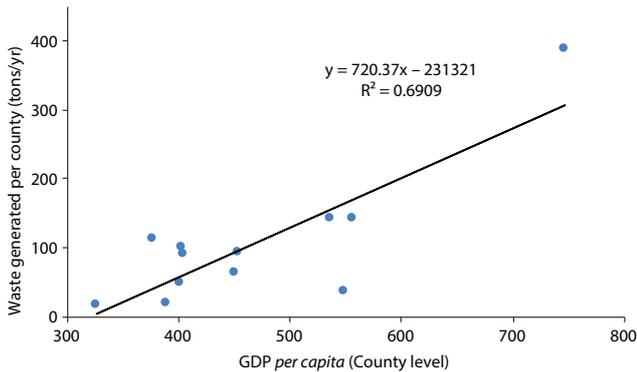


Fig. 7 Waste generated per county (tons/yr) against GDP *per capita* (county level).

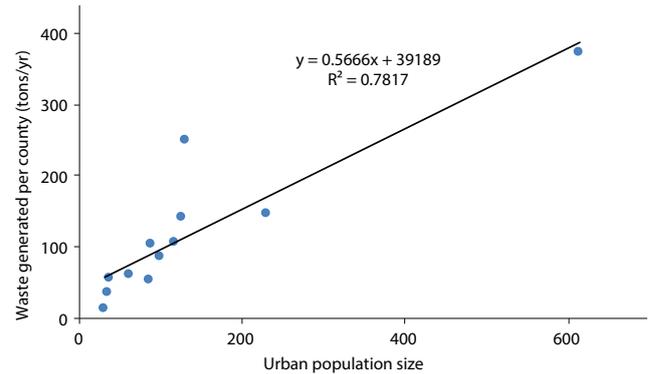


Fig. 8 Waste generated per county (tons/yr) against urban population size.

sults are not unexpected. This information can be used by policy makers to improve urban waste management, especially in areas where there is a high rate of urbanization.

Reflecting correlation results through interpolation map methods

The independent demographic and economic variables are for counties, so it is important to investigate their dynamics at the county level. As in any optimization process, the collection and analysis of data is of great importance because the accuracy of the optimization or simulation is only as good as the accuracy of the data (Monzambe et al. 2019). The analyses presented are based on data for the period between 2003 and 2018, for which there is sixteen sets of data for each of the counties. For two of them (Tirana, Durrës) there is a strong positive increase (Table 2) because they also show a positive correlation between (year – population) variables. In these two counties, there has been a steady and continuous increase in population growth. The correlations for the other ten counties are negative. This means that the dynamics at the county level differ from those at the country level. Generation of urban waste continues to increase in all the other counties despite the fact that their

population growth has decreased. Based on Table 2, we conclude this is mainly due to the effect their urban population, population density and GDP *per capita*. In addition, we should consider the role of the national policy on waste management.

Maps were produced using ArcGIS package and ArcMap 10.2.2 software. Source of data is the Albanian Institute of Statistics. We included a set of vector-based areas derived from GADM (Global Administrative Areas). The geographic coordinate system is GCS_WGS_1984 and datum D_WGS_1984. The scale of the maps is 1 : 2,000,000. Figs 9 and 10 present the quantity of urban waste produced in the different counties and dispersion of the population, respectively. Using inverse distance weighting (IDW) to overlap these two remaps (Fig. 11) provides an overview of the relationship.

Table 1 Correlation between the generation of SW and demographic and economic factors (the correlation is at county level).

Factor	Correlation (r)	Significance
Population	0.989	< .001
Urban population	0.884	< .001
Population density	0.924	< .001
GDP	0.831	< .001

Table 2 Correlations between the generation of SW per county and their urban population, population density and GDP *per capita*.

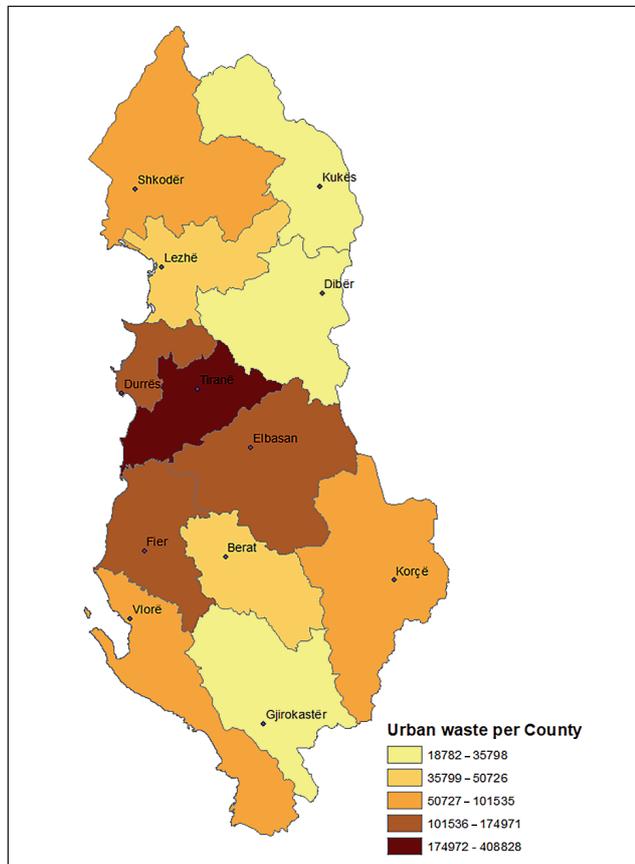
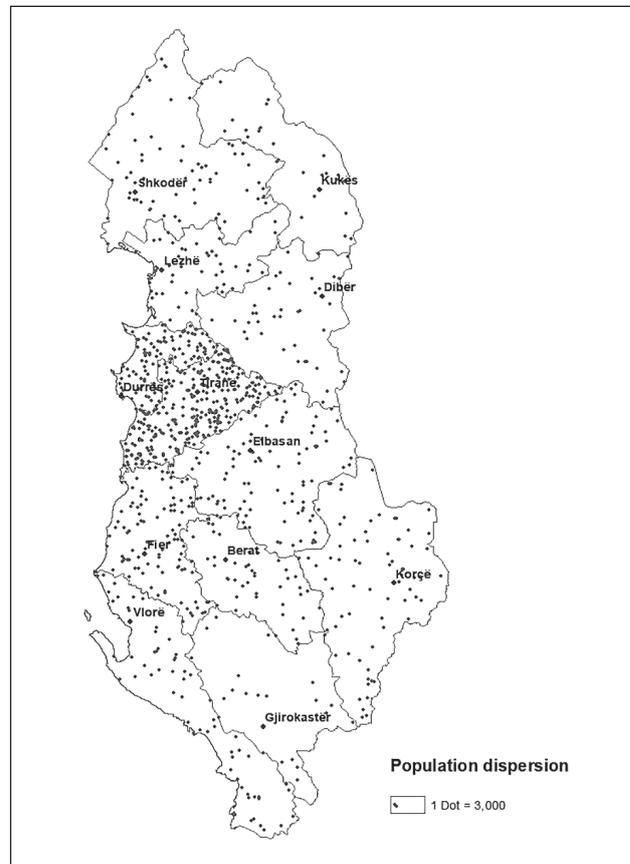
County	Correlation (R)	Urban Pop ¹	Pop. density ²	GDP <i>per capita</i> ³
Berat	-0.90	63395	70.9	451
Diber	-0.67	36375	46.8	389
Durrës	0.94	230938	378.1	536
Elbasan	-0.88	119006	87.1	377
Fier	-0.85	130659	157.7	556
Gjirokastrë	-0.70	37216	21.8	548
Korçë	-0.44	89262	56.6	405
Kukës	-0.52	30612	32.6	327
Lezhë	-0.86	86005	78.3	401
Shkodër	-0.90	100128	57.6	403
Tiranë	0.91	613518	538.0	747
Vlorë	-0.74	127034	69.9	454

¹ Data for column belong to 2015. ² Data for column belong to 2017. ³ (pers / km²), data for column belong to 2018.
Source: INSTAT

Based on the values in Table 2 column *correlation* the geographical dispersion (Fig. 12), the relationships between the correlations between *urban waste* and *population* were produced. There is a strong positive correlation in the area around the capital and a negative correlation in the rest of Albania.

Discussion

The reason for analysing SW data using ArcGIS mapping software is to provide forecasts supported by geospatial technology. Poor solid waste management leads to serious environmental issues. According to (Ardian-

**Fig. 9** Waste generation per county (tons/year).**Fig. 10** Population dispersion per county.

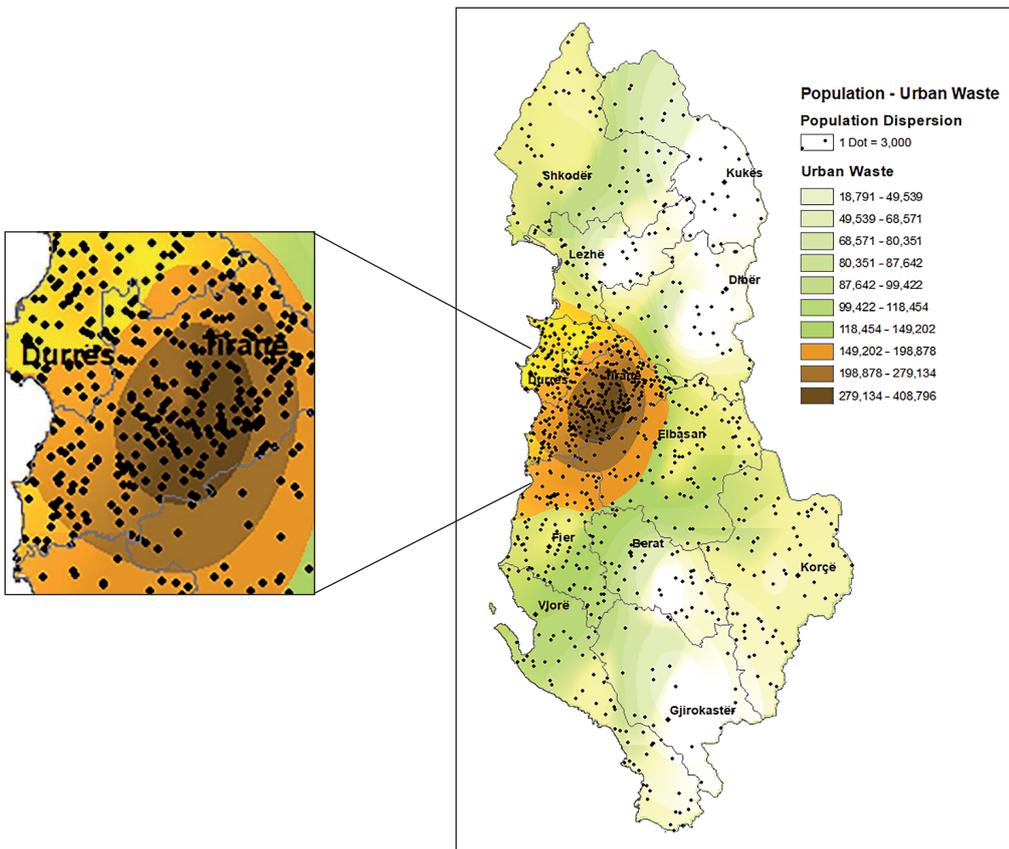


Fig. 11 Population dispersion – waste generation per county (tons/year), IDW interpolation.

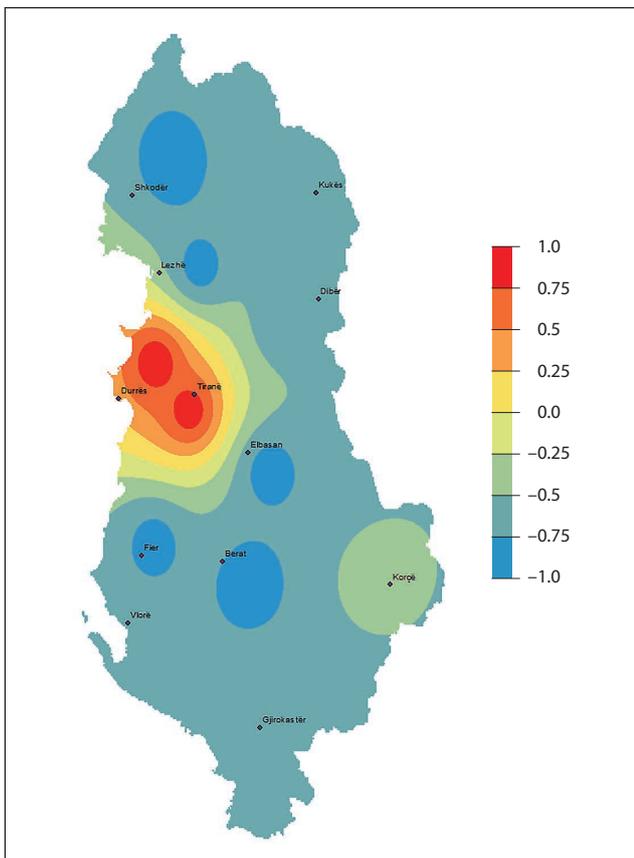


Fig. 12 Correlation between population dispersion and waste generation per county. IDW interpolation (time span 2003–2018).

syah and Maryono 2018), to be able to identify influential factors and the spatial distribution pattern of waste, it is necessary to use a statistical and spatial approach. The results presented, similar to those of Mushtaq et al. (2020), show that SW management varies from region to region, due to factors, such as demographic, socio-economic and accepted policy. Several studies (Kukula 2016; Ghosh and Ng 2021) indicate that waste management strategies depend on the approach of each administrative unit to the most decisive factors. We found a significant correlation in most of the administrative units, which can be used to implement decision-making strategies in each of them. The results indicate that correlation for waste generation *per capita* is stronger ($R = 0.989$) than for waste generation per county ($R = 0.233$). Although the correlation coefficients are both positive, they reveal that SW depends on geographical dispersion. In other words, SW is spatially dependent. The highlighted counties in Table 2 have the highest population densities and positive correlation coefficients for SW. The other administrative units had negative correlation coefficients due to lower population numbers. This study lacks data on sub administrative units, which would help us to better understand the detailed dynamics of waste management and the effect of socio-economic factors. Based on the data presented, policies can be implemented for counties, which may not be appropriate for sub administrative units. In the future, it would be of interest to expand the study to smaller ad-

ministrative units and then determine whether the correlation formula follows the same trend as the presented data or if it behaves differently.

Conclusion

This paper presents the results of a study on the characteristics and trends in the generation of solid waste in Albania. Geographic and mathematical methods were used to classify the first-level administrative division, the counties. The areas of interest are counties with positive correlation between year and population size. This study reveals the most important variables affecting the amount of waste generated and presents a predictive scenario for a spatiotemporal interpretation. At a comparative level, we focused this investigation on economic and demographic variables determining waste per capita and per county. There is a steady trend between waste generated and economy (moderate – strong), which indicates it is an important factor. The importance of demographic variables are divided into (low – strong) for the population variable and (moderate – strong) for urban and density variables. Although the dependent variable, waste per county, is strongly correlated with population density, the waste per capita is not associated with population growth.

The data analysed indicates the specific dynamics at the country level and also in each county. During the last fifteen years, there was a general increase the amount of SW generated in all counties, which is not reflected in population density. The population growth in the counties Tirana and Durrës was positive whereas in other counties it decreased. The linear correlations for the relationships are either strongly positive or negative. We conclude that both the demographic factor (pop. density) and economic factor (GDP per capita) play an important role in determining this relationship. In addition, the role of internal policy regarding waste management needs to be considered. The results of this study can be used to predict urban waste generated at the county level.

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