

LIFE+ LIMNOPIRINEUS: CONSERVATION OF AQUATIC HABITATS AND SPECIES IN HIGH MOUNTAINS OF THE PYRENEES

TECHNICAL REPORT



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~ Estanho de Vilac ~

ALTERATIONS AND CHANGES THAT AFFECT THE CONSERVATION OF THE BIODIVERSITY OF ESTANHO DE VILAC (ARAN VALLEY)

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ABSTRACT

Estanho de Vilac is located in a small endorheic basin in the Aran Valley (Catalonia) at an altitude of 1640 m. Originally, it was a small pond of fluctuating level, but due to livestock interests it was recreated with an external water supply. In recent years, with the modification of the bed and the continuous increase in the volume of water accumulated, it has also been used for trout fishing. As a result of these modifications, there has been a notable alteration of the system jeopardising the conservation of various amphibian species, especially *Alytes obstetricans* and *Rana temporaria*, as well as *Luronium natans*, an aquatic plant that throughout the Pyrenees can only be found in this lake. All three are species protected at European level by the Habitats Directive. The aim of this work is to improve the conservation status of these species and the ecosystem as a whole. For this, a study of the water cycle has been conducted, calculating the water balance of the basin and the changes in level caused by altering the inlet of artificial water have been monitored; the variability of the nutrient cycle and of the aquatic biodiversity and especially of the indicator organisms has been studied. The results show that Estanho is a temporary pond that could remain dry for some time during the summer, but that a water supply that exceeds the natural loss values can continuously increase the volume of water that it would have without external alterations. It is also noted that the presence of trout has significantly altered the diversity of fauna in the body of water and this also affects amphibian populations. In addition, the presence of a large number of equine cattle causes eutrophication of the water, as well as the disturbance of the population of *Luronium natans* and the integrity of the substrate where it grows. This population is also affected by the entry of *Potamogeton berchtoldii*, a water-based macrophyte common to alpine lakes. The actions carried out to reverse the ecosystem towards a more natural state, include the extraction of all trout and the installation

of a new watering hole with regulated water inlet. The elimination of trout has led to a rapid recovery of littoral macroinvertebrates and amphibians. Meanwhile, the decrease in the water level, caused by the decrease in the flow of the external contribution, has modified the extension of the *Luronium natans* population. This has been monitored in the two areas it occupies: in the area where the plant grows rooted to the floor, submerged with leaves floating on the surface of the water, and in the marginal area of the lake where the plant grows with amphibious forms that are exposed to the air. The decrease in water level has caused an increase in the density of the amphibious subpopulation, but in turn has been altered by the presence of livestock that deteriorates the edges of the lake. The aquatic subpopulation has been maintained, although it has low vitality. Plants have always been observed in a vegetative state and simultaneous flowering of multiple individuals was only seen in 2019. Based on these results, we propose that the existence of Habitats of Community Interest (HCI) 3150 and 3130 related to the water body and the two subpopulations of *Luronium natans* be recognized in the Estanho.

INTRODUCTION

Estanho de Vilac is a small lake of the Aran Valley corresponding to an old endorheic pond of glacial origin, whose ecological function should be conditioned by rainfall and probably maintained significant water fluctuation throughout of the year (Figure 1).



▲ Figure 1. Estanho de Vilac.

This lake is included in the list of Special Areas of Conservation (SAC code ES5130006) as a Site of Community Interest for its biodiversity. The greatest interest in terms of biodiversity lies on a stable population of *Luronium natans* (L.) Raf., an endangered macrophyte according to the Spanish Catalogue of Endangered Species (RDL 139/2011, of 4 February). In addition, several amphibians nominally protected in Catalonia (LD 2/2008, of 15 April) have been observed in the area, such as *Bufo spinosus* Daudin, 1803, *Lissotriton helveticus* (Razoumowsky, 1789), *Alytes obstetricans* (Laurenti, 1768) and *Rana temporaria* L., 1758. The last three species (Figure 2) also appear in the list of wild species under special protection of the National Catalogue (Directive 92/43/EEC), and *Luronium natans* and the last two amphibians in Annexes II, IV and V of the Habitats Directive, respectively.



▲ Figure 2. Species of the Habitats Directive present in Estanho: *Luronium natans*, submerged form (left) and amphibious form (right); *Alytes obstetricans* (lower left) and *Rana temporaria* (lower right).

However, this biodiversity could currently be compromised by the concurrence of a series of stressors that influence the variability of the ecosystem function.

Estanho de Vilac has historically been used as a water supply point for livestock (cattle and horses). Its fluctuating nature was an inconvenience for this use in the driest season, so a watering hole was incorporated with an artificial inlet of water from a ravine outside the basin. The excess water was guided to the pond in order to maintain its level. These modifications are old (probably

from the end of the 19th century) and over time they have been renewed and others have been incorporated, such as the excavation of the pond bed that was carried out around the 90s to expand the storage capacity. More recently, and facilitated by the hydrological changes derived from the watering hole, common trout, *Salmo trutta* L., 1758, was also introduced for sport fishing. Currently, the lake environment is still used for grazing of horse and cattle (Figure 3).



▲ Figure 3. Estanho de Vilac. Artificial water inlet and equine livestock.

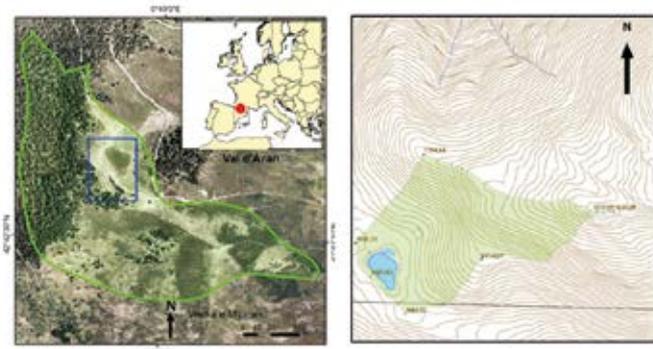
We hypothesise that the artificial water inlet and the extra supply of nutrients may compromise the survival of *Luronium natans*, and that the introduction of fish may affect some groups of organisms, especially the amphibians.

The importance of biodiversity at Estanho de Vilac together with the presence of *L. natans* (the only location of this aquatic plant in the Pyrenees) led to it being declared a Site of Community Importance (SCI) and Special Area of Conservation (SAC) specifically for this ecosystem, and to including it within the conservation objectives of the LIFE+ LimnoPirineus project (<http://www.lifelimnopirineus.eu>).

One of the actions of this project, which is reported in this work, is the improvement of the conservation status of *L. natans*, of two of the four amphibian species present at Estanho de Vilac, and of the ecosystem as a whole. This includes the study of the effect of hydrological changes and the elimination of introduced fish fauna.

GENERAL DESCRIPTION OF THE STUDY AREA

The area under study is Estanho de Vilac located in the Aran Valley, near Vielha, at an altitude of 1,640 m, on the western slope of Mont dera Solana, with coordinates 0° 48' 50.18" N and 42° 42' 37.43" E. The endorheic basin where it lies accounts for an area of 11.37 ha, and is mainly used by livestock (grazing by horses and cows) during the summer and occasional recreational use, given the easy access and relative proximity to populated areas (Figure 4).



▲ **Figure 4.** Location of Estanho de Vilac and SCI ES5130006 Estanho de Vielha (left) and Estanho basin (right).

In the Aran Valley the climate is mountain Atlantic, with relatively high rainfall distributed evenly throughout the year, and with mild temperatures in summer and cold temperatures in winter (in Vielha, 939.7 mm/year and 9.6 °C on average). Given the clear altitudinal gradient, both of precipitation and temperature, rainfall at Estanho is higher and temperatures are lower than the average values of the nearby Vielha station. The relatively high number of days without sunlight is notable, largely due to the presence of fog, which causes a decrease in evapotranspiration.

Estanho de Vilac is located in the syncline sector of the Aran Valley, dominated by Devonian outcrops that are mainly formed by schistose materials in this area. The natural floor of Estanho, of holocenic origin, should be flat and formed by lacustrine deposits of clays and silts rich in organic matter, which alternate with sandy levels (Colomer *et al.*, 2014). These deposits can be several metres strong, and fill and waterproof the bottom of lakes, both of glacial origin and lateral distension, as is surely the case here (Bordonau *et al.*, 1989). Estanho de Vilac has been transformed into a conical base structure, with a maximum depth in the centre of 2.7 m and a perimeter that has been enlarged to about 290 m in 2015. In 2015, it could already be considered a small-sized, elliptically shaped (approximately 100 m by 60 m) lake with the modified floor, probably formed by a mixture of the primitive materials present in the area, and with unknown permeability.

Hydrologically, Estanho is located in a small endorheic basin located above the aquifer of the Devonian metamorphic limestone of the Aran Valley, which can be up to 400 m in depth (ACA, 2015). It naturally receives rain and runoff water from the basin, which gives an irregular periodic regime of oscillation of level, both annual and interannual, determined by the meteorological conditions of the year (precipitation and temperature). In a period with low rainfall and high temperatures it would probably have been almost dry. The interest of a water point at medium altitude (1,640 m) for cattle had to be important at the time, as evidenced by the presence of old buildings (barn and cabin) in the meadows that surround it. To remedy the lack of water in the dry years, a fountain-watering hole with water channelled from a spring outside the basin was arranged. Over the years this guid-

ance has been renewed, each time with better materials and more efficiently. Since the last renovation (approximately in 2003), the surplus water from the watering hole has become a notable additional contribution to Estanho, causing its continued growth. Alongside this collection work, the floor of the pond was excavated to increase its capacity, without anticipating the possible alteration of the impermeability of the bed. During the LIFE+ LimnoPirineus project, a new watering hole has been built and the water inlet has been modified to control the flow, with the aim of reversing the expansion of the Estanho.

The soil dominating the basin is of dortent lithic type, which is characterised by having a low organic matter content and a depth limited to a maximum of 30 cm. It includes some rock outcrops and may be mixed with other types of soils similar to this one, either deeper or with more organic matter (Colomer *et al.*, 2014). Vegetation currently comprises some 80% of broom scrub (formed by *Genista balansae* (Boiss.) Rouy subsp. *europaea* (G. López & CE Jarvis) O. Bolòs & Vigo, HCI 5120), 10% of natural forest of *Pinus sylvestris* L. (HCat 42.5B11), 9% of mesophilic and acidophilic grasslands (HCat 35.122) and 1% of outcrops of schistose rocks (HCI 8220). The present dominance of *Genista balansae* subsp. *europaea* comes from its expansion over the space formerly occupied by pasture.

Inside Estanho we find a homogeneous environment with absence of stones and large amounts of silt deposits. In the centre, in the deepest part, there are only sediments. Around this area there is a macrophyte community consisting of three species: *Luronium natans* (L.) Raf., *Potamogeton berchtoldii* Fieber. and *Eleocharis palustris* (L.) Roem. & Schult. In addition to the macrophytes, filamentous algae have been observed of the *Spirogyra* Link. and *Mougeotia* C. Agardh genera.

Luronium natans is an aquatic species, with some phenotypic plasticity as it grows in permanently flooded or temporarily exposed beds. It has a rosette of linear submerged leaves and elliptical floating leaves. It produces rhizomes and long stolons that often break, so that the separated rosettes, pushed by the wind act as propagules and recolonise the shore. It blooms from June to August, although throughout the years of study it has practically not done so. The population of Estanho is the only one known in the Pyrenees and is probably a clonal population.

In the 80s, when *L. natans* was discovered in Estanho de Vilac, it covered practically the entire surface of the pond and was in bloom from June to September (Perdigó, 1983). At the beginning of the project, in the summer of 2014, *L. natans* covered almost half of Estanho's surface, about 2,462 m², with varying, but generally low densities depending on the areas. The most notable presence was in an inner strip that corresponded to the old pond shore. According to Lansdown & Wade (2003), in lacustrine environments *L. natans* tends to occupy the bank areas. Apparently, the species has been colonising the flooded parts more recently thanks to its ability to produce new stolons and fragments.

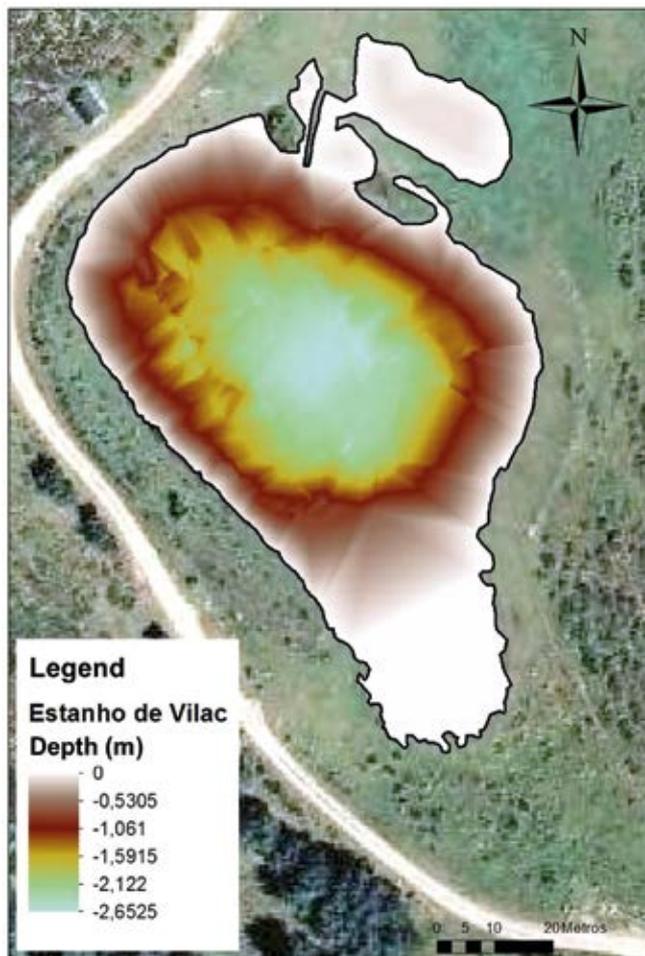
As for the other species, *Potamogeton berchtoldii* occupied 12% of the surface, about 588 m², and *Eleocharis*

palustris occupied just 0.3%, 15 m² in 2014. In fact, *Eleocharis palustris* formed a small stand that was originally on the shore of the lake, and as it increased in size, has ended up in the middle, surrounded by water. *Potamogeton berchtoldii*, more typical of lakes than of temporary ponds, appeared recently, and occupies the same areas as *Luronium natans*, except for the shore and the shallowest area. Filamentous algae grow as epiphytes on petioles and macrophyte leaves, so that they interfere with them in the occupation of space and the use of light. They form extensive floating carpets that were found in variable quantities throughout the study, mainly accumulated towards the leeward shore.

VARIABILITY STUDY OF THE HYDROLOGICAL CYCLE

Historical changes that affect the physical structure of the Vilac lake

Using the bathymetry of Estanho de Vilac, carried out in autumn 2014 with Sonda Echomap 50DV (Figure 5) and following a standardised protocol (Cooke et al., 1993), the area and volume have been calculated every 5 cm with the “Surface-Volume” tool of the ARC MAP 10 program and the Estanho area-volume ratio has been established.



▲ Figure 5. Bathymetry of Estanho de Vilac conducted in October 2014.

Historic aerial images of the ICGC (Institut Cartogràfic i Geològic de Catalunya [Cartographic and Geological Institute of Catalonia]) (<http://www.icc.cat/vissir3>) have been compiled from 1956 to 2014 and the perimeter of the Estanho has been digitised in different years. Actual data have been obtained from the lake area and the corresponding volumes have been determined based on the area-volume ratio established. This has led to historical real volume increases.

Water balance

Water balances have been carried out between 2003-2005, 2005-2007, 2007-2008, 2008-2009, 2009-2011, 2011-2012, 2012-2013 and 2013-2014 according to the available data on the increase of real volume of water in Estanho, in order to create a system operating model. This period has been chosen because it is assumed that bathymetry has not undergone changes.

Each water balance has been conducted matching the equation of inlets and outlets to the system:

$$P + EA = ED + ETR + ESC_s + GW \pm \Delta V$$

where P is the precipitation on the basin, EA is the artificial water inlet, ED is the direct evaporation from the surface of the lake, ETR is the real evapotranspiration of the basin vegetation, ESC_s is the surface runoff towards other basins, GW is groundwater and ΔV is the volume of increase or decrease in storage in the lake.

The baseline data for the calculation of water balances were: measurements *in situ* of artificial water inlet (with flowmeter) and climatological data of daily cumulative precipitation (p) and average daily temperature (t) at the Vielha-Mijaran station. Data from the stations at Sasseuva and Bonaigua have been used in cases of missing data. The p and t data were corrected by the difference in altitude at each of the stations with that at Vilac Estanho. The correction factor for annual precipitation is +30.1 mm every 100 m, applied by Del Valle (1997) for the basin of the Esera river in Benasque, and -0.49 °C every 100 m for the daily temperature, according to the recommendations of Lampre (2001).

The annual direct evaporation of water from the surface of the lake has been calculated using the Visentini equation (1963). In order to calculate the actual evapotranspiration, the monthly and annual evapotranspiration potential (ETP) has been calculated according to the Thornthwaite (1984) equation, which takes into account the average monthly temperature (T) and the latitude from the annual heat index (I), a monthly factor (a) that depends on this and a factor f that depends on the location.

Using the ETP , the ETR that is finally used in the balance of the basin has been calculated, providing the monthly hydrometeorological balance according to Thornthwaite for the water requirements of the vegetation. This method (Doorenbros & Kassam, 1979) takes into account a 42 mm water retention capacity (CAD), calculated according to the soil type in the basin, starting at a *mean* CAD = 1.4 mm cm⁻¹ for medium

texture soils, and considering a maximum depth of the root system (Z_r) of 30 cm.

For the hydrogeological balance of the basin, ETR and P were applied to the entire surface of the Estanh de Vilac micro-basin, which has 11.37 ha, and the lake surface ED in each period. The balance in the micro-basin has been closed calculating the volume of groundwater as the only unknown, since the basin is endorheic and there are no surface water outlets. Water that supposedly goes underground is treated as surplus.

Determination of flood level

To control the water level variations of the lake, a metallic rod was placed in the bed at a distance of 2 metres from the watering hole inlet channel. In the different campaigns, the distance from the end of the rod to the surface of the water was measured, taking the first as the zero point to assess the level changes. The same type of observation was conducted by taking another reference element (upper margin of the watering hole drainage channel) to corroborate the data. Data was collected until 2018.

Study of the nutrients cycle

During the summer and autumn of 2014, four samples of the water from Estanho de Vilac and three from the artificial water inlet were taken. Sampling include, among others, the collection of physicochemical data (temperature, turbidity, conductivity, pH and alkalinity). The water temperature was measured in a depth profile every 0.5 m. In addition, a data-logger was placed in the lake that measures continuous temperature to know the exact dates of freezing and defrosting. Turbidity was measured with Secchi's disc. The conductivity and pH were determined in the field, with manual multi-parameter probe, and in the laboratory. Alkalinity was determined by Gran's automatic potentiometric titration.

The water collection from both the inlet and the pond was carried out following standard protocols (ACA, 2005, 2006). Sampling of the pond was always conducted in the deepest place, taking representative samples of the entire water column.

In all samples, the main nutrients were analysed: nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}), total phosphorus (TP) and total nitrogen (TN), following the methods recommended by Ventura *et al.* (2000). NO_3^- was determined by electrophoresis with CIA-4000; NH_4^+ by spectrophotometry with the phenol-hypochlorite method; PO_4^{3-} with the malachite green stain method; TN was determined by persulfate digestion followed by ultraviolet spectrophotometry; and TP was oxidised to phosphate by persulfate digestion.

Lake water samples were taken and chlorophyll *a*, *b* and *c* were analysed as a surrogate of phytoplankton biomass. Filtering with 47 mm GF/F filter was conducted to saturation, noting the volume (minimum volume of 2.5 l). Chlorophyll was extracted by sonication with 5 ml at 90% acetone and measured with spectrophotometer. Chlorophyll concentrations were calculated with the equations

of Jeffrey & Humphrey (1975).

We estimated the DIN/TP molar ratio (DIN is the dissolved inorganic nitrogen) recommended by Bergstrom (2010) as one of the factors that can be related to phytoplankton biomass.

The main entries of nitrogen and phosphorus were studied, considering the contribution of direct rainfall on the pond and on the basin, the contributions that arrive through the artificial water inlet and the contributions derived from the presence of equine livestock in the basin.

The concentration of DIN, in the form of NO_3^- and NH_4^+ , that arrive through the rain, was taken from studies that include the area of Vielha (Catalan & Camarero, 1994). The TP in the rain was taken from Camarero & Catalan (2012). The amount of nitrogen and phosphorus that can be derived from equine livestock droppings was taken from agricultural studies (Iglesias, 1995; Boixadera *et al.*, 2000). Lastly, the DIN, TN, TON, TOP and TP means of the samples taken at the artificial water inlet for the pond were used.

To calculate the annual entry of nitrogen and phosphorus at Estanho de Vilac from contributions to the basin (not direct to water), it was taken into account that not all nutrients that enter the basin go to the pond, but instead a part thereof is retained by the vegetation. This occurs in percentages that depend on the vegetative period, and for nitrogen has also been seen to depend on the state in which it is found (organic or inorganic). The percentages for these calculations were taken from Iglesias (1995) and were applied to the estimated contributions of equine livestock (Iglesias, 1995; Boixadera *et al.*, 2000) and the amount of inorganic nitrogen and phosphorus from the average precipitation on the basin (Catalan & Camarero, 1994; Camarero & Catalan, 2012).

VARIABILITY STUDY OF THE BIODIVERSITY OF ESTANHO DE VILAC

To assess the variability in the biodiversity of Estanho de Vilac and the significance of the anthropogenic factors that affect it, analyses were conducted from two points of view. First, the trout community introduced in the lake and the stomach content thereof has been studied in order to assess the composition of its diet, since from the results obtained in other studies (Knapp, 2001; Jones & Sayer, 2003) it was considered that the predation by *Salmo trutta* of certain groups may be one of the determining factors in the loss of biodiversity at the Vilac lake. Second, a temporal analysis of the samples carried out with the LimnoPirineus project was conducted on several indicator groups: littoral macroinvertebrates, amphibians and crustaceans. These data were analysed in reference to the presence of trout introduced and in the case of crustaceans, the community has also been analysed with respect to the biomass of phytoplankton, using chlorophyll *a* as a surrogate of biomass.

Actions with the introduced fish

The trout detected during the study period were most likely introduced into the lake in 2011. One of the objectives of the LIFE+ LimnoPirineus project to eradicate introduced fish from high mountain lakes, was conducted in this regard at Estanho, by the company Sorelló S.L., project partner, through the installation of sampling nets (multi-mesh net; Nordic fishing standard). In July 2014, initial fishing had been carried out at Estanho, from where six specimens were extracted. Second fishing in October of the same year, estimated that almost all of the specimens had been eliminated, and in July 2015, the last specimen was recovered with a new net.

The 18 specimens caught were weighed, measured, and gutted. The stomach and intestines of each trout were preserved, first by freezing and then in ethanol for analysis. To assess the effect of *Salmo trutta* on the biodiversity of the lake, the stomach content of 12 individuals was studied. In the laboratory, cleaning and separation of these stomach contents was performed, and the identification and counting of organisms was conducted using a stereomicroscope with 6 to 40 magnifications. For the identification of macroinvertebrates, both in trout stomachs and benthos, the Puig (1999) illustrated identification guide was used.

Sampling and analysis of the variability in indicator groups

The temporal analysis of the abundances for each of the indicator groups was carried out based on data collected as of June 2014, and in the case of amphibians, also with information prior to the introduction of fish in Estanho (2008-2013). The data available and the type of sampling and analysis conducted for each group were the following:

Amphibians: Data from 16 censuses carried out over the different years of the project have been analysed. In all cases, the sampling consisted of counting the number of individuals in five 2 m transects, representative of the habitats on the pond shore. When different censuses were available for the same year, the highest data was used.

Benthic macroinvertebrates: Four sampling campaigns were performed (July, August, September and October 2014). Sampling of this group was carried out following a manual scanning methodology from the shore (littoral strip-sampling) in order to avoid damage to the *Luronium natans* macrophyte (20 sweeps of 1 m x 0.4 m). Samples of benthos collected were passed through a 1 mm sieve and the counts were carried out using a stereomicroscope with 6 to 40 magnifications.

Crustaceans: Six sampling campaigns were done (July, August, September and October 2014 and June 2015). For this, representative samples of the entire water column were filtered using a 200 µm conical net. Subsequently, the samples were separated under a microscope.

Sampling and analysis of the *Luronium natans* population

The *Luronium natans* population was differentiated into

three subpopulations according to rooting depth. The one that occupies the internal part of the lake (subpopulation C), the one that develops in the flooded area with depths between 10 and 100 cm (subpopulation B), and the one established at the perimeter edge that may emerge temporarily (subpopulation A).

For subpopulations A and B, sampling was carried out to determine its size at the beginning of the project (2014) and in the years after the modification of the water inlet flow (2016-2019). For this, 10 to 14 samples were considered in each zone, each corresponding to a surface area of 50 x 50 cm using a metal square, and the number of *Luronium natans* branches included in the square were counted. The samples were regularly and randomly distributed, following the edge of the lake. For subpopulation C, an estimate was made of the area covered by the plant, based on its abundance in 50 x 50 cm squares arranged on the water surface (values from 1 to 4 according to the occupation of the 4 quadrants) These samples were randomly distributed 10 times throughout zone C. An initial estimate (2014) was also made for biomass.

Phenology was monitored using the same number of samples and with the same arrangement as those used to assess the population size. Throughout the vegetative period, from May to September (October), there were monthly visits in 2014, 2015 and 2016, and more distanced visits from 2017 to 2019. The different forms of vegetative growth (linear basal leaves, spatulate basal leaves and floating elliptical leaves) and reproductive growth (flower, fruit) have been differentiated. No permanent samples were established due to the rapid change of the environment when uncovered by water. Good or bad state of growth and direct disturbances on the population have also been considered (livestock, tourism and occasional events). The spread of *Potamogeton berchtoldii*, which competes directly with *L. natans*, was simultaneously evaluated.

In order to verify the persistence of fruits and seeds in the lake sediment, in autumn 2014, (10/10/2014) a sampling was carried out of the sediment of the flooded area. A total of 16 samples were collected (10 cm from the top of a sediment cylinder extracted with a 7.5 cm diameter cylindrical probe) distributed at different depth points. The littoral zone was not sampled because it was a space of new colonisation where there has been no flowering or fruiting of the species. From the collected sample, the superficial 10 cm were selected and stored in the freezer until their study. Each sample was washed and screened to retain sediment components that were similar in size to fruits or seeds (*Luronium natans* produces monosperma achenes). Once this fraction was dried, the seeds were separated under a stereomicroscope and counted.

RESULTS

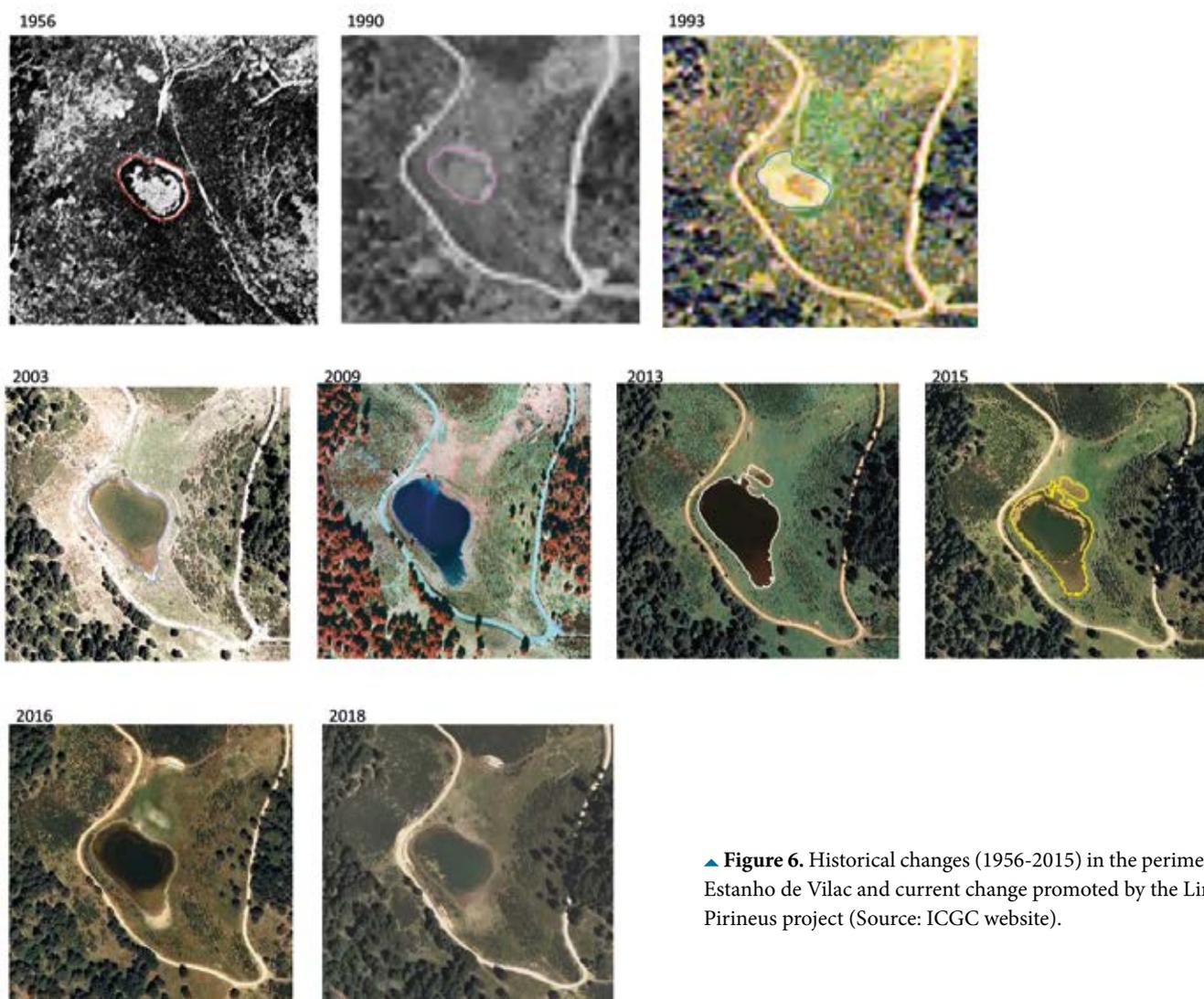
Hydrological variability: water balance

Estanho de Vilac has undergone very clear hydrological

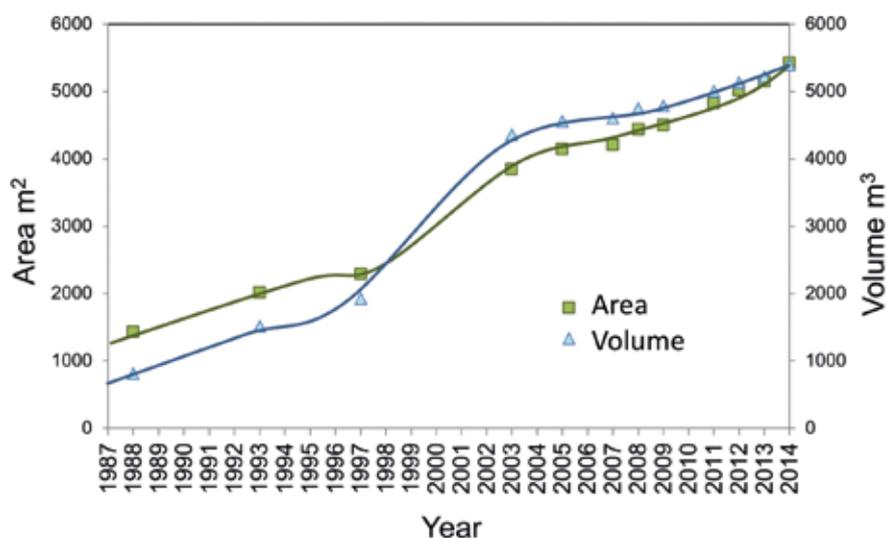
changes (Figure 6) that have been determined with the analysis of the historical aerial photographs that the ICGC (<http://www.icc.cat/vissir3>) has compiled. Mapping of the variation of the perimeter of the pond, together with the water balance, allows the representation of the historical evolution of the estimated surface area

and volume (Figure 7).

Hydrometeorological balances revealed an average real evapotranspiration (ETR) of 395.3 ± 65.1 mm/year. This represents 78% of the ETP (504.38 mm/year), which is consistent with climatological studies carried out in the

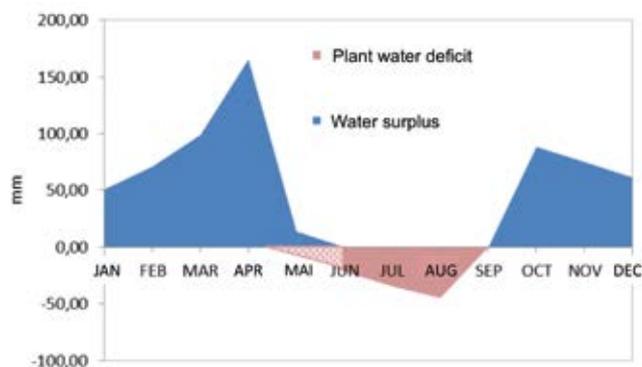


▲ **Figure 6.** Historical changes (1956-2015) in the perimeter of Estanho de Vilac and current change promoted by the Limno-Pirineus project (Source: ICGC website).



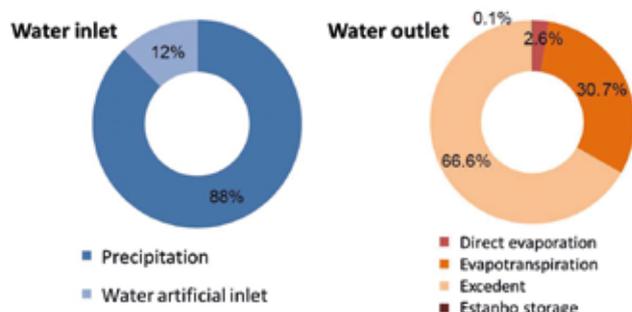
◀ **Figure 7.** Historical evolution of the surface and volume at Estanho de Vilac.

Aragonese Pyrenees (Lampre, 2001). The average precipitation (P) between 2003 and 2014 was 1112.68 ± 184 mm/year and the average direct evaporation from the lake surface was (ED) 842.26 ± 64.3 mm/year. Vegetation in the basin suffers water stress for 2 to 3 months a year, normally between June and August, and from September-October there is a water surplus (Figure 8).



▲ **Figure 8.** Hydrometeorological balance in an average year (balance 2011-2012) with indication of periods of surplus and plant water stress.

Applying the results of the hydrometeorological balances to the Estanho de Vilac basin and taking the artificial water inlet for the lake into account, we obtain that P is the main input of the balance in the basin (129.75 ± 21.01 hm³ per year) and GW the main output (98.55 ± 17.09 hm³ per year). EA is a constant value that represents 12% of the inlets (17.86 hm³ per year). It has a flow rate of 0.89 ± 0.127 l/s for 232 days when the water is not frozen. Taking P as the reference value, EA represents 14.06% of P . ETR represents 35% of P and 30.7% of the balance output (45.08 ± 7.32 hm³ per year). Figure 9 shows the distribution of balance components in percentages of total input and output.



The average storage of lake surface water (ΔV) is a very small value of the balance (0.1%), but it is always positive, which results in a constant increase in its volume. ED represents only 2.6% of the balance output but is $78.6 \pm 4.24\%$ of the volume of the lake (3.88 ± 0.44 hm³ per year). The excess (EXC) represents 66.6% of the balance output and 75.9% of P , and it is argued that its destination is mainly to recharge aquifers (GW).

Chemical variability. Input of nutrients

According to the data collected, the Estanho de Vilac water has an average conductivity of 113.2 $\mu\text{S}/\text{cm}$ and an average pH of 8.59. Water temperature varies a lot throughout the year. It remains below zero from mid-November to the end of March and a maximum value was measured during the sampling of 22.3 °C. The average is 15.98 °C. The artificial water inlet to Estanho freezes at the same time as the lake, but the rest of the year it remains at a constant temperature of 12.5 °C and has conductivity and pH values similar to those of Estanho (168.2 $\mu\text{S}/\text{cm}$, pH 8.6). Thermally, the lake is free of ice 7.74 months per year (232.25 days), freezing approximately in mid-November and defrosting at the end of March. The maximum temperature does not usually exceed 25 °C. In the four samples taken, the Secchi disk indicated that light penetrates to the bottom of the lake (2.7 m).

The average content in the main compounds of nitrogen and phosphorus, both in the Estanho water and the artificial inlet can be seen in Table 1, where there are notable differences between the composition of both, especially in terms of nitrate content.

		TON $\mu\text{g N}/\text{l}$	NO_3^- $\mu\text{g N}/\text{l}$	NH_4^+ $\mu\text{g N}/\text{l}$	TN $\mu\text{g N}/\text{l}$	DIN $\mu\text{g N}/\text{l}$	TOP $\mu\text{g P}/\text{l}$	PO_4^{3-} $\mu\text{g P}/\text{l}$	TP $\mu\text{g P}/\text{l}$
Artificial inlet	Average	0.00	378.62	1.51	313.35	380.13	0.65	0.78	1.42
	St. Dev.	0.00	105.48	2.10	138.43	105.77	0.77	0.03	0.79
Estanho	Average	397.08	5.98	5.13	406.54	11.11	15.86	2.79	18.65
	St. Dev.	130.83	3.24	5.32	132.16	8.56	8.34	1.89	9.96

▲ **Table 1.**

Average concentration and standard deviation of nitrogen and phosphorus compounds in the artificial water inlet to the lake and in the lake itself.

The inlet water through the tributary has an average NO_3^- concentration of 379 $\mu\text{g}/\text{l}$, while that of Estanho is only 5.98 $\mu\text{g}/\text{l}$. As for P, the differences are less pronounced, but the concentration is greater in the lake water, both for PO_4^{3-} and above all TOP (total organic phosphorus). There are also differences in TON (total organic nitrogen). While the water inlet does not have organic nitrogen, the lake water has an average concentration of 379.08 $\mu\text{g}/\text{l}$.

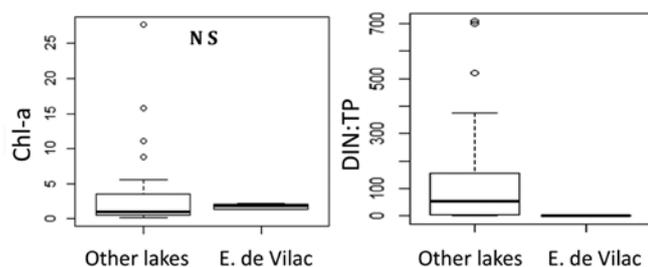
◀ **Figure 9.** Distribution in percentages of the water inlet and outlet balance in the system.

The ratio DIN/TP may indicate a limitation in Estanho primary production due to any of the nutrients (nitrogen or phosphorus) (Bergstrom, 2010). The values found in Estanho are shown in Table 2.

Date	DIN:TP	Limiting factor
16/07/2014	2.2	P
07/08/2014	1.1	N
15/09/2014	1.5	limit
10/10/2014	2.1	P
17/06/2015	0.6	N

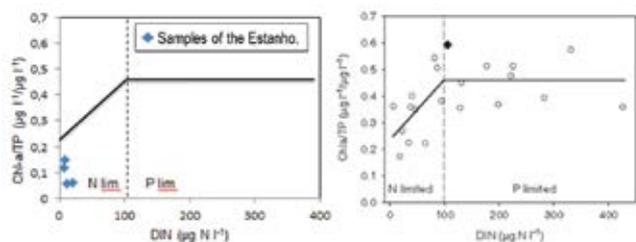
▲ **Table 2.** Limiting factor of phytoplankton production according to DIN: TP ratio (Bergstrom, 2010) in Estanho water samples

The *chlorophyll a* concentration was found at values between 1.19 µg/l and 2.18 µg/l, not being significantly different ($P = 0.262$; Mann-Whitney-Wilcoxon test) from the rest of the LIFE+ LimnoPirineus project lakes (40 lakes of different characteristics), although they are relatively low compared to these (Figure 10 left). If we compare the ratio DIN/TP of Estanho de Vilac with that of the rest of the lakes in the project, we find that the values in this case are significantly different from those of the whole ($P = 0.0218$; Mann-Whitney-Wilcoxon test), and lower compared to most of them (Figure 10 right).



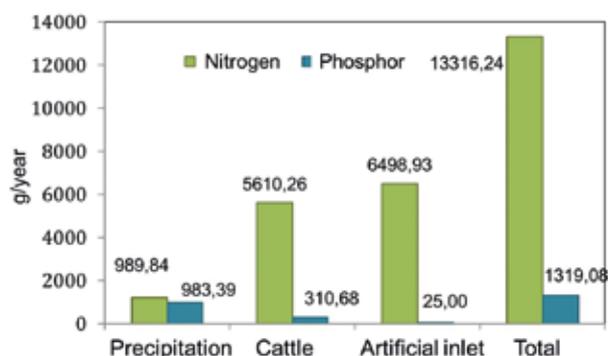
▲ **Figure 10.** Comparison between Estanho de Vilac and other 40 lakes in terms of chlorophyll *a* and DIN/TP, by means of the Mann-Whitney-Wilcoxon test for unpaired samples. NS= not significant; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

If we compare the relationship between phytoplankton biomass, TP and DIN with other studies carried out in the Pyrenees (Camarero & Catalan, 2012), the samples from Estanho de Vilac would be placed in a condition limited by N, with very little Chl *a* and DIN in relation to TP (Figure 11).



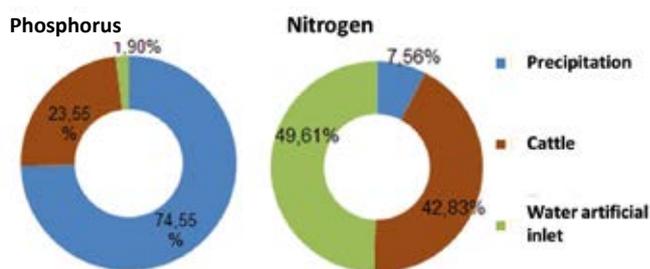
▲ **Figure 11.** Relationship between phytoplankton biomass, TP and DIN for the Estanho de Vilac samples (left) compared to the studies by Camarero & Catalan (2012).

In addition to the entry of artificial water, other sources of nitrogen and phosphorus entry into Estanho may be precipitation and the presence of equine livestock. Input for the entire Estanho microbasin have been calculated, taking a year as a reference period, and the results obtained can be seen in figure 12.



▲ **Figure 12.** Estimated amount of inorganic nitrogen and phosphorus that enters Estanho de Vilac in one year by different routes.

The origin of nutrient input as a percentage of the total input can be seen in figure 13. The main input of phosphorus, 75%, is produced through rain, while that of nitrogen is 92%, of local anthropic origin. 49.6% comes from the artificial water inlet and 42.8% from faeces of livestock present in the basin not retained by vegetation. It is necessary to consider that for the livestock, a conservative position has been proposed: 30 horses were counted grazing in a single month in summer in the area, that only half of the faeces are in the Estanho microbasin and that a large part is retained by the vegetation (Iglesias, 1995).



▲ **Figure 13.** Source of the nutrients that enter Estanho de Vilac according to percentage of entries.

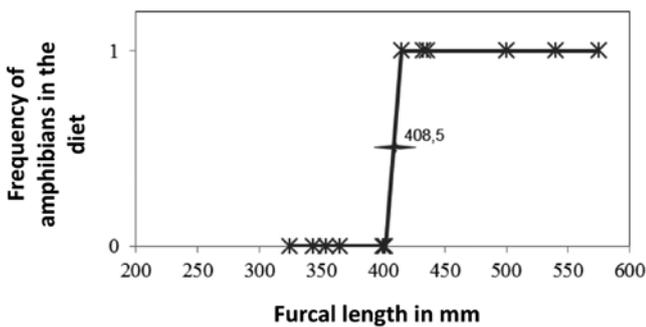
Variability of the biodiversity

A. Stomach content of introduced fish

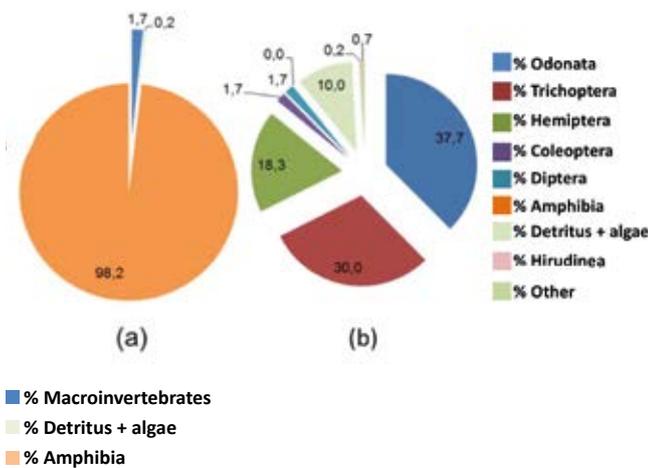
It is known that the trout at Estanho de Vilac were probably introduced in 2011. The age of the trout was determined by the otoliths of their inner ear (they were 3 years old in 2014) and the weight-length ratio of many

individuals agreed with the same, which is consistent with having specimens of the same age. Despite their young age, some of the trout caught reached a considerable size and weight (> 50 cm and > 3 kg).

The analysis of the stomach composition of the 12 trout caught in the October 2014 campaign revealed that their main diet consisted of macroinvertebrates and amphibians. A vast difference was observed between the diet of some trout and others. Some individuals seem to feed only on macroinvertebrates and others practically only on amphibians and this seems to be related to their size. Figure 14 shows how the logistic function indicates that small-sized *S. trutta* specimens feed almost exclusively on macroinvertebrates until they reach an average critical measurement of 408.5 mm. As soon as they exceed that length, amphibians become their preferred prey, representing almost 100% of their diet (Figure 15a). Up to 4 amphibians at once were counted in the stomach of a single trout. The analysis found a greater number of individuals (or parts of them) that belong to the Anura order than those that may belong to the Caudata order.



▲ **Figure 14.** Logistic function for the furcal length in mm of *Salmo trutta* after which amphibians appear in the stomach contents.



▲ **Figure 15.** (a) Average diet composition of large *Salmo trutta* specimens (FL > 408 mm) introduced in Estanho de Vilac. Macroinvertebrates included Odonata, Trichoptera and Coleoptera. (b) Average diet composition of small *Salmo trutta* specimens (FL < 408 mm) introduced in Estanho de Vilac.

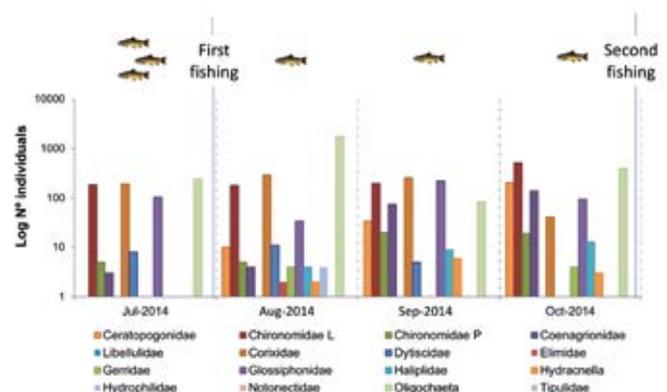
As for the macroinvertebrates found in the stomachs of small trout (Figure 15b), we can say that most stomach contents consist of *Odonata* (37.7%), *Trichoptera* (30%) and *Hemiptera* (18.3%) orders. Other orders that appeared in smaller proportions are *Diptera*, *Coleoptera* and *Hirudinea*. Crustaceans and some terrestrial invertebrates were also found. This type of content was counted as “Others”. Algae and sediment appeared in both large and small fish, sometimes in considerable quantity, although it is believed that they were collected involuntarily by trapping other prey.

Identification of the samples, in the case of amphibians, was only possible at the order level, since most were in an advanced state of digestion. In the identification of macroinvertebrates, however, it was possible to reach the level of families and subfamilies in some cases. We know that most *Hemiptera* in the trout diet are *Heteroptera* of the *Corixidae* family. And that all the *Diptera* found were from the *Chironomidae* family. From this family, both pupae and larvae were found and were counted separately in order to compare the results with benthos sampling. The larvae were mostly from the *Orthoclaadiinae* subfamily, while the *Tanypodinae* subfamily dominated for pupae.

B. Temporal analysis of macroinvertebrates

In most cases of littoral macroinvertebrate counts sampled in the benthos of E. de Vilac, it was possible to identify the family level, with the exception of mites and *Oligochaeta* that were counted at subclass level.

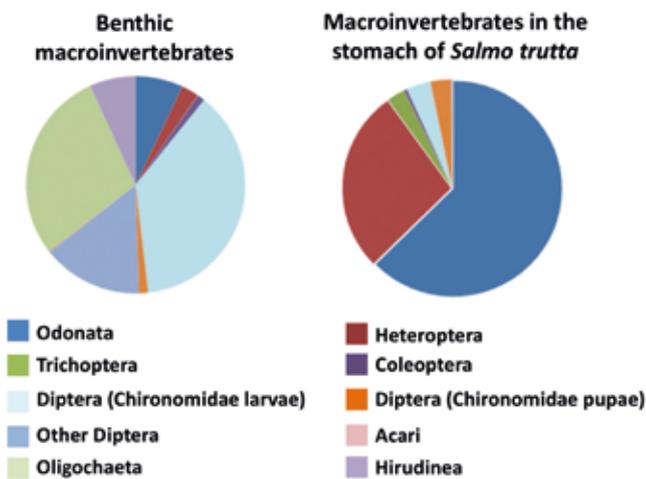
Figure 16 shows the evolution of the abundance of individuals in the families/subclasses over time using bar diagrams and reference is made to the *Salmo trutta* fishing carried out. The abundances are represented in a logarithmic scale so that all values are visible. In August there was an explosion of *Oligochaeta* that later disappeared.



▲ **Figure 16.** Evolution of the composition of the macroinvertebrate community from July to October 2014 expressed in abundance (in 8 m² of sampling).

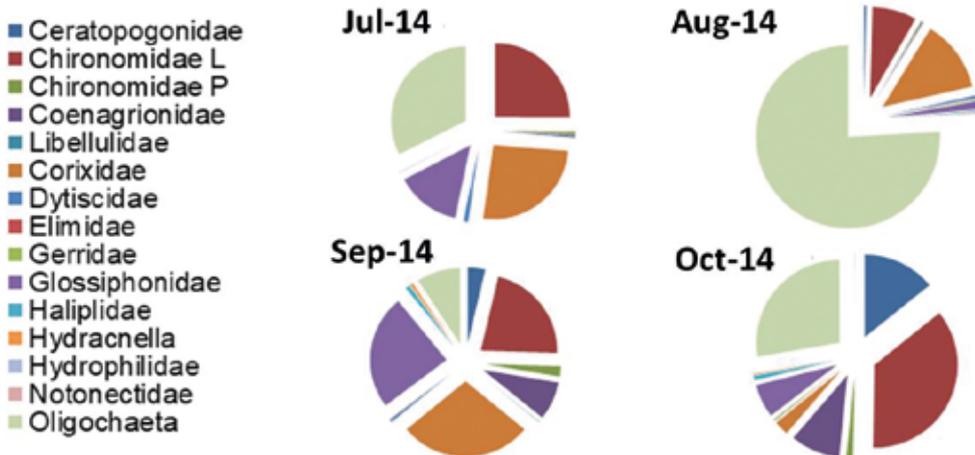
All macroinvertebrate samples were collected from an environment with trout, although in July 2014 the pressure from these should be greater as no fishing was carried out. In this case, there are no samples after the second fishing, which makes it difficult to assess the changes in the composition with respect to the presence of trout. However, we can compare the October sampling of macroinvertebrates with the stomach contents of the small trout that feed on them, since the sampling was done just a few days before the second trout fishing.

Figure 17, where the frequency of the macroinvertebrate groups in the benthos samples is compared to those from trout stomachs, reveals a vast difference between them that shows us the selection of prey by trout.



▲ **Figure 17.** Relative frequencies compared between the benthic macroinvertebrates sampled in the lake and in the stomachs of 6 trout.

Returning to the composition in the benthos, Figure 18 shows the evolution of the relative abundance of each family/subclass in the samples in percentages. Clear differences in time can be seen, but these may be due to seasonal changes in taxonomic groups.



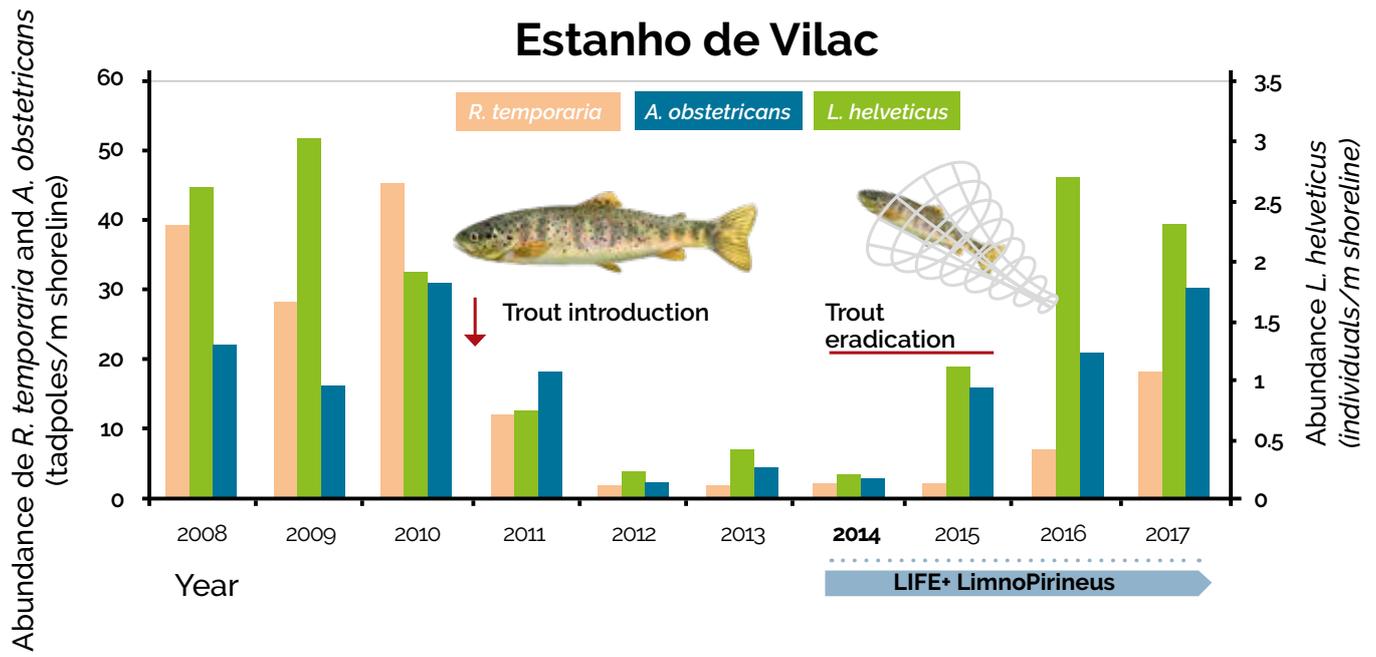
◀ **Figure 18.** Evolution of the composition of the macroinvertebrate community from July to October 2014 in terms of relative abundance of families/subclasses in the sample.

Some families of macroinvertebrates have specific increases in abundance, which may be due to seasonal hatching, as would be the case of *Oligochaeta* that have a very noticeable increase in August and a subsequent marked decrease. Other similar cases would be the *Corixidae* family of *Heteroptera* in August-September, and the *Glossiphoniidae* family of *Hirudinea* in September.

There are four families in which there is a progressive increase in relative abundance not attributable to seasonality. These are the *Diptera Ceratopogonidae* (from 0% in the sample in July to 14% in October) and *Chironomidae*, especially those in larval state, which go from 25% in the sample in July to 36% in October. The *Coenagrionidae* family of *Odonata*, go from 0.4% in July to 9.6% in October and the *Haliplidae* beetle family, which increases slightly but progressively from 0% to 0.9%. In the trout stomachs, only *Odonata* and *Chironomidae* were found in abundant proportions, so that not all of these changes can be explained by a lower pressure on behalf of the trout. A fact that attracts attention in this regard is the almost total absence of *Trichoptera* in benthos samples, when in trout it is a common prey.

C. Temporal analysis of the abundance of amphibians

The incorporation of data collected in previous projects allows us to verify that the trout introduced caused a significant decrease in densities, of 90-95% depending on the species, of amphibians present in the lake, which include the *Anura* of European interest *R. temporaria* and *A. obstetricans*, as well as the urodel *L. helveticus* (Figure 19). Once these trout were caught, amphibian populations recovered quickly and have been maintained at excellent levels throughout the project development.



▲ **Figure 19.** Evolution in the composition of the amphibian community at Estanho de Vilac. The estimated abundance of larvae or adults is represented per unit of coastline length.

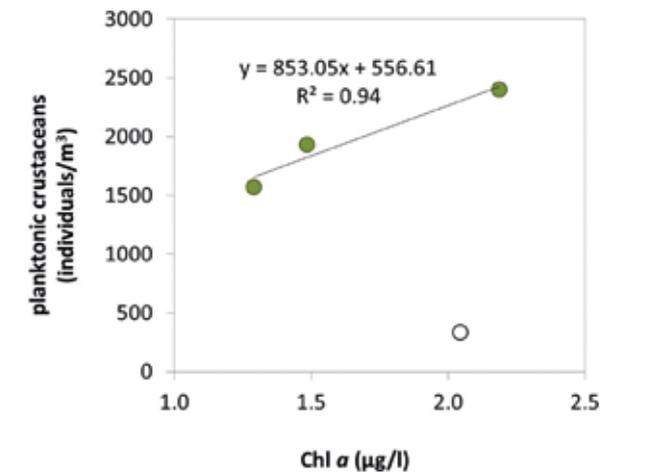
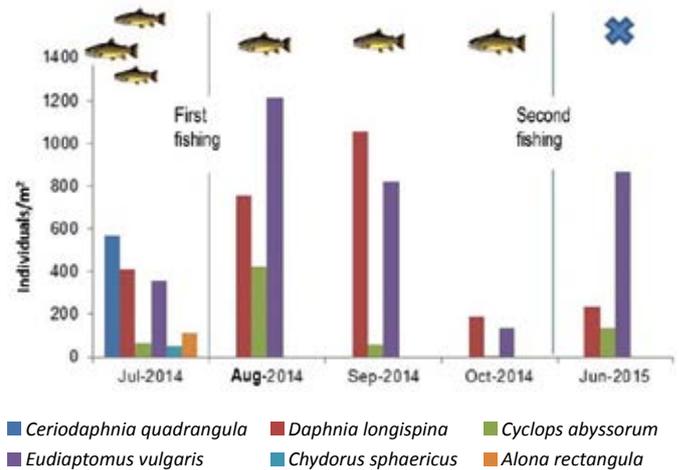
In the stomach of the trout caught in the second fishing, the two orders (*Anura* and *Urodela*) were identified, although it was not possible to reach the species level given the degree of decomposition of the amphibians.

D. Temporal analysis of the abundance of zooplankton

In the case of crustaceans, there is a sample of the crustacean community prior to fishing (July 2014), one after having eliminated all the fish, (June 2015) and two intermediate samples of the status with fish after the first fishing.

As shown in Figure 20a, which shows the evolution in the crustacean community, the first sample showed individuals of three species that have not been detected again in subsequent samples (*Ceriodaphnia quadrangula* OF Müller, 1785; *Chydorus sphaericus*, O.F. Müller, 1776 and *Alona rectangula* Sars, 1861). The three species not detected after trout fishing, have preference for eutrophic waters.

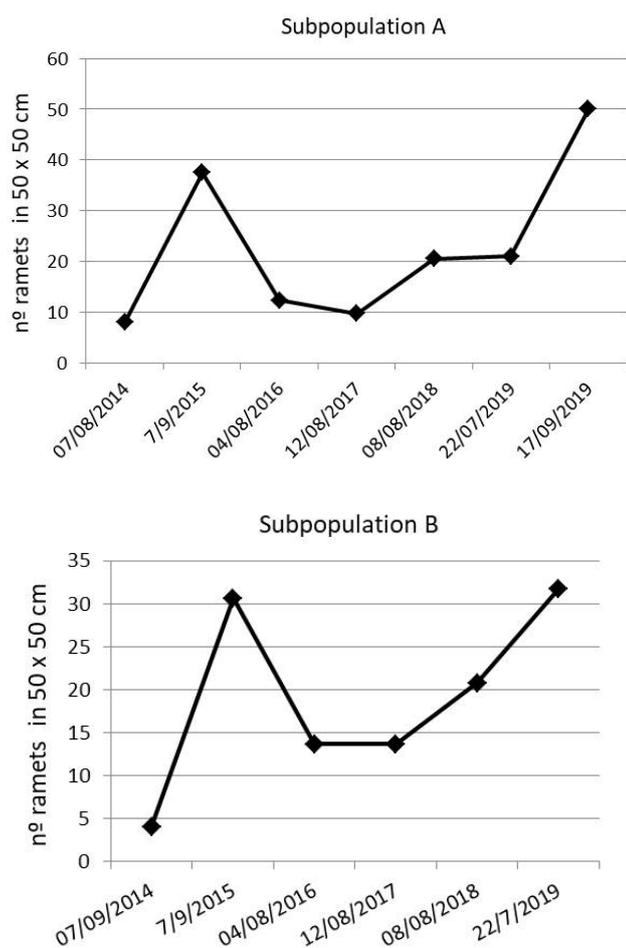
One of the factors that may be more related to the abundance of crustaceans is algal biomass. Figure 20 b shows the concentration of chlorophyll *a* (subrogating the algal biomass) against the abundance of crustaceans. The results indicate that between the months of July and September there is a positive relationship between algae biomass and the abundance of crustaceans, indicating that the abundance of crustaceans is related to the availability of food. In October, the abundance of crustaceans drops drastically, probably due to the phenology of these species, since they spend the winter in forms of resistance.



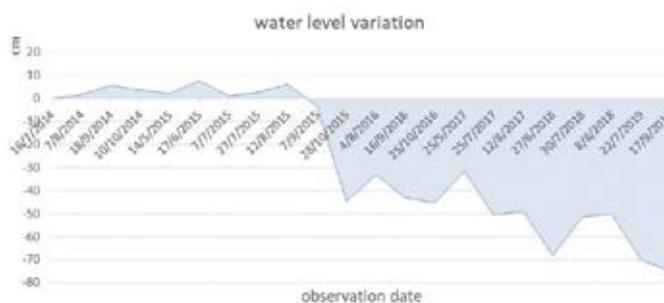
▲ **Figure 20.** Evolution in the composition of the crustacean community at E. de Vilac. (a) The number of individuals of each species is represented per estimated cubic metre. (b) Ratio of phytoplankton biomass estimated from chlorophyll *a* to the total abundance of planktonic crustaceans between July and September (green dots). In October the phytoplankton relationship is lost due to the seasonality of crustaceans.

E. Temporal analysis of the *Luronium natans* population

The *Luronium* population was monitored from July 2014 to September 2019. Of the three differentiated subpopulations, we were only able to establish continuous monitoring of subpopulations A (perimeter edge of the lake and temporarily exposed areas) and B (flooded area of low depth). The samples taken reflect the location status at each sampling moment (zone A or B) despite not having the same site. The withdrawal of the lake from the moment the artificial water supply is closed modifies the status of the sampling squares. Figure 21 shows how there is an increase in the number of individuals in the population as a whole at the beginning of September 2015, 11 days after the closure of the artificial water supply to carry out the installation works of a new watering hole. The water level has already dropped a few centimetres (Figure 22) and exposes an area with a large population of amphibious forms of *Luronium* and also improves the density of the flooded subpopulation. The 2016 sampling shows a sharp decrease in density, which slowly recovers in successive years.



▲ **Figure 21.** Evolution of the *Luronium natans* population according to the sampling carried out between July 2014 and July 2019 in subpopulations A and B, evaluated according to the number of branches per 0.25 m².



▲ **Figure 22.** Evolution of the water level between July 2014 and July 2019.

Regarding the development of the population's phenological cycle, since it is not possible to mark individuals, the data refer to observations based on both the density assessment samples and the set of different subpopulations.

Subpopulation A, scarcely developed in 2014 and widely extended in subsequent years, has always remained vegetative. No flower or fruit has ever been observed. Its growth status is variable according to soil moisture, showing larger sizes on silt with water at surface level or high water tables (saturated soils) and less developed forms in drier soils. The status of *Luronium natans* on these substrates recently emerged from the water is very vulnerable to trampling and disturbances caused by livestock, especially horses, both because of their weight and tendency to wallow and splash. Since the flood control started in 2016, subpopulation B has been moving towards the centre of the lake as its level decreased. In September 2019, it formed a dense crown of leaf rosettes that covered a wide area of the bottom of the Estanho edges.

Subpopulation B showed floating leaves and some rosettes with linear basal leaves during the first sampling years (2014-2016). In areas with less flooding, a remarkable expansive activity of the plant was observed, producing stolons and new rosettes. In deeper areas, shoots with long-petiolate leaves remained, with a tendency to decrease in size and vitality. This area is affected by occasional disturbances from livestock (they enter to drink water and cool off) and by direct competition from *Potamogeton berchtoldii* (macrophyte typical of mountain lakes that appeared in Estanho on an unspecified date). *Potamogeton* has expanded from the deepest waters of the centre of the lake (2014) to the edges (2018). Masses of filamentous algae that grow epiphytes on the plants detaching and floating agglomerates on the surface, also affect the growth of *L. natans*. Although the vegetative development is not good, the subpopulation of zone B has shown remarkable reproductive activity in terms of the formation of vegetative propagules (fragments of stolons with leaf rosettes) that the wind relocates within the lake; flower formation has been low. During 2014 and 2015, no flowering was observed. In 2016, 2017 and 2018, a few isolated flowers were seen, and in 2019,

there was abundant flowering (number of flowers already uncountable overall).

sample no.	depth (m)	location	n. of seeds
1	2.3	zone C	84
2	2.3	zone C	60
3	1.8	zone C	5
4	1.9	zone C	0
5	1.6	zone C	0
6	1.4	zone C	29
7	1.4	zone C	0
8	0.8	zone B	1
9	0.8	zone B	1
10	0.6	zone B	0
11	0.6	zone B	7
12	0.8	zone B	3
13	<0.4	zone B marginal	2
14	<0.4	zone B marginal	0
15	<0.4	zone B marginal	5
16	<0.4	zone B marginal	0

▲ **Table 3.** Evaluation of the *Luronium natans* seed bank in Estanho de Vilac (10/10/2014), based on the number of seeds detected in a total of 16 sediment samples, extracted from different depths.

Subpopulation C has remained more or less stable in status, although there is apparently a decrease in vitality (less leaf density and smaller leaves). In 2014, a central area in the shape of a crown was well identified in the lake, which in the years of monitoring has gradually disappeared.

The seed bank sampled in 2014 showed very variable densities according to the sample (Table 3). The results show that there were seeds in 62% of the samples, but these were only abundant in the samples obtained from the deepest part of the lake.

DISCUSSION

Hydrological variability

The hydrometeorological balance of the area indicates water stress for two months in summer. The balance in the Estanho basin gives an evapotranspiration of 35% P and a groundwater recharge of 75.9% P. This means that in the current climatic conditions and in a year without significant summer precipitation, the lake would dry within the period of plant water stress if there was

no artificial water inlet, recovering quickly towards the month of September. Despite being only 12% of the water entering the water balance, the entry of artificial water into Estanho is an imbalance that results in a continuous increase in the volume of the lake.

The changes in the hydrological regime of Estanho could affect *L. natans* compromising its survival. It has been observed that individuals in the deepest areas of the lake have a weaker appearance than those closer to the shore. Lansdown & Wade (2003), point out that this species has an ecological plasticity to adapt its way of life and reproduction to the environment in which it lives, so that there are three different adaptive typologies. The populations that grow in temporary water zones and in the area where permanent waters fluctuate, are annual and bloom abundantly. Plants that live in permanent waters are perennial and can give two other types. Those that live in bodies of water more than 2 m deep with little seasonal water variation are typically vegetative and only reproduce clonally from their rhizomes and stolons production. The last type presents the two types of reproduction, both sexual by flowering and clonal by stolons, and occurs in permanent water bodies with small level fluctuations.

The population of Estanho de Vilac would have evolved in conditions of significant water fluctuation (Perdigó, 1984) and since the modifications in the lake's artificial water inlet, the macrophyte tries to adapt to the new conditions, decreasing its sexual reproduction and increasing that of clonal type. This would explain the reduced flowering observed in recent years, and the current absence of this, in addition to the weakened appearance of individuals in the deepest areas. There is a very active clonal expansion towards the shore from the detachment of stolons and rosettes of individuals from the slightly flooded area. It is believed that some natural fluctuation of the water level would be adequate in this case, since sexual reproduction would help maintain genetic diversity, which would be fundamental in the Vilac population, which is isolated. Lansdown & Wade (2003) also point out that *L. natans* has more chance of survival in environments with permanent water than in seasonal water regime environments, so the best thing for the species seems to be to keep Estanho with permanent water at a level similar to the original (Perdigó, 1984) that allows a slight summer fluctuation of the level and exposes the flat bottom colonisable by the amphibious forms of *L. natans*.

Variability in the nutrients cycle

The calculation of nutrient input in the Estanho de Vilac ecosystem indicates a very high local anthropic input source, especially inorganic nitrogen (91%). It has been estimated that the annual amount of nitrogen entering Estanho is 10 times higher than P entering. With these data we would expect to find a clear limitation by phosphorus in the lake water and yet the DIN:TP ratio, Chl *a* and TP (Figure 11) shows that Estanho de Vilac is limited by nitrogen, with low phytoplankton biomass

values compared to other lakes studied in the area (Camarero & Catalan, 2001). According to the DIN:TP ratio (Bergstrom, 2010) the situation would change from one limiting factor to another over time (between nitrogen and phosphorus). These changes could be due to phosphorus input in the system due to atmospheric depositions that, as Camarero & Catalan (2001) point out, may become specific events that are heavily charged with phosphorus. In their article, the findings of Camarero & Catalan (2001) on recent anthropogenic changes in the depositions of atmospheric nitrogen and phosphorus and their consequences in the TN/TP ratio, lead them to argue in favour of the idea presented by Goldman (1988) that, contrary to what is classically thought, the limitation by phosphorus in mountain lakes is not natural but induced by the anthropogenic increase in nitrogen depositions.

One of the factors that may be limiting the growth of phytoplankton could be competition for light and nutrients with the community of macrophytes and epiphytic algae (Ventura *et al.*, 2008). It should be said that Estanho has significant seasonal differences in the state of development of floating algae, which could condition the entry of light and competition. In addition, both *L. natans* and *P. berchtoldii* are tolerant to high levels of nutrients, and in competitive circumstances this type of macrophyte usually has an advantage over the rest (Ventura *et al.*, 2008). With regard to the latter, it should be noted that *L. natans* has been described as intolerant of competition (Lansdown & Wade, 2003). The same authors point out that, in the event of eutrophication the flowering perennial forms of *L. natans* tend to reduce their coverage in favour of monocots and algae. During the project, considerable expansion of *P. berchtoldii* was observed from year to year, so it is possible that it is gaining ground against *L. natans* by competition.

Regarding zooplanktonic, there is a community of heleoplanktonic crustaceans in Estanho that may be helping to control phytoplankton biomass. It has been proven that in other target lakes, where there are no crustaceans, chlorophyll *a* content is much higher than that found in the Estanho. Phytoplankton biomass remains relatively low despite high nutrient input, as seen in previous sections, and crustaceans increase their abundance with phytoplankton biomass (Figure 20b). However, the amount of crustaceans present does not seem enough to explain low values of chlorophyll *a*, but they can serve as an indicator of a series of more complex processes in which other zooplanktonic and most likely heterotrophic organisms would also participate, which would help to keep phytoplankton biomass at low values despite large continuous nutrient input. Phytoplankton biomass could be limited by the combination of these factors (competition with macrophytes and feeding of zooplanktonic organisms) and most likely also by heterotrophic bacterial activity.

The high concentration of total organic nitrogen in the lake water, not being phytoplankton, may be organic matter in suspension. This TON would be scarcely available for the microbial loop as the lake is in conditions limited by nitrogen.

Variability of the biodiversity. Effects of the introduction of *Salmo trutta* in indicator groups

Trout are very selective predatory fish and, whenever possible, will prefer large sized prey that is easy to detect. This data has been confirmed by the study, since a critical size of around 40 cm has been found, after which trout go from basing their diet on macroinvertebrates to basing it on amphibians, since they acquire the ability to catch them when they grow larger. The high selectivity in catches made by the trout at E. de Vilac is not only seen among large and small trout, but in small trout that basically feed on macroinvertebrates, where a selection of some orders and families compared to others is clearly observed, since the differences between the composition of the macroinvertebrate community in the benthos and that found in the trout stomach is very different. In the stomach content of small trout, crustaceans also appeared, but to a much lesser extent than macroinvertebrates, it is assumed that with the same efficiency motivation. Crustaceans are likely to be more attractive to trout in case of population explosions.

Regarding the effect of salmonid predation on the indicator communities, a very significant effect on amphibian abundance has been proven, with the total abundance measured in Estanho decreasing by 90-95% in periods with trout versus periods without trout. The species most affected by the presence of trout were the common frog (*R. temporaria*), the common midwife toad (*A. obstetricans*) and the palmate newt (*L. helveticus*). The fourth amphibious species present in Estanho, the spiny toad (*B. spinosus*), is not predated by fish, possibly due to the toxicity of its skin (Miró *et al.*, 2018). However, the amphibian community seems to have recovered very quickly by eliminating trout and the four species that were found before the introduction of salmonids have reappeared in the usual abundance only a few months after their elimination. With the analysis of trout stomachs, it has been proven that they can be very voracious in terms of amphibians by concentrating on them as prey. The rapid recovery of amphibians has been undoubtedly favoured by the fact that the introduction of *Salmo trutta* had been recent and not all trout had yet acquired the ability to prey on amphibians, so the lake still had sufficient reserves of individuals to recover the community. The rapid and timely action of eradicating recently introduced trout in Estanho has prevented the disappearance of some amphibian species from the area in the medium term, as has been shown to happen in high mountain lakes where salmonids have been introduced, both in the Pyrenees and in other mountain ranges (Knapp 2005; Miró *et al.*, 2018).

As for the effect on macroinvertebrates, it is difficult to specify with the data available. It can be argued that the pressure exerted by salmonids has been greater for *Odonata*, *Heteroptera*, *Trichoptera* and *Diptera* of the *Chironomidae* family, because they are the ones found in trout stomachs, but we cannot confirm these results in the environment. It is possible that a recent introduction of *Salmo trutta* makes these effects still not noticeable,

since they mainly prey on amphibians. Over a longer period of time, it would be expected that by completely eradicating amphibians, as happens in all high mountain lakes with fish (Ventura *et al.*, 2008), the pressure would pass to the most conspicuous macroinvertebrate groups.

As for crustaceans, there are three species that disappear after the first trout fishing. One could argue from this, that trout exert pressure on larger crustaceans, since by decreasing this pressure these species prevail. However, trying to establish a relationship between changes in the crustacean community and the presence of salmonids is very complicated, since there may be many other influential factors such as food availability or environmental factors. It should be taken into account that some species such as *A. rectangula* and *C. sphaericus* have been reported as not very abundant and difficult to be continuously detected in the environment (Armengol, 1978).

Variability of the *Luronium natans* population

The population of *L. natans* is very conditioned by the morphology of the Estanho lake and the changes in its hydrological balance. The modification of the bed and the continuous growth of the volume of water from the end of the 90s until 2015 caused a change in the living conditions of *L. natans*, making deep water forms prevalent over amphibian forms. The geographical location of Estanho, expressed by altitude and other variables, is not favourable to the growth of this type of forms (Bardin *et al.*, 2012). The limitation of the artificial water inlet has caused the level to decrease returning the Estanho to its pond status. The amphibious forms of *L. natans* become dominant again, as when Perdigó (1983) discovered the species. From the conservation point of view, the artificial flow of water must be controlled to ensure the maintenance of the pond and also the passage and permanence of livestock. *Luronium natans* has a large ecological range within the aquatic environment, but it is limited by high concentrations (greater than 20 µg/l) of phosphorus (Bardin *et al.*, 2012), and due to its pioneer character, it is only competitive in habitats poor in nutrients (Willby & Eaton, 1993). Currently, Estanho de Vilac is an alkaline alpine pond according to the parameters evaluated (> 200 µeq l⁻¹). Although the concentration of chlorophyll in late summer is low (ca. 2.3 µg l⁻¹) as well as total nitrogen values (TN ca. 526 µg l⁻¹), it has high total phosphorus values (TP ca. 22.7 µg l⁻¹) that place it in the mesotrophic domain (9.3 µg l⁻¹ < TP < 31 µg l⁻¹).

The change of the environmental conditions promoted by the LIFE+ LimnoPirineus project at Estanho de Vilac and better knowledge of the habitat in which *Luronium natans* develops, leads us to propose the modification of its inclusion within the CORINE Habitat 22.433, as this has oligotrophic bodies of water. According to the characteristics of the water, the permanently flooded population corresponds to Habitat 22.431 (with a particular code, 22.431n) and should be included in HCI 3150. However, the temporarily flooded population

should be treated with the perennial amphibious communities of the Eurosiberian and Alpine region (22.31) with their own code that identifies them (22.31n amphibious populations of *Luronium natans*) included in HCI 3130. Similar formations of *Luronium natans* have been described from the Czech Republic as an association (*Luronietum natantis* Szankowski) within the *Littorelletea* class and the *Eleocharition acicularis* alliance (Chytrý, 2011).

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