

Szent István University

**IN SITU AND EX SITU
PILOT CONSERVATION PROGRAMME
OF THE EUROPEAN MUDMINNOW
(*UMBRA KRAMERI* WALBAUM, 1792)**

SÁNDOR TATÁR

Gödöllő

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Doctoral School

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1. Introduction and aims

In Europe nearly 80% of the native fish species are endemic and 37% of them are threatened, which is high compared to other taxonomic groups. There is evidence that 13 fish species have already gone extinct in Europe, with five others facing impending extinction. The greatest threats to native European fish are pollution, habitat fragmentation and destruction, loss and the spread of invasive alien species. The effects of climate change (e.g. drought) have been shown to drive the deterioration of freshwater ecosystems, and fish living in shallow waters of wetlands are in particular danger. Nonetheless, fish are under-represented in the conservation literature, with a clear bias in favour of birds and mammals.

Fen habitats are particularly exposed to environmental regulation by people, and in Europe their area has decreased dramatically. In Hungary, 97% of fens have been destroyed by regulation of watercourses, draining and ploughing. Small, isolated remnant marshland populations are particularly sensitive to environmental alterations (e.g. isolation), which often lead to a decrease in genetic diversity and an increase in mortality, as in the case of the European mudminnow *Umbra krameri*.

Freyhof & Brooks (2011) urged the establishment of Freshwater Key Biodiversity Areas and the development of species protection plans for European freshwater fish species. They also suggested establishing habitat monitoring and ex situ programmes, restrictions on settling of non-native fish species, and revision of the relevant legislation. One of the most important roles of a species protection plan is to identify and rectify factors that caused the initial extinction or decline.

The European mudminnow is small-bodied (7-8 cm), is the only native representative of the Umbridae family in Europe and is an endemic fish species of the Danube catchment area. It occurs sporadically along the Danube River between Vienna and the Danube Delta. Some populations live in the lower stretches of the River Dniester but the species' main range is in the Carpathian Basin. The European mudminnow lives in marshes, fens, vegetated backwaters and channels with clean water. The main threats to the species are habitat loss as a result of dredging of channels, the destruction of river and stream floodplains, the loss of fens and marshes and the spread of the invasive Chinese sleeper *Perccottus glenii*. This voracious competitor and predator of the European mudminnow is expanding its range in Eurasia and has colonized the catchment area of the Danube and Dniester rivers. It is estimated that populations of European mudminnow have declined by 30%. The species is categorized as vulnerable on the IUCN Red List and it also features on the Red Lists of seven European countries.

Although most of the reintroductions proved to be successful at a local scale in several countries, there is no detailed action plan for the conservation of declining populations of the European mudminnow across its diminishing and fragmented range.

The aim of the European mudminnow Conservation Programme (established in 2008) is to develop and test a comprehensive methodology to promote the rescue

and strengthening of populations of European mudminnow in Hungary. Accordingly, I implemented in situ and ex situ experiments including:

- (1) determining habitat characteristics (abiotic and biotic factors) required by the species, for consideration in the planning of surrogate habitats,
- (2) creating new fen and marsh surrogate habitats (“Illés Ponds”) to supplement lost native areas and harbour rescued populations and their offsprings,
- (3) rescuing broodstocks from habitats known to be threatened (e.g. dredging, filling up, draining, succession, exposed to contamination or desiccation) for propagation and preserving genetic diversity,
- (4) checking the quality of the created surrogate habitats (water quality, macroinvertebrates, macrophytes),
- (5) propagating and rearing European mudminnow under controlled laboratory conditions,
- (6) mudminnow stocking: creating new, self-sustaining populations in Szada Pilot Area for reintroduction and strengthening natural populations in the long term
- (7) monitoring the quality of the created habitats after stocking (abiotic and biotic factors).

2. Material and methods

2.1. Assessment of habitats and new surrogate habitats

The ecological requirements of the European mudminnow were determined based on a comprehensive literature review and field research in native habitats. In total, 11 natural habitats of the species were selected at random for surveys.

Water quality analysis (dissolved oxygen, pH and conductivity) were conducted using portable water quality meters (Votcraft) and other chemical properties (phosphate, ammonium, nitrite and nitrate ion concentrations) were measured using a VISOCOLOR[®] ECO test kit (Macherey-Nagel). Abundance and number of taxa of macroinvertebrates (i.e. food base of fish) were assessed using a kick-and-sweep sampling method. Botanical investigations included assessment of the macrophyte coverage and identification of all species and the dominant taxa at each site. Algal or bacterial blooms (cyanobacteria, sulphur bacteria) observed were recorded.

Fish assemblages were sampled using electric fishing (IG200, Hans Grassl) along randomly selected 150 m long transects. Samples were taken by wading upstream in streams and canals, and covering the whole volume of small, still waters (i.e. ponds and fens; < 500 m³). Fish were captured using electric fishing, placed in a plastic barrel filled with oxygenated water and transported to a laboratory at the Szent István University.

2.2. Szada Pilot Area

The 16 ha Szada Pilot Area (Fig. 1b) was chosen for the creation of new fen and marsh surrogate habitats (Illés Ponds) for *in situ* experiments based on the following criteria:

1. it is a drained wetland area with a few small and isolated native populations of European mudminnow (in Pócos Pond 1),
2. the groundwater level does not drop below 1.5 m even during droughts, thus facilitating the creation and maintenance of fen and marsh habitats,
3. this area is adjacent to the EU's Natura 2000 network of protected areas, and thus there should be no further risk of significant human impact and native species can colonize easily the new habitats,
4. it is close to the laboratory of Szent István University (Gödöllő) where *ex situ* experiments took place.

2.3. Creation of surrogate habitats

In total, 15 different natural habitats of European mudminnow (fens, vegetated ponds, streams and channels) in the three main regions of Hungary were selected at random for surveys, from a habitat list of National Parks and conservation literature (Fig. 1a).

During 2008-2013 we established six groundwater-fed ponds (i.e. Illés Ponds I–VIII; each with 50-60 m³ volume, 30-40 m² surface area, 1-1.5 and 2.5 m mean and maximum depths, respectively) by dredging degraded terrestrial habitats dominated by the invasive plants *Solidago* spp. (Fig. 1b).

Rather than creating one large lake we decided to design several smaller ponds with a high shoreline-to-surface ratio, which is important for the development of diverse and abundant macrophyte and macroinvertebrate assemblages. As we wished to preserve the genetic identity of each of the rescued broodstocks, Illés Ponds were constructed in such a way as to ensure the isolation from each other and from the surrounding aquatic habitats (except Illés Pond IV, which was created as a refuge extension of the over-vegetated and shallow natural Pócos Pond 2). Isolation of experimental ponds from surrounding watercourses also prevents immigration of invasive fish.

The ponds were created such that 50-70% of their surface area was in the shade of the surrounding trees and shrubs to prevent excessive warming and algal blooms, which are not tolerated by marshland fish. Shading was increased by introducing macrophytes (*Ceratophyllum demersum*, *Lemna minor*) to the Illés Ponds I, III, VI and VII. Aquatic vegetation help to settle macroinvertebrate species and inhibits the growth of algae by allelopathic effects and decreasing nitrate, nitrite and ammonium content of water.

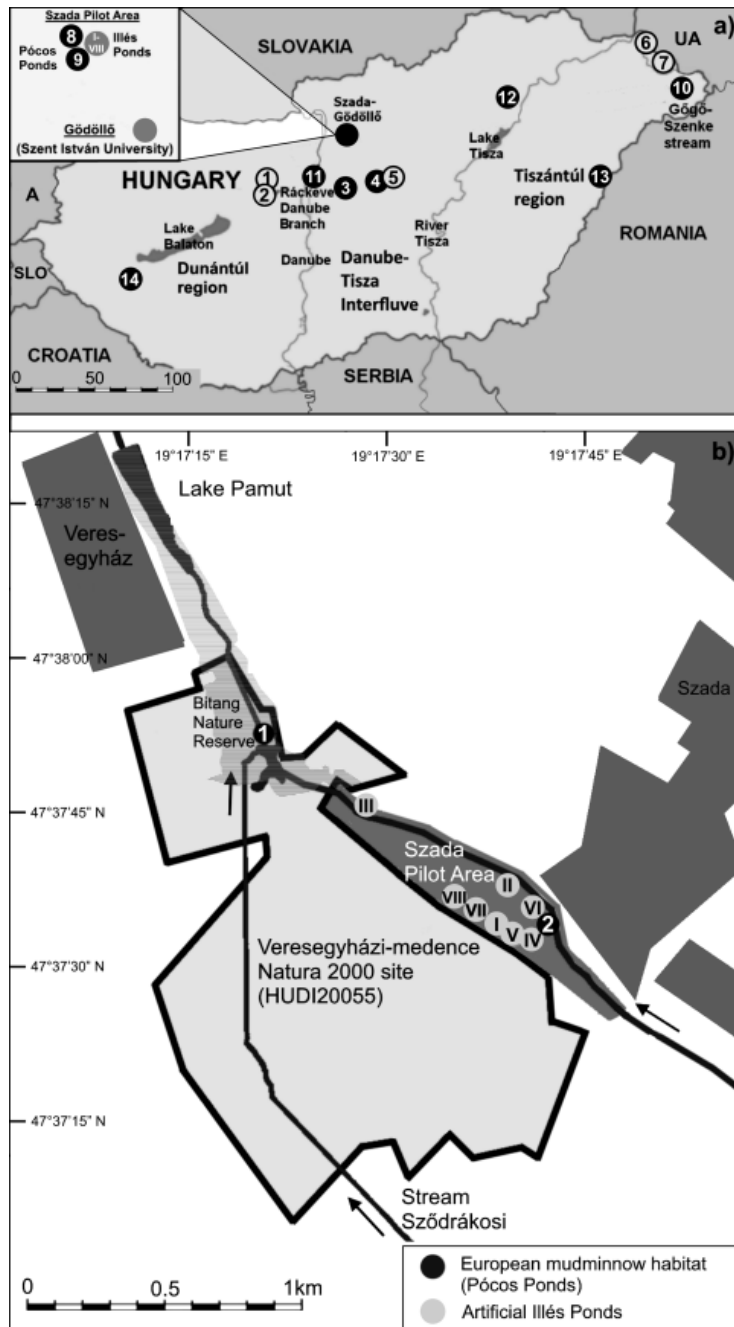


Fig. 1a. Sites of surveyed European mudminnow habitats in Hungary. White circles represent extinct populations. Habitats: 1. Császárvíz Stream, upper section, 2. Császárvíz Stream, lower section, 3. Ócsa Landscape Protection Area, 4. Felső-Tápió Stream, 5. Pond Farmos, 6. Csaronda River, 7. Lake Báb Nature Reserve, 8. Pócos Pond 1, 9. Pócos Pond 2, 10. Gógó-Szenke Stream, 11. Czuczor Island Nature Reserve and Csupics Island, 12. Hejő Stream, 13. Pocsaj Fen Nature Reserve, 14. Zala-Somogy Canal, 15. Hajta Stream.

Fig. 1b. Location of natural and artificial ponds (discovered Pócos Pond 1 and 2, Illés Ponds I-VIII respectively) in Szada Pilot Area (N47°37'35,82" E19°17'37,68").

2.4. Saving threatened stocks, captive breeding and stocking

In 2010 a total of 42 adult European mudminnow were rescued and transported to the laboratory (N=21) and Szada Pilot Area (N=21) from three threatened sites: the Pócos Pond 2, which nearly dried up in that year; the heavily polluted Gógó-Szenke Stream; and the Czuczor Island Nature Reserve in the construction area of the South M0 Bridge.

Propagation in a natural way

In 2010 in the laboratory broodstocks were held in separate aquaria to preserve their genetic identity. Aquaria (volumes: 100-700 l) were filled up with natural water originated from rescue sites. The bottom of the tanks was covered by a green plastic net to imitate spawning substrate (aquatic vegetation). Water temperature and a photoperiod were similar to that of the spawning season, in April. During 2011-2014 we continued captive breeding: a total of 50 mudminnows were captured in Illés Pond I, III, IV for propagation.

Induced propagation with hormone treatments

In 2010, 2011 and 2014 we attempt to induce propagation with carp pituitary extract (N=2) and human chorionic gonadotropin hormone (hCG; N=3).

Experiment on spawning substrate preference

In 2014 spawning substrate preference was tested to discover reproductive behaviour of mudminnow and improve the breeding method by experimenting. 3 females and 7 males were introduced into a 2 m³ plastic tank equipped with triplicates of 5 types of inorganic (artificial) spawning substrate, settled in plastic trays on the bottom: sand, gravel, artificial (plastic) plants, sand+artificial plant, gravel+artificial plant. Eggs were collected with a plankton net and transported in hatchery tanks (volume: 1.5 l).

Feeding experiments

At the beginning of the exogenous feeding, larvae were divided into two groups in plastic tanks for two different 21 day long experiments.

The aim of the first experiment was to investigate the effect of feeding frequency on the growth and mortality of the European mudminnow larvae. A total number of 240 larvae were stocked in six plastic tanks. Fish were divided into two experimental groups: Group "A" were fed with *Artemia* nauplii 4 times per day and Group "B" were fed with *Artemia* 6 times per day. Each treatment groups were stocked in three replicates.

In the second feeding experiment our aim was to determine whether *Artemia* can be replaced with dry food, which enable us to rear larvae under controlled conditions. Three tanks were randomly assigned into one of the three treatment groups so every treatment was applied in triplicate (40 fish/tank, N=360). The first group was fed 4 times a day exclusively with *Artemia* nauplii throughout the experiment (control group). The second group was fed with *Artemia* for the first 5 days. From day 6 fish were fed with *Artemia* and dry feed, and after 3-day transitional period, the amount of *Artemia* was gradually decreased. From day 9, fish were fed exclusively with dry feed (treatment A). Treatment of the third group was similar except that the transitional phase started at day 11 and the dry-feed-only period started on day 14 (treatment B).

The remnant part of mudminnow larvae (which were not participated in the experiments) were reared on a live food (*Artemia salina*, *Cladocera* sp., *Copepoda* sp., *Tubifex tubifex*, *Lumbricus terrestris*) for 3 weeks in aquaria linked to recirculation system (settling compartment, biological filter and sump). Larvae (N=203) were then reared in an artificial 9,8 m³ pond lined with foil. Starting from October there was not enough zooplankton in water, so we supplemented the diet with *Tubifex*.

Stockings

Captured fish, and their laboratory-reared offspring, originating from different populations were stocked in separate Illés Ponds (I, III, IV, VI, VII). Offspring from captive breeding and from natural spawning in Illés Ponds were used to supplement populations in native habitats where the parents originated. Laboratory-reared weatherfish (*Misgurnus fossilis*) and crucian carp (*Carassius carassius*) were also stocked before mudminnow stocking in order to test fish survival in Illés Ponds.

3. Results

3.1. Environmental characteristics of European mudminnow habitats

According to published data and the results of our surveys the European mudminnow generally occurs in shallow (0.5–1.5 m), shaded and often low-volume or stagnant small waterbodies. The physical and chemical water quality of natural habitats of the European mudminnow varies widely. For example the range of pH was 5.5-9.2, conductivity was 182-1180 µS, phosphate was 0.0-1.8 mg/l and nitrate was 0-35 mg/l. One third of habitats have a low (< 1 mg/l) dissolved oxygen concentration.

There was no difference between the naturalness of flora in mudminnow's present and former habitats. Our field surveys revealed that macrophyte coverage

in these habitats can vary widely (0–100%), with a mean value of 61% (free-floating + submerged plants: 52% + 9%).

Based on a literature review and field surveys of the natural habitats of the European mudminnow the most common macroinvertebrate taxa were Gastropoda (18%), Heteroptera (14%) and Coleoptera (12%). Number of taxa of macroinvertebrates was between 9 and 27 (mean value: 18), abundance of taxa was between 38 and 232 per sample (mean value: 107). The value of Simpson's Diversity Index was high (0.80; range: 0.73-0.87). Number of taxa was higher in habitats where mudminnows occurred (122 taxa) in contrast to waters where the species went extinct (64 taxa).

Introducing newly discovered Pócos Pond 1 and 2

In 2000 I discovered a new population of European mudminnow in Szada (Pócos Pond 1, Bitang Nature Reserve; water surface area: 150 m²; mean depth: 1.2 m; see Fig. 1b). The surrounding area is dominated by willows (*Salix* spp.), in summer the *Lemnaetum minoris* association usually reach 100% cover in the pond. I measured low dissolved oxygen concentration, the taxa number and abundance of macroinvertebrate assemblage is similar to other natural mudminnow habitats.

Pócos Pond 2 located 25 m far from Illés Pond 1 was discovered in 29 June 2009. We captured European mudminnows, weatherfish and crucian carps with electric fishing device. The water surface area of over-vegetated (cattail and reed dominated) and shallow (mean depth: 0.5 m) pond is only 105 m². Small cover of *Lemnaetum minoris* and *L. trisulcae* associations was observed. The habitat has reach vegetation and zooplankton, but the macroinvertebrate assemblage is poor. The nitrite concentration of water is high. Main threats to the pond are the marsh vegetation succession and drought.

3.2. Fish assemblages of European mudminnow habitats, current status of mudminnow populations

More than half (five of the nine investigated) of the previously known populations were not found in our surveys. We rescued mudminnows from one of these habitats (Pócos Pond 2) and further two threatened waters (Czuczor Island, Gógó-Szenke Stream).

In general, fish assemblages of still-water habitats comprised fewer species (1-4; mean: 1.5 species) than those of streams and canals (2-11; mean: 10.5 species). There was a negative relationship between the occurrence of the European mudminnow and the presence of invasive species. We found the European mudminnow in abundance only at five sites that lacked invasive species or had a low abundance of Prussian carp *Carassius gibelio*. In contrast, five former habitats of the European mudminnow where the species is no longer present are now populated by invasive fish, including the black bullhead *Ameiurus melas*, Prussian carp, pumpkinseed *Lepomis gibbosus*, Chinese sleeper *Perccottus glenii* and stone

moroko *Pseudorasbora parva*. The weatherfish was the most common native species captured over all sites (frequency of occurrence: 50%). The second and third most common species were Prussian carp (33%) and crucian carp (17%).

3.3. Establishment and monitoring of surrogate habitats

Illés Ponds I-VIII, created between 2008 and 2013 based on our experiences studying native habitats of the European mudminnow, revealed variable environmental characteristics.

Pre-stocking water quality, macrophyte and macroinvertebrate (i.e. food base) monitoring revealed that Illés Ponds I, III, IV, VI and VII met the species' conservation criteria [abiotic and biotic factors were within the range (reference values) of natural habitats] following a short primary succession period (8-23 months), and thus they were assigned for stocking with European mudminnow.

We planted the Illés Ponds I, III and VI with indigenous aquatic macrophytes (*Ceratophyllum demersum*, *Utricularia vulgaris*, *Lemna minor*) after their creation. The first two species established successfully and *Chara* sp. colonized the Illés Pond IV and VII spontaneously (these waters get more sunlight than others).

Water quality

Monitoring revealed that water quality parameters of Illés Ponds are mainly within the range of natural habitats. High dissolved inorganic nitrogen (DIN) content of ponds (≥ 40 mg/l) decreased to the range of natural waters (0-35 mg/l) in one year after their creation. I measured high values of phosphate ($\geq 0,3$ mg/l) which indicate hypertrophic conditions. Between 2008 and 2016 the concentration of phosphate increased significantly (+435%).

Illés Pond II, V and VIII had poor biological water quality, I observed regular blooms of cyanobacteria, and proliferation of iron and sulphur bacteria was also a common phenomenon. Illés Pond V dominated by the *Cladophora* sp. Therefore these ponds were excluded from mudminnow stocking.

Vegetation

The common reed *Phragmites australis* and other marsh plant species colonized the littoral of Illés Ponds spontaneously, but Illés Pond II, V and VIII has poor riparian vegetation because of significant shading. Cattail *Ceratophyllum demersum* disappeared from Illés Pond I and VII, and its coverage decreased significantly in Illés Pond V (*Cladpohora* sp. cover: 90%) in a few years after planting. We observed temporary growth of coverage of *Cladpohora* sp. (50-100%) in Illés Ponds, which decreased later to 0-3%, except Illés Pond V and VI.

Macroinvertebrate fauna

Our investigations revealed that macroinvertebrate abundance and number of macroinvertebrate taxa (38-323/sample and 12-19 taxa/sample) of Illés Ponds in Szada Pilot Area are mainly within the range of natural habitats. The values of Simpson's Diversity Index of Illés Ponds – except Illés Pond II, V and VIII, which have poor water quality – were higher than the lowest value of natural waters.

3.4. Saving threatened stocks, captive breeding

In 2010 a total of 42 adult European mudminnow were rescued from three threatened sites: the Pócos Pond 2, the Gögő-Szenke Stream, and the Czuczor Island (Fig. 2).

We attempted to propagate broodstock in a natural way and with hormone treatments in controlled conditions. Fish without any treatment bonded pairs under the green plastic net and laid eggs in the aquaria under temperature and light conditions similar to those in the spawning season. Hatching time were 8-13 days (13-14 °C), on the 23-24 days after spawning larvae started to feed exogenously. Our attempts to induce controlled propagation (with carp pituitary extract and hCG) of European mudminnow all failed.

In spawning substrate experiment all three females chose the gravel substrate with plastic plants, and two of them successfully spawned on them.

In our feeding frequency experiment growth rates of length of Group "B" (feeding 6 times/day) differed significantly from Group "A" (feeding 4 times/day), but there was no difference in mortality rates. The results of feeding protocols test show that European mudminnow larvae adapt poorly to commercial dry foods, the conversion from *Artemia* to dry feed caused significant reduction in growth rate of species.

Laboratory reared juveniles reached mean standard body length of 47 mm in aquaria by early August. In contrast, mean body length of the natural offspring of the same aged was only 27 mm in Illés Ponds. Five months old mudminnows reached a mean body length of 55 mm and sexual maturity (females visibly carried developing eggs) similarly to crucian carp and weatherfish. Based on Hungarian literature, mudminnows at the same age reached only 20-38 mm length in natural habitats.

During 2010-2014 we saved/captured 42+50 European mudminnows from natural habitats and Illés Ponds, thereafter we propagated them in a natural way. Altogether 1457 different aged juveniles were reared successfully on live food diet.



Fig. 2. Number of mudminnows saved for propagation in controlled conditions and stocked (captive-bred) individuals between 2010 and 2014. 378 individuals released in the Ráckeve Danube Branch were the natural offspring of saved adults released to Illés Pond III. 1186 captive-bred offspring (origin of fish: Gógó-Szenke Stream, Czuczor Island, Pócos Pond 2) were released in Illés Pond I, III, IV, VI and VII

* 13 parent fishes were captured from Illés Pond IV, which is connected to Pócos Pond 2. ** In total we transported 37 parent fishes from Illés Pond I, III, VI and VII

3.5. Stocking surrogate habitats with marshland fish, results of monitoring

During 2009-2013 we stocked induce propagated and captive bred weatherfish (470) and crucian carp (470) juveniles for test survival into Illés Pond I, II, III, V, VI, VII. Monitoring results revealed that these fish survived in most of the waters – except in Illés Pond II and V – consequently, we launched European mudminnow stockings in Illés Ponds I, III, IV, VI and VII. Between 2010 and 2014 we released a total of 1186 captive bred mudminnow into Szada Pilot Area.

Introduced fish survived in Illés Ponds I, III, IV, VI and VII assigned for stockings. Observed mean recapture rates were the same (14%) for European mudminnow and crucian carp, and 7% for weather-fish.

Our survey results showed natural recruitment of mudminnow and crucian carp in most of the Illés Ponds. Recruitment was most abundant in the densely vegetated Illés Pond III, which has a high shoreline to surface ratio. Between September 2010 and 2015 we caught 368 mudminnow and 57 crucian carp juveniles in the Illés Ponds. Recapture data of Illés Pond I showed regular propagation and establishment of permanent self-sustaining stock of mudminnow.

3.6. Stocking for the maintenance of natural mudminnow populations

Between 2010 and 2012 we released 528 European mudminnow juveniles into natural habitats.

After removal of pollution, we introduced 100 offspring and 50 adult mudminnow into Gógó-Szenke Stream in 2010. In June, 121 juveniles of parents

originated from Czuczor Island were released into a nearby habitat, Csuplics Island. Further, 257 ind. of natural recruitment of Illés Pond III were reintroduced to the preserved remnant of Czuczor Island in 2011.

Whereas, threatened population of Pócos Pond 2 was reinforced indirectly by ensuring its connectivity with Illés Pond IV in September 2012 (refuge extension). During 2010-2012 we stocked 404 juveniles into Illés Pond IV.

4. Theses – new scientific results

Summary of my new scientific results related to appointed aims:

1. I have determined the ecological features (abiotic, biotic and anthropogenic factors) of European mudminnow habitats for consideration in the planning of surrogate habitats and explore ecological requirements of species,
2. I discovered two new natural habitats of mudminnow in Szada (Pócos Pond 1 and 2),
3. I have created suitable surrogate habitats in Szada Pilot Area for rescued mudminnow stocks,
4. I elaborated natural propagation methodology for large number of mudminnow larvae,
5. I have examined for the first time the effects of feeding (on *Artemia* nauplii and dry feed) on the growth and mortality of the mudminnow larvae,
6. Establishing self-sustaining stocks in Szada Pilot Area for reinforce and reintroduce populations of European mudminnow in the natural habitats where parent fish had originated.

5. Conclusions and proposals

5.1. Environmental characteristics of European mudminnow habitats

Water quality

In one third of investigated habitats we recorded extremely low (<1 mg/l) dissolved oxygen concentrations, which is a common feature in shallow, muddy still water with high retention time. Nevertheless this circumstance is tolerated by the European mudminnow, which uses its swim-bladder as an auxiliary breathing

organ. This ability confers a competitive advantage over other fish species in marsh and fen environments.

Vegetation

Typhaetum latifoliae was the most common marsh plant association in investigated Hungarian habitats. This finding contradicts information found in the literature, which mentions *Phragmites communis* dominance. We observed common duckweed *Lemna minor* in all the sites, but there is no data in literature about its occurrence. Based on scientific papers frogbit *Hydrocharis morsus-ranae*, European white water lily *Nymphaea alba* and greater bladderwort *Utricularia vulgaris* are common aquatic species of Hungarian waters.

Macroinvertebrate fauna

The most abundant taxa were chironomids *Chironomidae* sp. (pollution tolerant family), *Gammarus roeseli* and waterlouse *Asellus aquaticus* (moderate pollution tolerant species) in mudminnow's habitats. The most common taxa were *Asellus aquaticus*, *Chironomidae* sp. and *Cloeon dipterum*.

5.2. Fish assemblages of European mudminnow habitats, current status of mudminnow populations

Less than half of the previously known populations were found in our surveys, indicating the species may be at considerable risk of extinction even today. Other studies have reached similar conclusions and identified two main sources of threats.

Habitat loss is illustrated by the case of protected Czuczor Island, where construction work is resulting in the loss of fen habitat. The stream bed of upper section of Császárvíz Stream was filled up partially in 2011 and the lower section is lined with concrete.

The second evident threat is the spread of invasive fish species. Invasive species were abundant in all former habitats of the European mudminnow where we failed to capture any individuals. Conversely, where the European mudminnow was present invasive species were generally absent or occurred only in small numbers. The most voracious invader in these habitats is the Chinese sleeper. The European mudminnow may coexist with most invasive fish species in diverse habitats (e.g. in densely vegetated Felső-Tápió Stream) but in the long term it does not survive the settlement of the Chinese sleeper. The proliferation of invasive species in wetland habitats could be an indicator of environmental degradation (e.g. regulated water level, increased nutrient load, extinction of macrophytes), and thus their effect on the European mudminnow could be considered to be indirect. Specialist fen fish species can not compete with invasive species under altered habitat conditions, therefore, it is important to conserve and restore wetland

habitats in their original form. Both natural fen habitats in the Szada Pilot Area (i.e. Pócos Ponds 1 and 2) contained European mudminnow populations and were free of invasive species and human impacts, which supports the relevance of this area for a species conservation experiment.

Still water habitats of the European mudminnow generally contain less rich fish assemblages than watercourses and canals presumably because of isolation and low oxygen level. Fish assemblages of European mudminnow habitats are poor, among the most common associate species are the weatherfish and the crucian carp. All the three species are adapted to survive periods of extremely low oxygen concentrations.

5.3. Establishment and monitoring of surrogate habitats

Although Illés Ponds are just slightly different in morphology and located close together, they vary significantly in development (e.g. water quality, macrophyte flora and macroinvertebrate fauna) because of effects of different ecological factors.

The creation of several small, isolated habitat patches (i.e. ponds) instead of a more extended and connected fen system has several advantages, especially in monitoring and controlling ongoing biological and environmental processes, preventing the spread of disease, and keeping invasive species out of the system. Further benefits are small cost of monitoring, planning and easier testing of output of conservation activities.

Water quality

The main sources of groundwater pollution (increased DIN and phosphate loading) in Szada Pilot Area (and throughout Hungary) are the lack of sewage cleaning in the last few decades, using chemical fertilizers and atmospheric sedimentation of traffic air pollutants.

Primary succession (intercepting of nutrients of settled algae, bacteria and macrophytes) generally decreased DIN level in Illés Ponds. The main source of phosphorus is groundwater in the newly created ponds, but later external loading (e.g. decomposition of fallen leaves) can increase phosphate level. In the first year of ponds mean concentration of phosphate was 0.3 mg/l. This level increased several times over time, which is in line with published observations that lakes serve as a trap for phosphorus. Hypertrophic conditions of Illés Ponds frequently caused high coverage (>90%) of aquatic vegetation and *Cladophora* sp. or blooms of cyanobacteria. I observed the highest increasing phosphate level in Illés Pond II and VIII (+1100 and 900%), where proliferation of sulphur and iron bacteria is a common phenomenon too. This is supported by evidence that low oxygen concentration helps to release phosphorus from sediment into water (internal loading).

Until May 2016 DIN concentration of Illés Ponds – except Illés Pond IV and V – decreased under 1 mg/l. It has to be noted that *Utricularia vulgaris* (Illés Pond I) and *Ceratophyllum demersum* (Illés Pond VI) are floating macrophytes, therefore they do not facilitate releasing nutrients from sediment, which helps to keep nutrition level low. Furthermore the latter species is able to uptake a large amount of nitrate and ammonium with their leaves. However, I observed decreasing DIN level both in macrophyte and algae dominated ponds. The continuation of decline may become a limiting factor for algae and aquatic vegetation too.

Alternative stable states in ponds of Szada Pilot Area

Different groups of primary producers may dominate shallow lakes within a wide range of nutrients due to multiple abiotic and biotic effects, and such states dominated by a particular group may often represent alternative stable states. Several positive and negative feedbacks play a role in shifting to alternative stable states and stability of freshwater ecosystems. During 2009-2015 we observed the following 5 alternative stable states in the ponds of Szada Pilot Area:

*1. Clear state with dominance of submerged macrophyte *Ceratophyllum demersum* or *Utricularia vulgaris* and poor phytoplankton: Illés Pond I, III, VI*

Limiting growth of algae by intercepting DIN, shading and allelopathy is a considerable effect of *Ceratophyllum demersum*. Small, fish-poor ponds usually have small number of benthivorous species, which support clear state. This is because feeding of benthivorous fish (e.g. bream *Abramis brama*) can contribute to phosphorus enrichment/planktonic eutrophication and thus to a shift to turbid state. We have not observed declining macrophytes and algal blooms in those Illés Ponds which have aquatic vegetation, despite high phosphorus concentration and stocking partially benthivorous crucian carp and weatherfish. Rich vegetation can stabilize clear state under increasing nutrient level, which is favourable for fish. This is confirmed by our results, which showed that nutrient range of clear and turbid states significantly overlapped in the ponds of Szada Pilot Area, nonetheless clear state could remain for a long time.

*2. Clear state with *Chara* sp. and poor phytoplankton: Illés Pond IV, VII*

Illés Pond IV and VII get more sunlight than other ponds, therefore *Chara* sp. colonized spontaneously the bottom of these waters. *Chara* sp. is able to decrease the bicarbonate level of the water, thus their competitive ability is stronger than most macrophytes. Consequently carbon-limited environment suppresses algae and other aquatic plants.

3. Turbid state with cyanobacterial dominance (dense phytoplankton): Illés Pond II, VIII

Strong light limitation of phytoplankton and shading of the riparian trees and shrubs are the key mechanisms to inhibit the settlement of submerged macrophytes. This results in a turbid state in shallow lakes. Intensive nutrient uptake of cyanobacteria resulted in lowest mean DIN concentration (0,8 mg/l) among Illés Ponds. Decomposition of dead phytoplankton causes oxygen depletion in the upper sediment layer of ponds, which is a common phenomenon in Illés Pond II and VIII.

4. Dominance of *Cladophora* sp. and low density of phytoplankton: Illés Pond V

There are no records of a *Cladophora* sp. dominated alternative stable state in the literature, but my monitoring data show that it could be a new type of stable state in small ponds. Illés Pond V is the shallowest water in Szada Pilot Area, where *Cladophora* sp. permanently occurs. *Cladophora* sp. often reach an 80-100% coverage, which inhibits colonization of phytoplankton and macrophyte species by shading. High cover of floating filamentous algae mat causes extreme oxygen dynamics [i.e. changes from very high (daytime) levels to oxygen deficiencies (at night)].

5. Dominance of floating plants (*Lemnetum trisulcae* association) and low density of phytoplankton: Illés Pond II

This alternative stable state can remain for a long time in shallow waters, but we observed the *Lemnetum trisulcae* association only in one year (2013), when DIN level decreased and phosphate concentration extremely increased. In the previous year the pond was in a turbid state (cyano-, iron- and sulphur bacteria dominance). In 2014 DIN level increased fivefold, phosphate content fell, consequently pond shift to turbid state again.

Macroinvertebrate fauna

Ponds dominated by aquatic vegetation had richer macroinvertebrate assemblages than waters dominated by *Cladophora* sp. and cyanobacteria. Highest differences were revealed in Heteroptera, Diptera and Ephemeroptera taxa.

The primary cause of high or low macroinvertebrate abundance and taxa number outside the range of natural habitats was extremely bad water quality of Illés Pond II and V (e.g. water blooms, periodic releasing of toxic ammonia and hydrogen sulfide, oxygen depletion).

Survival of fish in surrogate habitats

Based on results of monitoring, 5 of the 8 ponds of Szada Pilot Area proved to be suitable for marshland fish, because they survived, and European mudminnow and crucian carp reproduced successfully. Nevertheless weatherfish could not reproduce. It is likely that the main reason for it is the low water temperature of the groundwater (annual fluctuation between 2.9 - 16.3 °C) which inhibits to reach spawning temperature (18-21 °C) of species permanently in ideal depth of ponds.

Our results highlight that planted (*Ceratophyllum demersum*, *Utricularia vulgaris*) or spontaneously settled (*Chara* sp.) macrophytes have important role in the development of newly created ponds by improving water quality and helping to survive marshland fish. On the one hand, they inhibit planktonic eutrophication by shading, allelopathy and intercepting nutrients, on the other hand, aquatic vegetation is a hiding and reproducing place for fish and macroinvertebrates. For example, recruitment was most abundant in the densely vegetated Illés Pond III in 2011. Although we released more adults into Illés Pond I than into Illés Pond III, reproduction success was weaker in the former pond.

Captive bred offspring was abundant enough for stocking waters in Szada Pilot Area. The pilot programme is successful, because saved mudminnows survived in medium term (2010-2016), thus we can continue releases in the future.

5.4. Saving threatened stocks

We believe that the majority of the threatened populations of Gőgő-Szenke Stream, Czuczor Island and Pócos Pond 2 might have gone extinct without our rapid action/rescue. These cases occurred within one year, which shows clearly the vulnerability of mudminnow populations and highlights the necessity of synthesising conservation actions.

Isolated populations have unique genetic pools representing Evolutionary Significant, Conservation and Management Units - these aspects were taken into consideration during saving, captive breeding and releasing.

5.5. Captive breeding of threatened stocks

Ex situ rearing (e.g. for propagation) of the European mudminnow is easy despite their need for live food.

Our attempts to induce controlled propagation of European mudminnow failed. Hormone treatment would probably have been successful if we had more parent fish and tried various doses.

The mudminnows' preference for gravel and artificial plant spawning substrates was surprising given that there are no gravel bottom in their natural habitats. We propose that a supernormal stimulus causes this behaviour. The speed of embryonic and larval development of mudminnow was similar to data reported in the literature.

We supposed that the European mudminnow is a typical diurnal predator so they do not forage during the night. Nevertheless, our results showed that the mean growth rate of Group “B” (fed with *Artemia* 6 times per day) was significantly higher than that of Group “A” (*Artemia* 4 ×) right from the first week to the end of the experimental period. Consequently the mean final length was greater in Group “B”. The mean body weight of Group “A” was also smaller than that of Group “B”. However, this difference was not significant at the end of the experiment. The likely reason is that it was impossible to measure the individual weights of fish, so only feeding groups were compared, resulting in low power.

The mean growth rate of individuals bred in the laboratory was higher than that of mudminnows of natural waters. This facilitated the success of stocking, because fish stocked in Autumn were able to reproduce in the new habitats the next year.

5.6. Reinforcement of natural European mudminnow populations

The ideal outcome for a conservation programme is that threatened populations recover in their original restored habitat or in an equivalent natural or artificial surrogate habitat within the range of the conservation unit. We deemed our efforts to conserve populations of European mudminnow as successful when we reintroduced all three threatened populations to their original habitats while preserving their genetic identity.

During the implementation of the European mudminnow Conservation Programme, pollution of Gógő-Szenke Stream was eliminated and most of the fen on Czuczor Island was retained. We introduced laboratory reared and natural offspring from Illés Ponds to both sites. The threatened population of Pócos Pond 2 was reinforced indirectly by ensuring its connectivity with Illés Pond IV, which is a suitable refuge for marsh fish assemblages in dry periods.

6. General conclusions and suggestions

The range of the European mudminnow has declined significantly over the last 150 years. Nowadays the main threats to the species are habitat loss, disturbance and the spread of invasive fish species.

The European mudminnow Conservation Pilot Programme (2008-) is the first comprehensive species conservation programme that includes rescuing individuals from threatened populations, captive breeding and rearing, creation of new habitats, introduction of saved and captive-bred stocks to surrogate habitats, and reinforcement of threatened parent populations.

Our new ex situ methods for propagation and larvae rearing are effective tools for population reinforcement and reintroduction of mudminnow in suitable sites. The mean growth rate of individuals bred in the laboratory was significantly higher

than that of mudminnows of natural waters. Therefore they are able to reproduce the next year.

We created new surrogate habitats containing self-sustaining stocks with unique genetic pools, which on the one hand enable further propagations and research, and on the other hand help to reinforce natural populations.

Marshland fish were settled in 5 Illés Ponds initially determined to be suitable environments for fish conservation. We observed three generations of mudminnow (saved parent fish, captive-bred and released individuals, and offspring born in the new habitat) together in the Illés Ponds several times. Our results shows that permanent self-sustaining populations established in ponds.

Laboratory-bred offspring and fry from surrogate habitats were sufficient to reinforce threatened populations in natural habitats. The number of mudminnows (captive bred: N=271, offspring of Illés Pond III: N=257) introduced to the parent's original habitat was higher than that of the number of saved individuals (N=42). In addition, we released a further 1186 captive bred fish into waters of the Szada Pilot Area. The conservation value (CV) of the released mudminnow (N=1714) was EUR 1,375,072 (CV: 802.26 EUR/individual), but the total cost of European mudminnow Conservation Pilot Programme (2008-2016) was below EUR 25,672.

To facilitate conservation actions, further research and management actions are needed, such as:

- improving our knowledge of the environmental conditions in which the European mudminnow can survive (e.g. ammonia, hydrogen sulfide and nitrite tolerance),
- studying competition and predation between the Chinese sleeper and European mudminnow,
- identifying the most threatened populations, elaborate conservation and restoration protocols for threatened and degraded habitats,
- creation of new surrogate habitats for saving more broodstocks,
- improving initial evaluation method of surrogate habitats to increase safety of stocking,
- developing induced propagation and cage spawning methods (for wild spawning),
- developing methodology for sperm cryopreservation,
- continuing experiments on spawning substrate preference and feeding,
- reinforcement of natural populations with captive-bred juveniles and natural offspring of Szada Pilot Area for conserving genetic diversity and preventing inbreeding,
- involving further marshland fish (e.g. sunbleak *Leucaspius delineatus*) in the conservation programme,
- synthesizing domestic and foreign projects related to European mudminnows, and developing a comprehensive programme, taking into consideration the genetic guide on relevant conservation and management units.

Recommendations for habitat reconstructions

I recommend creation of shallow and wide littoral zone and high shoreline-to-surface ratio for new surrogate habitats, for the development of diverse and abundant marsh/fen macrophyte and macroinvertebrate assemblages. In addition, nutrition uptake of littoral vegetation decreases external loading.

In the future, it would be necessary to create further experimental systems with multiple similar-sized surrogate habitats to enable testing the effect of different habitat structures, water chemistry and ecological interactions on the European mudminnow and other threatened marshland fishes.

Stocking strategy of Szada Pilot Area

We suggest to capturing parent fish from Szada Pilot Area and threatened natural habitats within conservation and management units (a circle of 80 km radius) to increase genetic diversity (i.e. prevent inbreeding and genetic rift) of three broodstocks (originated from Gőgő-Szenke Stream, Czuczor Island and Pócos Pond 2) of Szada Pilot Area. Following propagation, juveniles must be stocked in Illés Ponds and in natural waters within the conservation unit for the purpose of strengthening populations and prevent inbreeding in both sites.

Stocking strategy for large natural water bodies

For sites that are apparently suitable for mudminnow, but where the species is absent, the first suggested step is to clarify the causes of disappearance by researching literature data and the habitat characteristics of the sites. The next step is to compare obtained data with reference values (range) of natural habitats. Habitats with phosphate and ammonia concentration outside the reference values for cyanobacterial, sulphur bacterial and *Cladophora* sp. blooms, lack of aquatic vegetation, low diversity of macroinvertebrate assemblage, high taxon number or abundance of invasive fish species are not suitable for stocking. We also suggest not releasing mudminnows in sites where Chinese sleepers occur. In order to increase the survival rate of the stocked fish, it is not advisable to stock water bodies > 0.5 ha because it is not possible to produce sufficient (large) number of juveniles. Before stocking mudminnows, I recommend fencing 100-200 m³ of the water body with a dense fish grid. Then survival experiment may start with stocking weatherfish and crucian carp. It is also possible to release the parent fish into a relatively small, largely closed corner of the large water body or artificially created, closely related habitat, where they can reproduce. If the above mentioned associate species survive for a year, the next step is stocking mudminnows. If the mudminnows survive, the grid can be removed after one year, and further stockings conducted. During the survival experiment, it will be useful to monitor the fish fauna and other environmental characteristics over several seasons, and after the spawning season, I recommend investigating the fenced water body to assess whether the marshland fishes have reproduced.

7. Publications related to the topic of the dissertation

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