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Development of eDNA methods for monitoring two stygobiotic species of the Dinaric Karst, *Proteus anguinus* and *Congeria jalzici*, using digital PCR

Razvoj metod eDNA za monitoring dveh stigobiontov Dinarskega krasa, človeške ribice (*Proteus anguinus*) in Jalžićeve jamske školjke (*Congeria jalzici*), z uporabo digitalne PCR

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The welfare of species and ecosystems are assessed by biological field surveys. Without knowing about the existence of particular species, we cannot protect them. Environmental DNA (eDNA) methods detect species at very low densities. When carefully validated and appropriately interpreted (see e.g. Cristescu & Herbert 2018), eDNA-based inventories improve biological surveys of ecosystems and are used to guide conservation efforts (e.g. Zaiko et al. 2018). Apart from establishing the presence of endangered or rare species, eDNA methods are also applied for monitoring the spread of alien invasive species and infectious agents such as fungal or bacterial spores, viruses and parasites. Analyses of total eDNA in a sample (i.e. environmental metagenome) are utilized to obtain information on the species community structure, the relative abundance of different trophic levels, and their interactions. However, a number of properties of eDNA molecules, such as differences in their release rates by different species during different life-history stages, their persistence under different environmental conditions and their variable detection rates using different techniques, are still insufficiently understood. Consequently, while eDNA methods are becoming wildly popular for establishing the presence of species in diverse environments, few studies to date have attempted to use eDNA quantification for determining population sizes and trends in these species (e.g. Doi et al. 2015a, 2017, Buxton et al. 2017, Chambert et al. 2018 and references therein).

Recent records of the olm *Proteus anguinus* outside its historically known range, discovered through detection of its DNA dissolved in groundwater, introduced the eDNA methodology to the study and conservation in the cryptic subterranean environment (Gorički et al. 2017). Since then, we have been able to detect the presence of *P. anguinus* in karst groundwater by analyzing samples of water collected on the surface – at springs or in wells. An upgraded technology, droplet digital PCR (ddPCR), which is reportedly more sensitive, accurate and resistant

to PCR inhibitors than classical qPCR (Doi et al. 2015b, Taylor et al. 2017, Baker et al. 2018), is being tested for direct quantification of eDNA molecules in groundwater. We are using the large, accessible and relatively well-characterized natural *P. anguinus* population inhabiting the Planinska jama Cave (southwestern Slovenia) as a model. The size of this population was recently estimated using a genetic mark-recapture method, although only the number of animals actually caught was reported (Zakšek & Trontelj 2017). In succession of the named survey, we sampled water at several sites along the subterranean Pivka River and recorded representative stream profiles and flow velocities at or near the sampling sites (Fig. 1). Thereupon we estimated the number of eDNA molecules specific to *P. anguinus* in three 100 m long sections of the stream (B, F and I of Zakšek & Trontelj 2017). Based on the data reported by Zakšek & Trontelj (2017), we also estimated the number of eDNA molecules per individual animal in the selected stream sections.

Until now we have tested several sampling and filtration strategies and found that their success greatly depends on the amount of suspended particles in the water, which in turn reflects wet weather conditions during a yet undetermined period of time before sampling. On the other hand, during more stable, i.e. better hydrological conditions for the eDNA method, we were able to obtain comparable molecule counts in two consecutive years in all three river sections that were sampled twice. The relative number of eDNA copies per individual animal was the lowest in section F, where the highest number of animals had been observed, but where the slowest water flow was measured. This may indicate a higher rate of eDNA sedimentation (sinking), although we failed to detect more eDNA copies closer to the stream bottom than on the water surface. Alternatively, our result might reflect differences in eDNA release rates between animals from different sections. This explanation is even more plausible when we compare the other two sections with greater hydrologic similarity, yet we consistently detected more eDNA copies in section B than in I – both in absolute and relative terms – which apparently cannot be explained by transfer from the upstream sections alone. Our results indicate the need to investigate and characterize the Planinska jama Cave and its resident *P. anguinus* population in greater detail, as well as to measure the many variables in the eDNA model in a more controlled, experimental setting.

Figure 1. Stream profiling of the Pivka channel in the Planina Cave (photo: C. Mayaud).
Slika 1. Meritev pretoka na Pivškem rokavu Planinske jame (foto: C. Mayaud).

In another line of eDNA research, the utility of ddPCR is being explored for improved detection of the much smaller and rare stygobiont, the cave clam *Congeria jalzici*. The clam is known from only one site in Slovenia and three sites in Croatia (Hudoklin & Ilenič 2012, Bilandžija et al. 2013). The spring of the Krupa River in southeastern Slovenia (Fig. 2) is another presumed site of *C. jalzici*, although only shells, but no living animals were found in it (Sket 1971 in Hudoklin & Ilenič 2012). This spring is the site of one of the greatest ecological disasters in the Dinaric Karst. Between 1962 and 1984, dozens of tons of pure polychlorinated biphenyls (PCBs) were dumped at waste disposal sites and in nearby dolines by a local condenser factory. The undegradable carcinogenic chemicals are still gradually released from the sediment into the groundwater and were detected in the tissues of *P. anguinus* residing in the aquifer (Pezdirc et al. 2011). We detected eDNA of *C. jalzici* in the Krupa spring water and thereby confirmed the presence of a living population there. In the future, we will survey additional sites in the area potentially inhabited by the mollusk to assess the presence and vulnerability of any newly discovered populations, and to monitor the spread of the alien invasive zebra mussel *Dreissena polymorpha*, which may soon become a new major threat to the Slovenian populations of *C. jalzici*.

In conclusion, eDNA holds great potential for monitoring and conservation of fauna in inaccessible subterranean habitats. In the future, the eDNA methodology might be applied in the estimation of *P. anguinus* population sizes without having to see, mark or otherwise disturb the animals themselves. In parallel to eDNA assay development for various stygobiotic species of the Dinaric Karst, a groundwater-sample library is being created. This collection of samples will be available for future analysis of their species composition, once a reference sequence database for known Dinaric Karst species is created. It will serve as a source of information on any changes in species distribution over time, or for some species, as a record of their existence before they are lost forever.

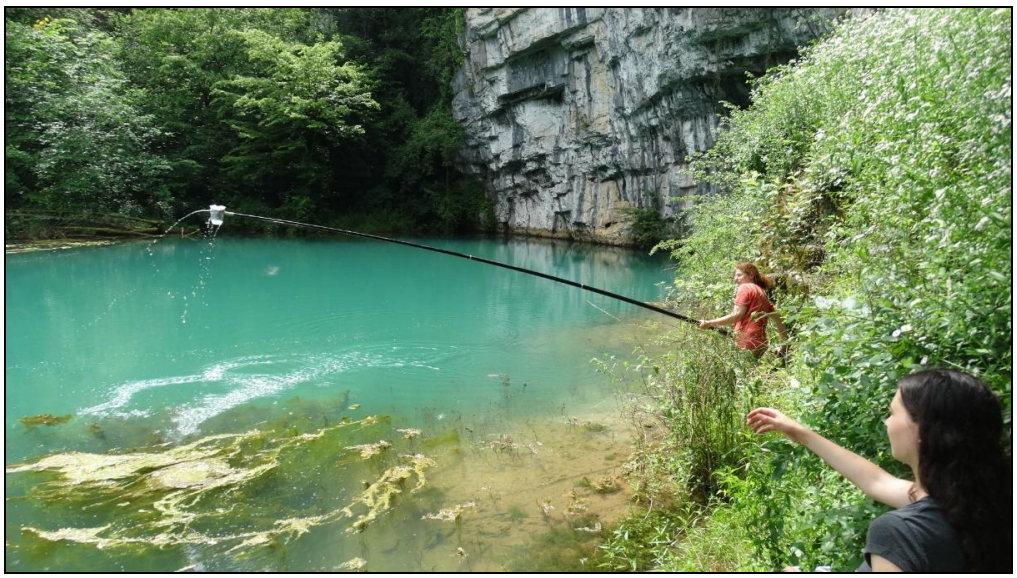

Figure 2. Water sampling at the Krupa spring (photo: P. Presetnik).
Slika 2. Vzorčenje vode na izviru Krupe (foto: P. Presetnik).

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