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Original article

Evaluation of Benthic Macroinvertebrate Diversity in a Stream of Abandoned Mine Land Based on Environmental DNA (eDNA) Approach

Mi-Jung Bae (0000-0003-4286-1119), Seong-Nam Ham (0000-0002-2256-0880), Young-Kyung Lee (0000-0002-6926-7323) and Eui-Jin Kim* (0000-0002-6528-9699)

Nakdonggang National Institute of Biological Resources, Sangju 37242, Republic of Korea

Abstract Recently, environmental DNA (eDNA)-based metabarcoding approaches have been proposed to evaluate the status of freshwater ecosystems owing to various advantages, including fast and easy sampling and minimal habitat disruption from sampling. Therefore, as a case study, we applied eDNA metabarcoding techniques to evaluate the effects of an abandoned mine land located near a headwater stream of Nakdonggang River, South Korea, by examining benthic macroinvertebrate diversity and compared the results with those obtained using the traditional Surber-net sampling method. The number of genera was higher in Surber-net sampling (29) than in the eDNA analysis (20). The genus richness tended to decrease from headwater to downstream in eDNA analysis, whereas richness tended to decrease at sites with acid-sulfated sediment areas using Surber-net sampling. Through cluster analysis and non-metric multidimensional scaling, the sampling sites were differentiated into two parts: acid-sulfated and other sites using Surber-net sampling, whereas they were grouped into the two lowest downstream and other sites using eDNA sampling. To evaluate freshwater ecosystems using eDNA analysis in practical applications, it is necessary to constantly upgrade the methodologies and compare the data with field survey methods.

Key words: environmental DNA, field sampling, functional feeding group, non-metric multidimensional scaling

INTRODUCTION

Headwater streams are important sources of biota, organic matter, and water downstream (Clarke *et al.*, 2009). However, many headwater streams are affected by anthropogenic disturbances, such as urbanization, agriculture, and mining. These disturbances near headwater streams can severely influence freshwater biodiversity and water quality downstream. Therefore, it is essential to interpret the impacts of various anthropogenic disturbances on headwater streams. However, the assessment of headwater streams (i.e., creeks, tributaries, and small rivers) for biodiversity conservation has been relatively neglected in favor of that of main streams and/or rivers. Because headwater streams occupy more than three-quarters of the stream channel in watersheds (Benda *et al.*, 2005; Clarke *et al.*, 2009), it is practically difficult to monitor all these streams. Thus, it is necessary to establish a more efficient and time-saving procedure to investigate river health through biodiversity monitoring.

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* Corresponding author: Tel: +82-54-530-0860, Fax: +82-54-530-0869
E-mail: ejkim@nnibr.re.kr

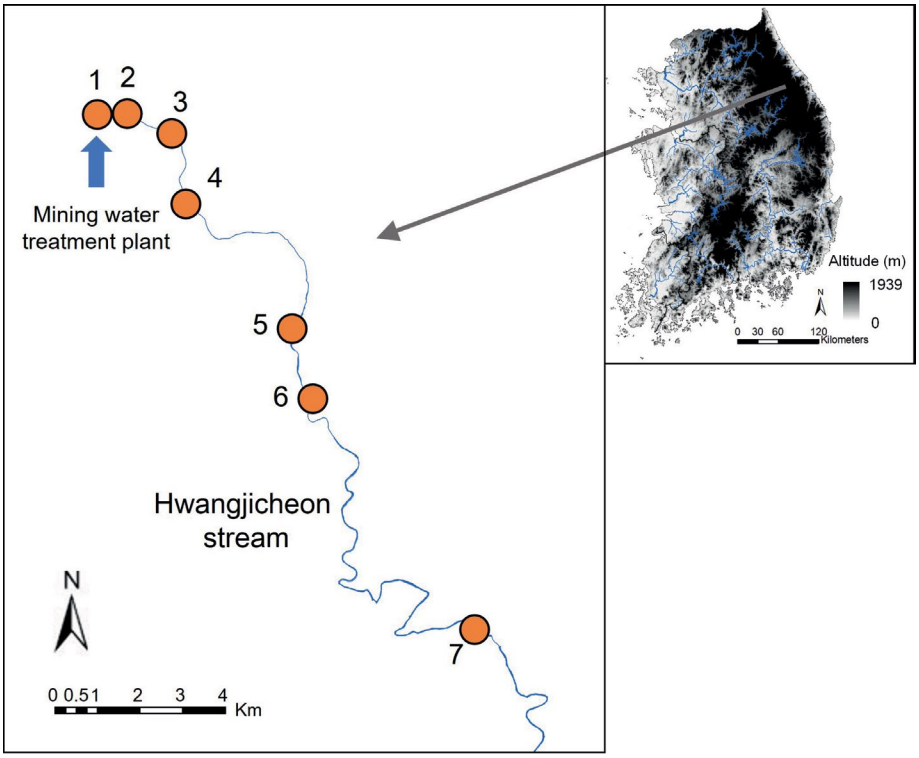

Fig. 1. Sampling sites in Hwangjicheon stream.

Among the various anthropogenic disturbances, mining activity (past or present) is presumed to have severe and long-lasting effects on streams and rivers (Marqués *et al.*, 2001). The biodiversity of streams near mining areas is easily affected by changes in physical and chemical factors (e.g., organic matter breakdown, increased conductivity, erosion or deposition, and sediment contamination) (Bae *et al.*, submitted). In particular, mining activity often causes heavy metal contamination near streams, and the heavy metal concentration in the water generally exceeds the recommended limits for drinking or agricultural use, resulting in the disruption of freshwater biodiversity (Loayza-Muro *et al.*, 2010). Moreover, in areas with abandoned mines, heavy metals that have accumulated in stream sediments from past mining activities cannot be completely eliminated, causing long-lasting damage to freshwater ecosystems, even though they usually do not exceed the permissible limits for water quality. Thus, benthic macroinvertebrate communities in streams near mines or abandoned mining areas are frequently disturbed or even destroyed, leading to a severe disruption of the functional connectivity of ecological networks and a decrease in species diversity (Romero *et al.*, 2008).

The diversity of benthic macroinvertebrate communities is an essential biotic indicator in freshwater ecosystems because they can perceptively reflect environmental alterations (e.g., land-use changes, water quality, and food web) in their habitat (Tzafesta *et al.*, 2021). In recent years, with the advancement of next-generation sequencing technologies, environmental DNA (eDNA)-based metabarcoding approaches for benthic macroinvertebrate communities have gained increasing attention as a convenient assessment technique for evaluating the status of freshwater ecosystems (Mächler *et al.*, 2016). This technique possesses several advantages such as fast and easy sampling, applicability to various pending ecological issues (e.g., evaluating biodiversity and detecting endangered species or invasive species), and minimal habitat disruption from sampling (Díaz-Ferguson *et al.*, 2014; Thomsen *et al.*, 2015; Harrison *et al.*, 2019; Coble *et al.*, 2019). It is expected that eDNA-based assessment techniques can be improved through continuous comparison with field sampling.

In this study, we employed eDNA metabarcoding techniques to evaluate the effects of an abandoned mine land located near a headwater stream of the Nakdonggang Riv-

er, South Korea, by examining benthic macroinvertebrate diversity. Subsequently, we compared the results of the eDNA-based assessment with those of the traditional Surber-net sampling method.

MATERIALS AND METHODS

1. Ecological data

We collected benthic macroinvertebrates using a Surber net (30 cm × 30 cm, 250 μm mesh) (i.e., the original field survey method) and water samples for eDNA analysis at seven sampling sites in the Hwangjicheon stream in 2019 (Fig. 1). The Hwangjicheon stream is located in Taebaek-si, where South Korea's representative coal mines exist. In 2010, heavy metal concentrations (Cd, Pb, Fe, and Mn) in mining water and groundwater near the Hwangjicheon stream were reported to exceed the water quality limits (MIRECO, 2018). Substrates at sites 1 to 3 are acid-sulfated, and the mining water treatment plant is located next to site 1.

Using a Surber net, each site was sampled in triplicate in the riffle area within a 50-m range (Bae *et al.*, 2016). Collected samples were preserved in 99% ethanol in the field, which was replaced with 70% ethanol in the laboratory. We then sorted and identified macroinvertebrates at the genus level based on Quigley (1977), Pennak (1978), Brighnam *et al.* (1982), Yun (1988), and Merritt and Cummins (2006) to compare the results with those of eDNA analysis.

Using a sterile bottle at each site, water samples were collected from the stream bottom without disturbing the sediment. Water samples (2 L in each replicate) were collected in triplicate at the same sites as Surber net sampling. All water sampling was conducted immediately before Surber net sampling. The 6-L samples were vacuum filtered using a Supor® 200 Membrane Filter (0.2-μm pore size; Pall Corporation, Ann Arbor, MI, USA). Filters were placed into 50-mL tubes using sterile forceps and stored at −20°C until DNA extraction. DNA was extracted from the filters using a PowerWater® DNA Isolation Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Subsequently, PCR amplification was conducted for the eukaryotic V4 region of the small subunit ribosomal DNA (18S rRNA gene) using the universal primers Uni18SF and Uni18SR (Zhan *et al.*, 2013). Next-generation sequencing and bioinformatics analyses were performed as described by Fernández *et al.* (2018).

We also measured 24 environmental factors, including

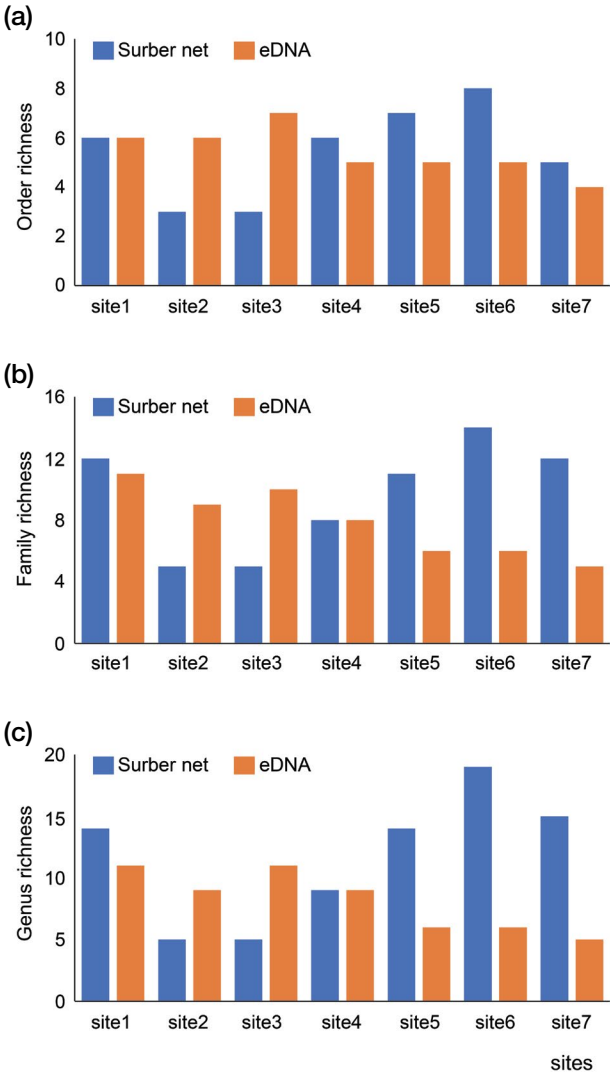

Fig. 2. Comparisons of order (a), family (b) and genus richness (c) between a Surber net sampling and eDNA analysis.

geographical factors (i.e., latitude, stream order, distance from source) and land use (%), which were extracted from a digital map using ArcGIS 10.6 (ESRI, Redlands, CA, USA). Whereas the substrate composition, dissolved oxygen, pH, and conductivity were measured in the field, biological oxygen demand (BOD), total nitrogen (TN), ammonia (NH_4^+), nitrate (NO_3^-), total phosphorus (TP), orthophosphate (PO_4^{3-}), and chlorophyll-a (Chl-a) were measured in the laboratory according to APHA (2005); water samples (4 L at each site) were obtained from the field using a sterile bottle.

2. Data analysis

First, as descriptive measures, genus richness and func-

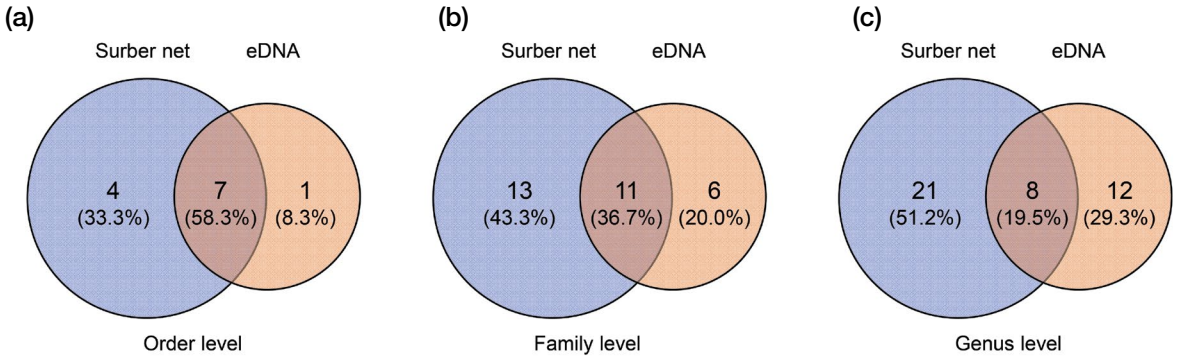

Fig. 3. Venn diagram analysis in order (a), family (b) and genus richness (c) using a Surber net sampling and eDNA analysis.

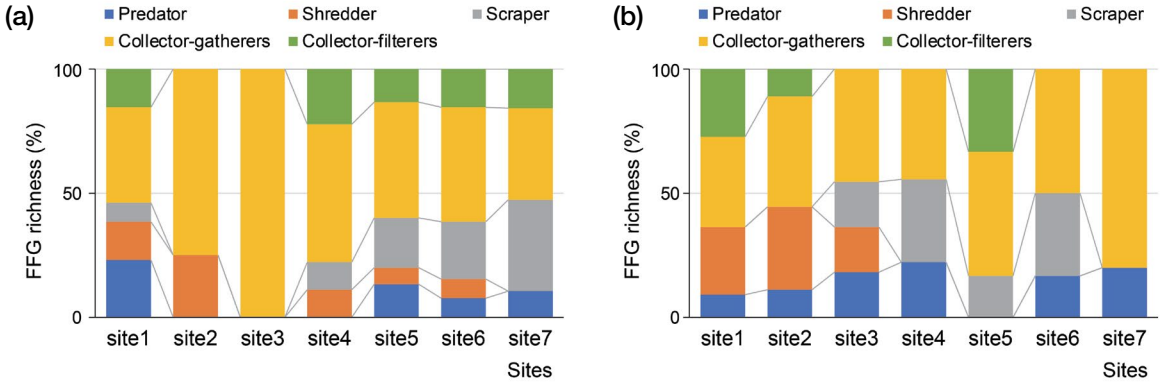

Fig. 4. The relative ratio (%) of functional feeding groups (FFG) based on a Surber net (a) and eDNA (b).

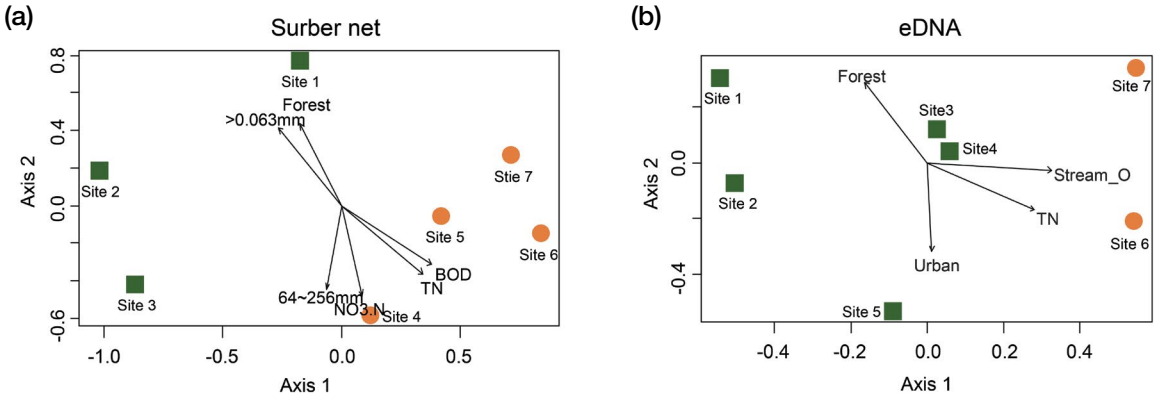

Fig. 5. Non-metric multidimensional scaling ordination based on benthic macroinvertebrate community with a Surber net sampling (a) and eDNA analysis (b). Environmental factors with $p < 0.05$ are represented in the figure. Different colors and symbols indicate the result of cluster analysis: green square, cluster 1 and orange circle, cluster 2.

tional feeding groups (FFGs) were compared between Surber net sampling and eDNA analysis. In addition, Venn diagrams were constructed to determine overlapping genera, families, and orders between the Surber net sampling and eDNA analysis. Second, cluster analysis (CA) and non-metric multidimensional scaling (NMDS) were conducted to outline benthic macroinvertebrate community compositions as well as to check whether each evaluation method accurately re-

flected the situation at the abandoned mining areas. After CA, multi-response permutation procedures (MRPP) were conducted to determine significant differences between clusters defined by CA. Venn diagrams, CA, and NMDS were analyzed using the 'vegan' package (Oksanen *et al.,* 2011) in R (R Core Team, 2016).

RESULTS AND DISCUSSION

In total, 29 genera (24 families and 11 orders) and 20 genera (16 families and 8 orders) were found in the Hwangjicheon stream using Surber net sampling and eDNA analysis, respectively. When we collected samples using a Surber net, genus richness decreased at sites 1 (14) to 3 (5), where substrates at the stream bottom were acid-sulfated, and then increased at sites 4 (9) to 7 (15) (Fig. 2). In contrast, for eDNA analysis, the genus richness tended to decrease from upstream (sites 1 and 11) to downstream (sites 7 and 5). Venn diagram analysis revealed that eight genera overlapped between the Surber net sampling and eDNA analysis (Fig. 3). More genera were collected using Surber net sampling (21) than those detected by eDNA sampling (12).

The relative FFG ratios based on genus richness revealed that, in Surber net sampling, the ratio of collector-gatherers was highest (41.4%), followed by scrapers (27.6%), collector-filterers (13.8%), predators (10.3%), and shredders (6.9%) (Fig. 4). On the other hand, when using eDNA analysis, the ratio of collector-gatherers was also highest (30.05%), followed by shredders (20.0%), scrapers (20.0%), collector-filterers (20.0%), and predators (10.0%). Considering FFGs at each site in Surber net sampling, the ratio of scrapers (e.g., *Psilotreta*) decreased from site 1 (7.7%) to site 3 (0.0%) and then tended to increase from site 4 (11.1%) to 7 (36.8%), including *Semisulcospira*, *Ecdyonurus*, *Epeorus*, and *Drunella*. Only collector-gatherers were observed at site 3. In contrast, using eDNA analysis, scrapers, including *Fossaria*, *Physa*, *Ecdyonurus*, and *Epeorus*, increased from site 3 (16.0%) to site 6 (33.3%).

CA grouped the sites into two clusters based on similarities in macroinvertebrate composition obtained by the Surber net and eDNA sampling methods. MRPP showed significant differences between the two clusters (i.e., using a Surber net: $A = 0.07$, $p < 0.05$; eDNA sampling: $A = 0.08$, $p < 0.05$). NMDS also reflected differences in benthic macroinvertebrate community composition (i.e., using a Surber net: stress value = 1.7 for the first two axes and eDNA sampling: stress value = 2.3 for the first two axes) (Fig. 5). In the Surber net method, sites with acid-sulfated substrates and a high percentage of forest in terms of land use were located in the left part of NMDS ordination (the sites included in cluster 1), whereas sites with high TN, NO_3^-, and BOD values were located in the right part (the sites included in cluster 2). Factors that influenced macroinvertebrate communities included TN (0.892), BOD (0.865), the ratio of 0.063-mm in substrate composition (0.859), NO_3^- (0.849), the ratio of forest (0.780), and the ratio of 64~256 mm (0.720) (Table 1). For eDNA analysis, the sites with high values in stream order and TN were located in the right part of the NMDS ordination, whereas other sites (sites 1 to 5) were located in the left part. The influential factors on the macroinvertebrate community were the percentage of forest (0.881), TN (0.856), stream order (0.851), and the ratio of urban in land use (0.810).

We observed differences in the benthic macroinvertebrate community between Surber net sampling and eDNA analysis in the abandoned mining area. Although the results based on eDNA analysis did not seem to reflect the impact of the abandoned mining area on benthic macroinvertebrates, the approach could be improved by considering the following factors. First, contrary to other studies using eDNA analysis, we observed a lower number of genera. To evaluate the status of freshwater ecosystems using eDNA analysis based on benthic macroinvertebrate diversity, several approaches using the mitochondrial cytochrome c oxidase subunit I (*cox*1 or COI) gene as a biomarker have been proposed in addition to the 18S rRNA gene marker (Dowle *et al.*, 2016; Fernández *et al.*, 2018; Elbrecht *et al.*, 2019; Fernández *et al.*, 2019; Meyer *et al.*, 2021). When the COI gene was employed as a marker, more benthic macroinvertebrate species were detected compared with those detected using 18S rRNA gene markers and the traditional field survey such as Surber net sampling (Fernández *et al.*, 2018). In addition, the highly specific COI marker for benthic macroinvertebrates can reduce the probability of non-target (e.g., protozoa, phytoplankton, fungi, bacteria) amplifications (Gleason *et al.*, 2021; Leese *et al.*, 2021).

Second, eDNA analysis results can differ depending on the water sampling depth, especially for benthic macroinvertebrate communities. We tried to collect stream water for eDNA analysis at the stream bottom because of the behavior and living habits of "benthic" macroinvertebrates, although the stream water depth (< 30 cm) in our research stream was

Table 1. Relationships between environmental factors and the non-metric multidimensional scaling (NMS) ordination of benthic macroinvertebrate assemblage using a Surber net sampling and eDNA analysis.

Environmental factors		Using a Surber net				Using eDNA analysis			
		Axis 1	Axis 2	r^2	*p* values	Axis 1	Axis 2	r^2	*p* values
Geography	Altitude (m)	−0.912	0.410	0.653	0.121	−0.990	0.140	0.809	0.051
	Stream order	0.970	−0.245	0.677	0.103	**0.997**	**−0.082**	**0.851**	**0.025**
	Distance from sources (km)	0.981	0.192	0.514	0.206	0.953	0.303	0.635	0.145
Land use (%)	Urban	0.567	−0.824	0.236	0.627	**0.038**	**−0.999**	**0.810**	**0.007**
	Agriculture	0.461	−0.887	0.121	0.736	0.808	0.590	0.762	0.052
	Forest	**−0.376**	**0.927**	**0.780**	**0.046**	**−0.492**	**0.870**	**0.881**	**0.015**
	Grassland	−0.699	−0.715	0.269	0.494	−0.972	0.236	0.088	0.843
	Wetland	0.908	−0.419	0.276	0.607	0.780	−0.626	0.390	0.469
	Bareland	0.185	−0.983	0.619	0.153	0.952	−0.306	0.391	0.366
Substrate composition (%)	0.063 mm	**−0.539**	**0.842**	**0.859**	**0.017**	−0.869	0.495	0.620	0.147
	0.063∼2 mm	−0.335	0.942	0.662	0.137	−0.493	0.870	0.417	0.360
	2∼4 mm	−0.948	−0.318	0.075	0.845	0.377	0.926	0.368	0.437
	4∼64 mm	0.250	0.968	0.681	0.056	−0.928	0.372	0.120	0.874
	64∼256 mm	**−0.143**	**-0.990**	**0.720**	**0.029**	0.758	−0.652	0.207	0.637
	256 mm	0.078	0.997	0.471	0.226	−0.597	0.803	0.208	0.651
Water quality	Dissolved oxygen (mg L^{-1})	0.721	0.693	0.335	0.453	0.267	0.964	0.162	0.707
	Conductivity (μS cm^{-1})	−0.221	−0.975	0.619	0.111	0.656	−0.754	0.169	0.706
	Biological oxygen demand (mg L^{-1})	**0.771**	**−0.637**	**0.865**	**0.017**	0.847	−0.532	0.689	0.107
	Ammonia (mg L^{-1})	0.264	−0.964	0.366	0.408	0.960	−0.279	0.039	0.923
	Nitrate (mg L^{-1})	**0.180**	**−0.984**	**0.849**	**0.011**	0.883	−0.469	0.291	0.448
	Total nitrogen (mg L^{-1})	**0.686**	**−0.728**	**0.892**	**0.025**	**0.856**	**−0.516**	**0.856**	**0.008**
	Ortho-phosphate (mg L^{-1})	0.447	−0.895	0.580	0.205	0.224	−0.975	0.619	0.173
	Total phosphorus (mg L^{-1})	0.952	−0.307	0.705	0.091	0.592	−0.806	0.716	0.081
	Chlorophyll-a (mg L^{-1})	0.025	1.000	0.185	0.725	−0.945	0.327	0.199	0.649

P values lower than 0.05 are indicated in bold.

shallower compared with the main streams. However, it has been proposed that the community composition of freshwater organisms determined using eDNA analysis significantly differs depending on water depth (i.e., the bottom, middle, and surface of stream water) (Zeng *et al.*, 2021). Therefore, eDNA analysis from water sampling at different depths is recommended.

Third, the behavior (i.e., generation, migration diffusion, and degradation) of eDNA in freshwater ecosystems should be considered (Carraro *et al.* 2020). Despite the several advantages and considerable potential of eDNA techniques in evaluating the status of freshwater ecosystems, several prerequisites, including consideration of quantitative decay time (half-life) of eDNA (Tzafesta *et al.*, 2021), eDNA diffusion rate due to hydrological factors (Carraro *et al.*, 2020), minimizing non-target amplification (Leese *et al.*, 2021), and normalization between actual populations (or biomass) and detected signals (Dowle *et al.*, 2016; Pereira-da-Conceicoa *et al.*, 2021), still need to be addressed to completely replace existing field survey methods. Thus, integrated eDNA metabarcoding approaches, conducted parallel to traditional field surveys, are required for eDNA technology to eventually overcome the current limitations.

CONCLUSIONS

As a case study, we employed eDNA metabarcoding tech-

niques to evaluate the effects of abandoned mining areas on benthic macroinvertebrate diversity in the Hwangjicheon headwater stream, South Korea. The eDNA analysis data were subsequently compared with those obtained using the traditional Surber-net sampling method. The number of genera was higher in Surber-net sampling (29) than in eDNA analysis (20). In addition, only approximately 20% of the detected genera overlapped in the Venn diagram analysis. The genus richness tended to decrease from headwater to downstream when using eDNA analysis, while the genus richness from Surber-net sampling reflected the acid-sulfated sediment area generated by mining. Using CA and NMDS, acid-sulfated sites were separated from the other sites in Surber-net sampling, whereas the sites were grouped into the two lowest downstream sites and other sites when using eDNA sampling. Although eDNA techniques have considerable potential for application in evaluating freshwater ecosystems, it is necessary to constantly improve the methodologies by comparing them with field survey data.

Author information Mi-Jung Bae (Nakdonggang National Institute of Biological Resources, Senior Researcher), Seong-Nam Ham (Nakdonggang National Institute of Biological Resources, Researcher), Young-Kyung Lee (Nakdonggang National Institute of Biological Resources, Researcher), Eui-Jin Kim (Nakdonggang National Institute of Biological Resources, Principal Researcher)

Author contributions Conceptualization, M.-J.B. and E.-J.K.; Field survey: M.-J.B. and Y.-K.L.; Literature survey: M.-J.B., S.-N.H. and E.-J.K.; Writing, Review and Editing, M.-J.B., E.-J.K. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES

Benda, L.E.E., N.L. Poff, D. Miller, T. Dunne, G. Reeves, G. Pess and M. Pollock. 2004. The network dynamics hypothesis: how channel networks structure riverine habitats. *BioScience* **54**(5): 413-427.

Brigham, A.R., W.U. Brigham and A. Gnilka. 1982. Aquatic Insects and Oligochaetes of North and South Carolina. Midwest Aquatic Enterprises, Mahomet, IL. 837 pp.

Brinkhurst, R.O. 1986. Guide to the freshwater aquatic microdrile Oligochaetes of North America. *Canadin Specil Publication of Fisheries and Aquatic Sciences* **84**: 259.

Carraro, L., E.Mächler, R. Wüthrich and F. Altermatt. 2020. Environmental DNA allows upscaling spatial patterns of biodiversity in freshwater ecosystems. *Nature Communication* **11**: 3585.

Clarke, A., R. Mac Nally, N. Bond and P.S. Lake. 2008. Macroinvertebrate diversity in headwater streams: a review. *Freshwater Biology* **53**(9): 1707-1721.

Coble, A.A., C.A. Flinders, J.A. Homyack, B.E. Penaluna, R.C. Cronn and K. Weitemier. 2019. eDNA as a tool for identifying freshwater species in sustainable forestry: A critical review and potential future applications. *Science of the Total Environment* **649**: 1157-1170.

Díaz-Ferguson, E.E. and G.R. Moyer. 2014. History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments. *Revista de Biologia Tropical* **62**: 1273-1284.

Dowle, E.J., X.C. Pochon, J. Banks, K. Shearer and S.A. Wood. 2016. Targeted gene enrichment and high-throughput sequencing for environmental biomonitoring: A case study using freshwater macroinvertebrates. *Molecular Ecology resources* **16**: 1240-1254.

Elbrecht V., T.W.A. Braukmann, N.V. Ivanova, S.W.J. Prosser, M. Hajibabaei, M. Wright, E.V. Zakharov, P.D.N. Hebert, D. Steinke. 2019. Validation of COI metabarcoding primers for terrestrial arthropods. *PeerJ* **7**: e7745.

Fernández, S., S. Rodríguez, J.L. Martínez, Y.J. Borrell, A. Ardura and E. García-Vázquez. 2018. Evaluating freshwater macroinvertebrates from eDNA metabarcoding: A river Nalón case study. *PLoS One* **13**: e0201741.

Fernández, S., S. Rodríguez-Martínez, J.L. Martínez, E. Garcia-Vazquez and A. Ardura. 2019. How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain). *Environmental DNA* **1**: 385-401.

Gleason, J.E., V. Elbrecht, T.W. Braukmann, R.H. Hanner and K. Cottenie. 2021. Assessment of stream macroinvertebrate communities with eDNA is not congruent with tissue-based metabarcoding. *Molecular Ecology* **30**: 3239-3251.

Harrison, J.B., J.M. Sunday and S.M. Rogers. 2019. Predicting

the fate of eDNA in the environment and implications for studying biodiversity. *Proceedings of the Royal Society B* **286**: 20191409.

Leese, F., M. Sander, D. Buchner, V. Elbrecht, P. Haase and V.M. Zizka. 2021. Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification. *Environmental DNA* **3**: 261-276.

Loayza-Muro, R.A., R. Elías-Letts, J.K. Marticorena-Ruíz, E.J. Palomino, J.F. Duivenvoorden, M.H. Kraak, W. Admiraal. 2010. Metal-induced shifts in benthic macroinvertebrate community composition in Andean high altitude streams. *Environmental Toxicology and Chemistry* **29**(12): 2761-2768.

Mächler, E., K. Deiner, F. Spahn and F. Altermatt. 2016. Fishing in the water: effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. *Environmental Science & Technology* **50**: 305-312.

Marqués, M.J., E. Martínez-Conde, J.V. Rovira, S. Ordóñez. 2001. Heavy metals pollution of aquatic ecosystems in the vicinity of a recently closed underground lead-zinc mine (Basque Country, Spain). *Environmental Geology* **40**: 1125-1137.

Merritt, R.W. and K.W. Cummins. 2006. An Introduction to the Aquatic Insects of North America. Hunt Publishing Company, Dubugue.

Meyer, A., F. Boyer, A. Valentini, A. Bonin, G.F. Ficetola, J.N. Beisel, J. Bouquerel, P. Wagner, C. Gaboriaud, Fl. Leese, T. Dejean, P. Taberlet and P. Usseglio-Polatera. 2021. Morphological vs. DNA metabarcoding approaches for the evaluation of stream ecological status with benthic invertebrates: Testing different combinations of markers and strategies of data filtering. *Molecular Ecology* **30**: 3203-3220.

Oksanen, J., F.G. Blanchet, R. Kindt, P. Legendre, P. Minchin, R.B. O'Hara, G. Simpson, P. Solymos, M.H.H. Stevens, H. Wagner. 2011. Vegan: Community Ecology Package. R Package Version.

Pennak, R.W. 1978. Freshwater Invertebrates of the United States. John Wiley and Sons, Inc., New York.

Pereira-da-Conceicoa, L., V. Elbrecht, A. Hall, A. Briscoe, H. Barber-James and B. Price. 2021. Metabarcoding unsorted kick-samples facilitates macroinvertebrate-based biomonitoring with increased taxonomic resolution, while outperforming environmental DNA. *Environmental DNA* **3**: 353-371.

Quigley, M. 1977. Invertebrates of Streams and Rivers: a Key to Identification. Edward Arnold, Ltd., London.

R Core Team. R. 2017. A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria, Available online: https://www.R-project.org/ (accessed on 1 Aug 2021).

Romero, A., R. Medina and S. Flores. 2008. Estudio de los metales pesados en el relave abandonado de Ticapampa. *Revista del Instituto de Investigación de la Facultad de Ingeniería Geológica, Minera, Metalúrgica y Geográfica* **11**(22): 13-16.

Thomsen, P.F. and E. Willerslev. 2015. Environmental DNA-An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* **183**: 4-18.

Tzafesta, E., F. Zangaro, V. Specchia and M. Pinna. 2021. An Overview of DNA-Based Applications for the Assessment of Benthic Macroinvertebrates Biodiversity in Mediterranean Aquatic Ecosystems. *Diversity* **13**: 112.

Yoon, I.B. 1988. Illustrated Encyclopedia of Fauna and Flora of Korea. Vol. 30. Ministry of Education, Seoul. (in Korean)

Zeng, C., Y. Wen, X. Liu, J. Yu, B. Jin and D. Li. 2021. Impact of anthropogenic activities on changes of ichthyofauna in the middle and lower Xiang River. *Aquaculture and Fisheries*. In press.

Zhan, A., M. Hulák, F. Sylvester, X. Huang, A.A. Adebayo, C.L. Abbott, S. Adamowicz, D. Heath, M. Cristescu and H.J. MacIsaac. 2013. High sensitivity of 454 pyrosequencing for detection of rare species in aquatic communities. *Methods in Ecology and Evolution* **4**: 558-565.