2025 Vol. 68 | Št. 4

ABS

Acta Biologica Slovenica

DBS Društvo biologov Slovenije

BF UNIVERZA V LJUBLJANI Biotehniška fakulteta

Acta Biologica Slovenica, 2025, 68 (4)

Založila/Published by
Založba Univerze v Ljubljani / University in Ljubljana Press
Društvo biologov Slovenije / Slovenian biological society

Za založbo/For the publisher
Gregor Majdič, rektor Univerze v Ljubljani / the Rector of the University of Ljubljana
Anita Jemec Kokalj, predsednica Društva biologov Slovenije / Chairman of Slovenian Biological Society

Izdala/Issued by
Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za biologijo /
University of Ljubljana, Biotehnical Faculty, Department of Biology

Za izdajatelja/For the Issuer
Marina Pintar, dekanja Biotehniške fakultete UL / Dean of Biotechnical Faculty

Naslov uredništva/Editorial Office Address
Univerza v Ljubljani, Biotehniška fakulteta, Acta Biologica Slovenica,
Večna pot 111, 1000 Ljubljana, Slovenija

Oblikovanje/Design
Ajda Fortuna

Naslovnica/Cover page
Hribski urh, avtor/author: Tina Klenovšek

Izdajanje revije sofinancira Javna agencija za znanstvenoraziskovalno in inovacijsko dejavnost Republike Slovenije (ARIS)
The journal is co-financed by Slovenian Research and Innovation Agency (ARIS)

Publication is free of charge.

ISSN 1854-3073 (spletna verzija/online version) UDK 57(497.4)
DOI: 10.14720/abs.68.4
http://journals.uni-lj.si/abs/
Acta Biologica Slovenica je indeksirana v – is indexed in: CAB Abstracts, Web of Science Clarivate

Table of Contents

ABS

Original Research

The effect of different herbicides and soil moisture regimes on Camelina performance

Asghar Ebadi-Segherloo [1], Mohsen Janmohammadi [2,*], Naser Sabaghnia [2], Mehdi Mohebodini [3]

Abstract

This experiment aimed to evaluate the effect of different herbicides, including H_0: no application of herbicide, H_1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-R-methyl, and H_4: pinoxaden as post-emergence herbicides, on weed growth and camelina performance characteristics under well-irrigated (I_1) and rainfed (I_2) conditions. The response of the studied traits to herbicides varied across moisture regimes. The lowest population density was recorded under conditions of I_1H_2 (8 plant m-2), I_2H_3 (7 plant m^{-2}), and I_2H_4 (6.33 plant m^{-2}). The lowest weed biomass was obtained under rainfed conditions with the use of cycloxydim. The tallest plants were recorded under I_1H_2 conditions (110.96 cm), and the shortest plants were recorded under I_2H_0 conditions (51.87 cm). The highest amount of chlorophyll was observed in plants grown under the conditions of I_1H_4 (58.83 SPAD unit), I_1H_2 (56.70), and the lowest amount under the conditions of I_2H_0 (36.33) and I_2H_1 (39.00). Seed yield under rainfed conditions decreased by 33% compared to well-irrigated conditions. The highest seed yield was obtained by using cycloxydim (1135 kg ha^{-1}) and pinoxaden (1110 kg ha^{-1}). Although weed populations were low under rainfed conditions, the effectiveness of herbicides was more prominent under fully irrigated conditions than under rainfed conditions.

Keywords

Camelina sativa, pinoxaden, post-emergence herbicide, pre-emergence herbicides, haloxyfop-R-methyl

1 Moghan College of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

2 University of Maragheh, Faculty of Agriculture, Department of Plant Production and Genetics, Maragheh, Iran

3 Department of Horticultural Science, Faculty of Agricultural Science and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

* **Corresponding author:**
E-mail address:
mjanmohammadi@maragheh.ac.ir

Citation: Ebadi-Segherloo, A., Janmohammadi, M., Sabaghnia, N., Mohebodini, M., (2025). The effect of different herbicides and soil moisture regimes on Camelina performance. Acta Biologica Slovenica 68 (4)

Received: 11.06.2025 / **Accepted:** 15.07.2025 / **Published:** 18.07.2025

https://doi.org/10.14720/abs.68.4.23011

Vpliv različnih kemičnih herbicidov in režimov vlažnosti tal na uspešnost kameline

Izvleček

Namen tega poskusa je bil oceniti učinek različnih herbicidov, vključno s H_0: brez uporabe herbicida, H_1: trifluralinom kot herbicidom pred vznikom, H_2: cikloksidimom, H_3: haloksifop-R-metilom in H_4: pinoksadenom kot herbicidi po vzniku na rast plevela in proizvodne značilnosti rička v dobro namakanih (I_1) in deževnih (I_2) pogojih. Odziv preučevanih lastnosti na herbicide se je razlikoval glede na režim vlažnosti. Najnižja gostota populacije je bila zabeležena v pogojih I_1H_2 (8 rastlin m^{-2}), I_2H_3 (7 rastlin m^{-2}) in I_2H_4 (6,33 rastlin m^{-2}). Najnižja biomasa plevela je bila dosežena v deževnih pogojih z uporabo cikloksidima. Najvišje rastline so bile zabeležene v pogojih I_1H_2 (110,96 cm), najkrajše rastline pa v pogojih I_2H_0 (51,87 cm). Najvišjo količino klorofila so opazili pri rastlinah, gojenih v pogojih I_1H_4 (58,83 SPAD enote), I_1H_2 (56,70), najnižjo pa v pogojih I_2H_0 (36,33) in I_2H_1 (39,00). Pridelek semen v deževnici se je v primerjavi z dobro namakanimi pogoji zmanjšal za 33 %. Najvišji pridelek semen je bil dosežen z uporabo cikloksidima (1135 kg ha^{-1}) in pinoksadena (1110 kg ha^{-1}). Čeprav so bile populacije plevela v deževnici nizke, je bila učinkovitost herbicidov izrazitejša v pogojih polnega namakanja kot v deževnici.

Ključne besede

Camelina sativa, pinoxaden, herbicid po pojavu plevela, herbicid pred pojavom plevela, haloksifop-R-metil

Introduction

Water shortage is a major factor limiting crop yields in semi-arid regions. Climate changes in recent decades and a significant increase in air temperature have aggravated the water shortage in these areas (Morante-Carballo et al., 2022). *Camelina sativa*, an emerging oilseed crop from the Brassicaceae family, has a long history of cultivation but has recently regained attention for its potential agricultural value. *Camelina sativa* can produce an acceptable yield with low inputs, and it also has high relative resistance against pests, diseases, and adverse environmental conditions such as high temperature, drought stress, and salinity (Obour et al., 2015). The main producers of camelina include the United States, Canada, Slovenia, Ukraine, China, Finland, Germany, Austria, and New Zealand (FAOSTAT, 2024). Weeds represent another significant challenge in the cultivation of Camelina. Weeds significantly reduce the growth and quality of crops by competing with crops for light, water, and nutrients. Not controlling weeds in crops of the Brassicaceae family can reduce the crop yield by about 50% (Ullah et al., 2020). Crop production systems have conventionally prioritised plant modification for higher yield over other characteristics, so any procedure of weed conquest must deliberate its net effect on efficiency, given that decreased seed yield in a weed-free environment can be compensated by the yield advantage provided by operative weed control (Ghidoli et al., 2023). Although the area under Camelina cultivation is expanding in semi-arid regions, there is still not much information about the efficacy of herbicides in Camelina production systems. Currently, to control rapeseed weeds, trifluralin as the pre-plant soil incorporated herbicide, clopyralid for control of broadleaf weeds, and cycloxydim, haloxyfop-R-methyl and sethoxydim for control of narrow leaf weeds are recommended for the regions of Iran (Shimi et al., 2014).

A large portion of Iran is characterised by a semi-arid climate where plant growth is constrained by several environmental and edaphic limitations. These include low annual precipitation, irregular and unpredictable rainfall patterns, and the concentration of precipitation during the cold months, when many plants are in the rosette stage and exhibit minimal growth. Additional challenges include low winter temperatures in high-altitude areas, deficiencies or limited availability of essential nutrients in the rhizosphere, low soil organic matter content, high soil pH, and prolonged periods of dryness and low rainfall during the spring growing season (Modarres and da Silva, 2007; Janmohammadi and Sabaghnia, 2023; Fattahi et al., 2023; Janmohammadi et al., 2024). Although several studies have examined the effects of water deficit on Camelina plants, reporting significant reductions in vegetative growth, seed yield, and

oil quality (Borzoo et al., 2021; Bukhari et al., 2022; Hazrati et al., 2024), the effect of water deficit conditions on the effectiveness of herbicides has not been well studied yet.

Studies investigating the use of herbicides such as quinclorac, S-metolachlor, dimethenamid-P, pendimethalin, and pyroxasulfone herbicides during the pre-planting stage in the Camelina fields have shown that early Camelina growth is sensitive to these soil-applied chemicals. However, as the plants develop, their sensitivity decreases, and the negative effects of the herbicides are progressively reduced. The application of quinclorac sought the highest safety in the product (Jha and Stougaard, 2013). Propaquizafop and quizalofop-p-ethyl as systemic herbicides can be absorbed by foliage and roots and translocated through the phloem, and finally inhibit fatty acid biosynthesis (inhibitor of Acetyl-CoA Carboxylase; ACCase). The use of the mentioned herbicides on various varieties of Camelina indicated the different effects of these herbicides on the chlorophyll fluorescence and the functioning of photosystem II; however, after 42 days, the adverse effects of these herbicides were removed. However, spray of clopyralid and picloram as selective herbicides for control of broadleaf weeds or woody plants (act as auxin mimics) had little effect on Camelina photosynthetic characteristics (Sobiech et al., 2020). Regarding the effect of water shortage conditions on the effectiveness of herbicides, some reports showed that the effectiveness of clodinafop-propargyl (as an inhibitor of ACCase) and mesosulfuron-methyl + iodosulfuron-methyl sodium (as an inhibitor of amino acid biosynthesis through the inhibition of acetolactate synthase) decreased significantly under drought stress conditions, and this was attributed to the decrease in stomatal conductance and the decrease in the absorption of herbicides (Alizade et al., 2020). Trifluralin is a soil-applied pre-emergent herbicide used to control a wide range of broadleaf and fine-leaf weeds. It inhibits germination or root growth by inhibiting cell division (Li et al., 2022). However, there is no information available on trifluralin's effectiveness in *Camelina sativa* fields. Additionally, it appears that the function of herbicides and their effects on weeds can be affected by soil moisture regimes. Because under water stress conditions, plants may close the stomata and change the cuticle thickness. Although a few studies have examined the use of certain herbicides in Camelina production systems, the effectiveness of these herbicides under varying soil moisture conditions remains largely unexplored. This study aimed to evaluate the effects of trifluralin as a pre-emergence herbicide, and cycloxydim, haloxyfop-R-methyl, and pinoxaden as post-emergence herbicides, on weed suppression and Camelina seed yield components under both well-irrigated and rainfed conditions in the Maragheh region of northwestern Iran.

Materials and Methods

Crop Growth

A field trial was carried out in the Margheh region, East Azarbaijan, Iran, during the 2023-2024 crop season. This area is located in Northwest Iran, on the southeastern slopes of Sahand Mountain at an altitude of 1490 meters. The climate of the studied area is cold semi-arid (with latitude and longitude 37° 23'N; 46° 16'E) with predominant winter rainfall, and the amount of rainfall during the growing season (September-July) was estimated at 294 mm. Before conducting the experiment, random samples were taken from the field soil at a depth of 10-30 cm, and some of its physical and chemical properties were examined (Table 1). The studied area is located on the southern slopes of Mount Sahand, where most of its annual precipitation occurs during the cold seasons.. The cultivated area in the present experiment was fallow during the previous cropping season. In mid-October 2023, the farm soil was ploughed using a moldboard plough, and immediately, 10 t ha^{-1} of cow manure fertilisation was spread on the soil surface and mixed using a sweep to a depth of 15 cm. Final seedbed preparation was carried out through secondary tillage using a rotavator and a furrower in early March 2024. Inter-row spacing was 50 cm. Before planting seeds, 60 kg of phosphorus was consumed through triple super phosphate fertiliser and 100 kg of nitrogen through urea, based on the suggested dose. In the middle of March, the farm was divided into main and sub-plots.

Table 1. Soil texture and chemical characteristics of soil samples in the studied field.

Tabela 1. Tekstura in kemijske značilnosti vzorcev tal na preučevanem polju.

Soil texture	Clay (%)	Silt (%)	Sand (%)	N (%)	K (mg kg^{-1})	P (mg kg^{-1})	OC (%)	pH	EC (ds m^{-1})	$CaCO_3$ (%)
silty loam	23	49	28	0.12	282	11	0.24	7.82	0.92	10.8

Experimental Details

The experimental design was a split-plot (2×5) based on a randomised complete block design with three replications. The main factor at two levels included irrigation regimes (I_1: well irrigated and I_2: rainfed). In well-irrigated conditions, the field was irrigated 8 times from the time of planting to harvest, and a total of 550 mm of water was consumed. Sub-plots were assigned to chemical herbicides. Herbicide treatments were H_0: no application of herbicide, H_1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-p-methyl, and H_4: pinoxaden as post-emergence herbicides. Trifluralin herbicide (Treflan, CAS 1582-09-8) is a selective pre-emergent herbicide (EC 48%) obtained from Elixir Agricultural Company, Yazd, Iran. After ploughing and disc operations, and 10 days before seed planting, treflan herbicide was used at the suggested rate of (1.5 L ha^{-1}) along with spraying 300-500 litres of water evenly and immediately mixing it with the soil using a light disk. This herbicide disrupts the germination and death of weed seedlings by disrupting mitosis and inhibiting microtubule synthesis.

Cyclohexidim (Focus, CAS 66-81-9, 10% EC, BASF, Germany) is a systemic and selective herbicide from the cyclohexane oxime group, which is used to control narrow-leaved weeds in canola fields. The selective properties of this herbicide are excellent, and it can be used in all stages of the growth of crops. Focus contains the active ingredient cyclohexadim from the group of cyclohexanidones, which is used according to the recommendation after germination and after the four-to-five-leaf stage of narrow-leaved weeds in the amount of 2 L ha^{-1}, with the dysfunction of Acetyl-CoA carboxylase (ACCase) preventing the synthesis of fatty acids and causing the death of weeds. Haloxyfop-R-methyl (CAS Number 72619-32-0, EC 10%) is a systemic herbicide that was produced under the brand name of Super Gallant from Jahan Chemi-Iran. This selective herbicide controls a wide range of one-year and multi-year narrow-leaf weeds in agricultural fields, and it was used at the rate of 1 L ha^{-1} at the 2-5 leaf stage of the weeds. Haloxyfop belongs to the aryloxyphenoxypropionate group of herbicides, which is absorbed by the plants within one hour through the leaves, and by inhibiting the ACCase enzyme, prevents fatty acid biosynthesis and inhibits the growth of weed plants' meristem tissues.

Pinoxaden herbicide (Axial, CAS 243973-20-8, and EC 5%) is the only available herbicide from the Pinoxaden group. This poison inhibits the growth of narrow-leafed grasses. This herbicide was used at the stage of 4-6 narrow leaves in the amount of 1.2 L ha^{-1}. In control conditions or without chemical herbicides, the weeds in the experimental plot were sprayed with distilled water. All the herbicides were sprayed according to the manufacturer's recommendations in the vegetative stages of weeds with the help of a motorised backpack sprayer (Solo model 433). Post-emergence herbicides were applied to the above-ground parts in the early hours of the day. We tried to cover all the weed shoots with herbicides, but the herbicide drops did not flow on the leaves.

The seeds of *Camelina sativa* "Sohail cv." were obtained from the oil seeds department of the Dryland Agricultural Research Institute, Maragheh, Iran. The mentioned variety has a relatively high adaptability to different weather and soil conditions, and compared to other oilseed plants, it needs less water, fertiliser, and pesticides, and is more resistant to cold (Rostami Ahmadvandi et al., 2021). The seeds were planted manually on top of the ridges at a depth of 1.5 cm. Intra-row spacing was 5 cm. The planting date coincided with the beginning of the rains. The amount of the first rain was enough for seed germination and seedling establishment.

Data Recorded

After applying post-emergence herbicides and in the middle of the main stem elongation stage (BBCH= 35; Martinelli and Galasso, 2011), the surviving weeds between the rows and on the rows were counted using 1 m^2 quadrate from each experimental unit and reported as weed populations density (Hussain et al., 2022), and after cutting them above the ground surface, they were dried in an oven at 70 °C for 48 hours and then weighed (weed biomass).

At the early stage of flowering (BBCH=60; First flowers open), the chlorophyll of the upper and developed leaves of Camelina was measured using a manual chlorophyll meter (SPAD 502, Konica Minolta, USA). In the physiological maturity stage of Camelina (BBCH=89; nearly all siliques were ripe, the crop was ready to be harvested), using an arbitrarily 1 m^2 quadrate from each experimental unit, plant height was measured. The lateral growth of the canopy was calculated by measuring the diameter of the canopy from left to right. After harvesting Camelina plants and drying them in the oven, seed yield components such as

the silique number per plant, number of seeds per silique, weight of 1000 seeds, yield per unit area, biological yield, and harvest index (ratio of grain yield to biological yield) were calculated.

Statistical Analysis

The experiment was analysed as a split-plot based on a block design. Before performing the ANOVA, the normality of the data was checked and, in some cases, data transformation was performed. One-way analysis of variance was performed using SAS software, and comparison of means was performed using LSD. To interpret the similarity between treatment compounds and their effect on the evaluated biological traits, Principal Component Analysis was performed using Minitab software version 19.2.

Results

Wedd population and biomass

The results of the variance analysis indicated that the interaction between irrigation (I) and herbicide (H) treatments had a statistically significant effect on weed population density at the 1% level. The highest geometric mean weed density under well-irrigated conditions without herbicide application (I_1H_0). In contrast, the lowest weed densities were recorded with pinoxaden (6.33) and haloxyfop-R-methyl (7.00) under well-irrigated conditions, and with cycloxydim (8.00) under rainfed conditions. Among the herbicides tested, trifluralin demonstrated the least effectiveness in reducing weed population density under both irrigation regimes (Figure 1).

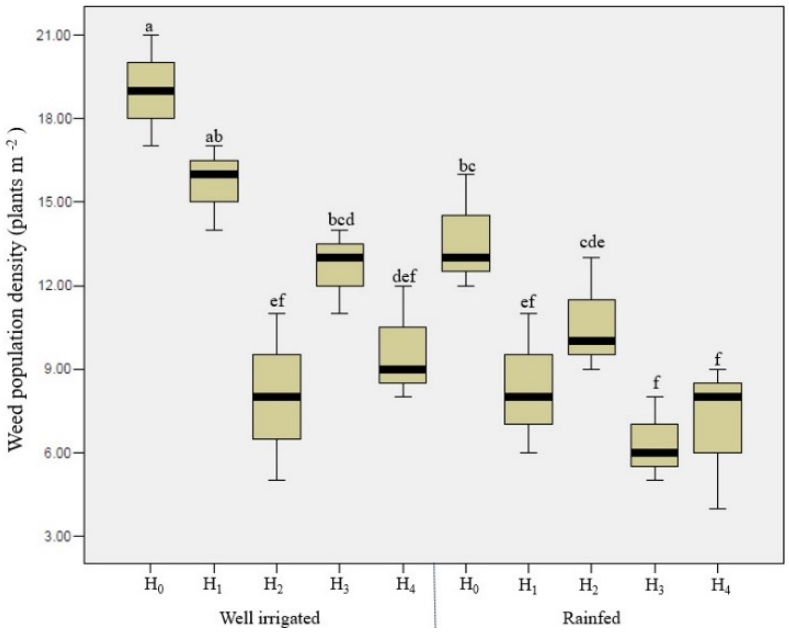


Figure 1. Evaluation of weed population density in *Camelina sativa* field with different herbicide applications (H_0: no application of herbicide, H_1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-p-methyl, and H_4: pinoxaden as post-emergence) under well-irrigated and rainfed conditions in the Maragheh region, northwestern Iran. The dashed line inside the box indicates the mean of the treatment combination, and the bar lines above and below the box indicate the distribution of the data. Boxes with similar letters do not have statistically significant differences at the 5% level.

Slika 1. Ocena gostote populacije plevela na polju *Camelina sativa* z različnimi uporabami herbicidov (H_0: brez uporabe herbicida, H_1: trifluralin kot herbicid pred vzklitjem, H_2: cikloksidim, H_3: haloksifop-p-metil in H_4: pinoxaden po vzklitju) v dobro namakanih in deževnih razmerah v regiji Maragheh, severozahodno od Irana. Črtkana črta znotraj okvirja označuje povprečje kombinacije obdelave, črte nad in pod okvirjem pa označujejo porazdelitev podatkov. Okvirji z enakimi črkami nimajo statistično pomembnih razlik na ravni 5 %.

Under well-watered conditions, the application of cycloxydim, haloxyfop-R-methyl and pinoxaden reduced weed population density by 57%, 33%, and 49%, respectively, compared to the untreated control. Under rainfed conditions, these herbicides reduced weed density by 22%, 53%, and 48%, respectively, relative to plots without herbicide application. Weed population density was reduced by 29% under rainfed conditions compared to well-irrigated conditions. Among the herbicides tested, cycloxydim and pinoxaden were the most effective in reducing weed population density. The evaluation of the above-ground biomass of weeds showed that the lowest biomass was obtained under rainfed conditions with the application of I_2H_4 (10.33g) and I_2H_2 (11.00 g). The highest amount of biomass was recorded under well-irrigated conditions without the use of herbicides (48.33 g) and the use of trifluralin (34.00 g). The most effective herbicides in reducing weed biomass compared to herbicide-free conditions were cycloxydim (65%), pinoxaden (55%), haloxyfop-R-methyl (46%) and trifluralin (35%), respectively. Additionally, water deficit under rainfed conditions reduced weed biomass by 45% compared to well-irrigated conditions (Figure 2).

Chlorophyll content and growth characteristics of Camelina

The interaction effects of irrigation (I) and herbicide (H) treatments on the chlorophyll content of Camelina leaves were statistically significant at the 1% level. The lowest amount of chlorophyll was recorded in herbicide-free conditions or in plots under rainfed conditions (Table 2). The highest amount of chlorophyll was recorded under well-watered conditions with the application of cycloxydim (56.70). Water shortage under rainfed conditions resulted in a 21% reduction in chlorophyll content compared to well-irrigated conditions. Plots treated with acetyl-CoA carboxylase (ACCase)-inhibiting herbicides exhibited higher chlorophyll content than those subjected to other treatments. Assessment of plant height revealed that the interaction effects of I × H are significant at the 1% level. The tallest plants under well-watered conditions

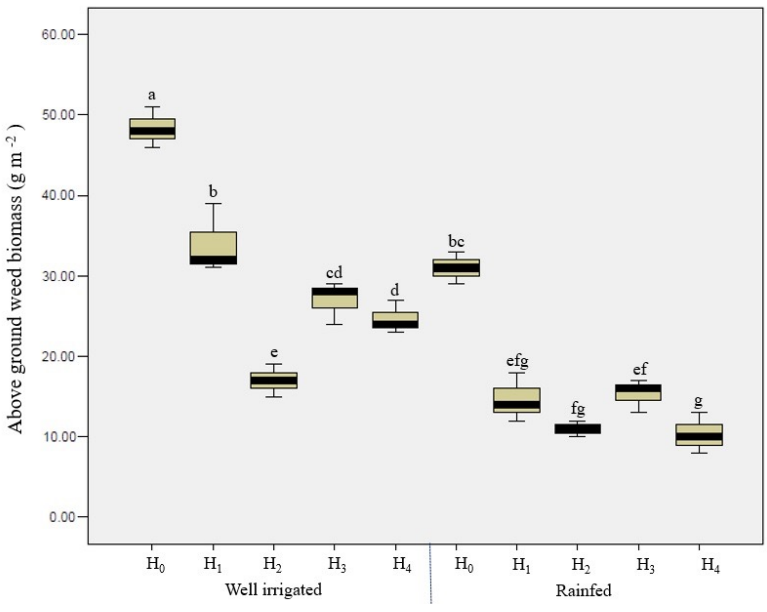


Figure 2. Mean comparison of above-ground biomass of weed under different chemical weed control methods (H_0: no application of herbicide, H_1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-p-methyl, and H_4: pinoxaden as post-emergence) under different soil moisture regimes in the semi-arid region of the Mragheh, Iran.

Slika 2. Povprečna primerjava nadzemne biomase plevela pri različnih kemijskih metodah zatiranja plevela (H_0: brez uporabe herbicida, H_1: trifluralin kot herbicid pred vzklitjem, H_2: cikloxidim, H_3: haloksifop-p-metil in H_4: pinoxaden po vzklitju) pri različnih vlažnostnih razmerah tal v polsušni regiji Mragheh v Iranu.

were recorded with the application of cycloxydim (110.96 cm) or the application of haloxyfop-R-methyl (101 cm). The shortest plants were obtained under the rainy conditions without herbicide application (51.82 cm) or trifluralin application (59.13 cm). Water stress resulting from the absence of irrigation under rainfed conditions reduced plant height by approximately 35% compared to well-irrigated conditions. The effect of the herbicide application on Camelina height was higher under well-irrigated conditions, and the use of trifluralin, cycloxydim, haloxyfop-R-methyl, and pinoxaden increased plant height by 18%, 42%, 31%, and 8%, respectively, compared to herbicide-free conditions (Table 2). The highest lateral growth of the canopy was recorded under well-irrigated conditions without herbicide application (67.33 cm) or haloxyfop-R-methyl (64.00 cm) application. However, the lowest canopy width was recorded for plants grown under rainfed conditions without herbicide application (42.33 cm) or with the spray of haloxyfop-R-methyl (47.66 cm). Among Camelina seed yield components, the seed number per silique (NSS) was significantly affected by I×H effects. The highest amount of NSS was obtained under well-watered conditions with cycloxydim (15.95) application. The lowest NSS value was recorded under rainfed conditions without herbicide application (6.67). The silique number in plants grown under rainfed conditions was 56% lower than in well-irrigated conditions. There was no difference in the number of siliques per plant among the levels of chemical control, but the plants grown under chemical weed control conditions showed a higher number of siliques per plant compared to herbicide-free conditions (Table 2).

Table 2. Mean comparisons of yield components of *Camelina sativa* under different irrigation regimes and herbicide utilisation in Northwest Iran, Mragheh.

Tabela 2. Povprečne primerjave komponent pridelka *Camelina sativa* pri različnih režimih namakanja in uporabi herbicidov v severozahodnem Iranu, Mragheh.

Irrigations (I)	CHL	PH	LG	SNP	NSS	TSW	SY
Well-irrigated (I1)	53.85a	93.12a	59.09a	135.13a	15.10a	0.840a	1273.53a
Rainfed (I2)	42.86b	60.21b	45.34b	59.72b	8.78b	0.665b	852.07b
Herbicides (H)							
Control (H0)	64.67c	44.25b	46.06c	88.66c	10.65b	0.718b	984.17c
Trifluralin (H1)	75.36b	44.94b	49.03c	94.65bc	12.38a	0.718b	1018.33c
Cycloxydim (H2)	86.26a	51.35a	53.83b	102.50a	12.48a	0.800a	1110.83a
Haloxyfop-R-methyl (H3)	81.56a	49.83a	54.83a	101.66ab	11.75a	0.766a	1065.33b
Pinoxaden (H4)	75.48b	51.41a	57.33a	99.66ab	12.38a	0.762a	1135.33a
$I_1 H_0$	51.17b	77.48d	51.80cd	124.33c	14.53b	0.787a	1183.33c
$I_1 H_1$	50.89b	91.60c	52.33c	132.66bc	14.93ab	0.812b	1206.00c
$I_1 H_2$	56.70a	110.96a	60.00b	145.66a	15.93a	0.917a	1308.00b
$I_1 H_3$	51.66b	101.96a	67.33a	139.00ab	14.70ab	0.841b	1281.33b
$I_1 H_4$	58.83a	83.74d	64.00ab	134.00bc	15.43ab	0.845b	1389.00a
$I_2 H_0$	37.33e	51.87g	40.33g	53.00e	6.76d	0.649cd	785.00f
$I_2 H_1$	39.00e	59.13fg	45.73ef	56.63de	9.83e	0.623d	830.67ef
$I_2 H_2$	46.00cd	61.56ef	47.66de	59.33de	9.03c	0.684c	913.67d
$I_2 H_3$	48.00bc	61.29ef	42.33fg	64.33d	8.80c	0.692c	849.33e
$I_2 H_4$	44.00d	67.23e	50.66cd	65.33d	9.47c	0.679cd	881.67de
	Significance level						
I	0.05	0.0018	0.008	0.0005	0.009	0.006	0.0026
H	<0.0001	<0.0001	<0.0001	0.0002	0.0035	0.0028	<0.0001
I×H	0.003	<0.0001	0.0003	0.157	0.05	0.171	0.011
CV%	4.50	5.76	4.97	6.13	7.49	3.51	4.79

CV: The coefficient of variation (%), Chl: leaf chlorophyll content (SPAD unit), PH: Plant height (cm), LG: lateral growth of canopy (cm), SNP: silique number per plant, NSS: seed number per silique, TSW: thousand seed weight (g), SY: seed yield (kg ha-1), At the significance level, p values less than 0.05 ($p < 0.05$) and 0.01 ($p < 0.01$) are significant at 95% and 99%, respectively. The means with various letters in each trait (column) are statistically different.

Evaluation of the thousand seed weight revealed that the interaction between irrigation and herbicide treatments was statistically significant at the 5% level. Plants grown under rainfed conditions exhibited a 21% reduction in seed weight compared to those grown under well-irrigated conditions. Camelina plants grown under ACCase-inhibiting herbicide conditions (H_2, H_3, and H_4) had more seed weight compared to other treatments. Assessment of seed yield showed that the mutual effects of irrigation and herbicide on this trait are significant at the 5% level. Plants grown under rainfed conditions had 35% less seed yield compared to well-irrigated conditions (Table 2). The highest seed yield (1389 kg ha^{-1}) was recorded under well-irrigated conditions with the application of pinoxaden. The lowest seed yield was observed under rainfed conditions without the use of herbicide (785 kg ha^{-1}) or with the use of pre-plant herbicide (830 kg ha^{-1}).

Biological yield

The calculation of the biological yield of Camelina indicated that the mutual effects of irrigation and herbicide are statistically significant on this trait at the 1% level. The highest biological yield was recorded with the application of cycloxydim under well-irrigated conditions (4249 kg ha^{-1}). Haloxyfop-r-methyl and pinoxaden herbicides were used in second place and produced the same biological yield. The lowest biological yield (2592 kg ha^{-1}) was recorded under herbicide-free and rainfed conditions (Figure 3). The effect of haloxyfop-r-methyl herbicide on improving biological yield in well-irrigated conditions was more significant than in rainfed conditions.

The interaction effects of I×H were significant at the 5% level. The harvest index under rainfed conditions was 3.54% less than under well-irrigated conditions. The

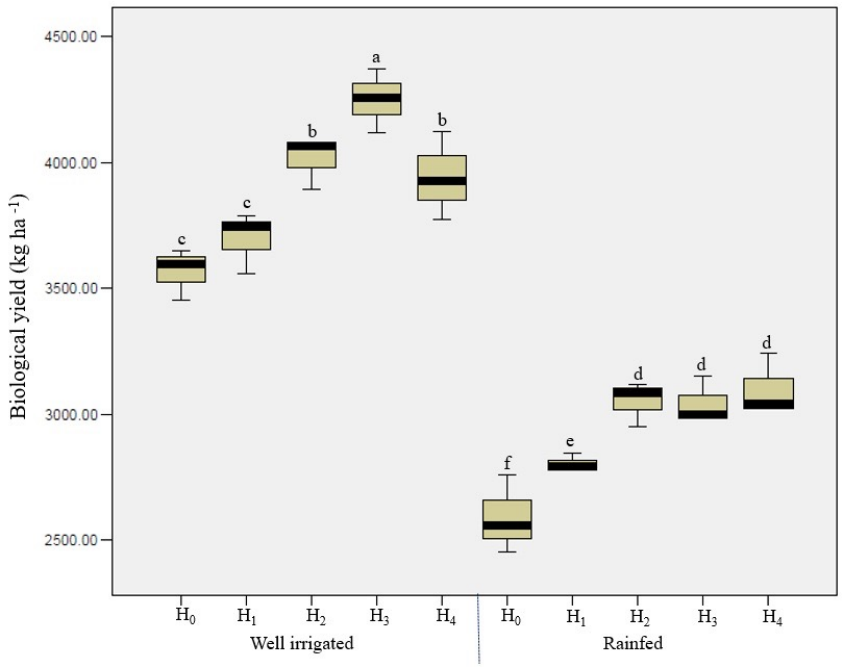


Figure 3. Biological yield of *Camelina sativa* field affected by different herbicides (H_0: no application of herbicide, H_1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-p-methyl, and H_4: pinoxaden as post-emergence) under well-irrigated and rainfed conditions in Maragheh region, northwestern Iran.

Slika 3. Biološki pridelek poljene kamelije (*Camelina sativa*) pod vplivom različnih herbicidov (H_0: brez uporabe herbicida, H_1: trifluralin kot herbicid pred vzklitjem, H_2: cikloksidim, H_3: haloksifop-p-metil in H_4: pinoxaden kot herbicid po vzklitju) v dobro namakanih in deževnih razmerah v regiji Maragheh, severozahodno od Irana.

highest harvest index was recorded under well-irrigated conditions with the use of pinoxaden (35.27), trifluralin (33.17), and without the use of herbicides (32.62). Under rainfed conditions, the lowest harvest index was obtained with the use of chemical herbicides. This indicates that the effect of herbicides on improving vegetative growth and biological yield was more prominent than seed yield. The herbicide-free treatment under both irrigation conditions caused a significant increase in the harvest index (Figure 4).

Cluster analysis and PCA

However, under well-irrigated conditions, application of pinoxaden resulted in the highest HI. The clustering of combined treatments according to the similarity in affecting the studied traits showed that the least effective treatments included no use of herbicide and the application of trifluralin under rainfed conditions (cluster I) and no use of herbicide under well-irrigated conditions (cluster III) (Figure 5). Under well-irrigated conditions, Cycloxydim, Haloxyfop-R-methyl, and pinoxaden (H_4, H_3, and H_2) herbicides were included in cluster II, which were the most effective treatments. However, under rainfed conditions, the effectiveness of all herbicides was almost similar and less than in well-irrigated conditions (cluster IV).

The plots obtained from Principal Component Analysis (PCA) showed that the first component was able to differentiate the soil moisture conditions from each other. The second component also separated the herbicides in terms of effectiveness (Figure 6). The plot obtained from PCA showed that the effectiveness of herbicides under well-irrigated conditions is different from rainfed conditions, and ACCase-inhibiting herbicides were more effective than pre-plant herbicide (Trifluraline). Furthermore, under well-irrigated conditions, the effects of Trifluraline on investigated traits were not prominent when compared with control conditions. Pre-plant herbicide could not affect the weed population under well-irrigated conditions.

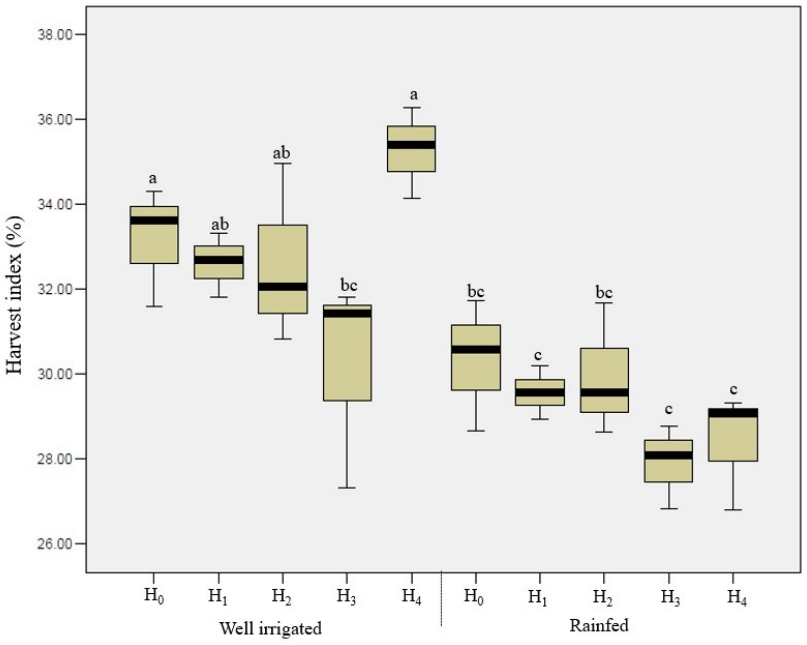


Figure 4. Assessments of harvest index of Camelina under different chemical weed control methods (H_0: no application of herbicide, H_1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-p-methyl, and H_4: pinoxaden as post-emergence) under different soil moisture regimes in the semi-arid region of Mragheh, Iran.

Slika 4. Ocene indeksa pridelka kamelije pri različnih metodah kemičnega zatiranja plevela (H_0: brez uporabe herbicida, H_1: trifluralin kot herbicid pred vzklitjem, H_2: cikloksidim, H_3: haloksifop-p-metil in H_4: pinoxaden kot herbicid po vzklitju) pri različnih vlažnostnih razmerah tal v polsušni regiji Mragheh v Iranu.

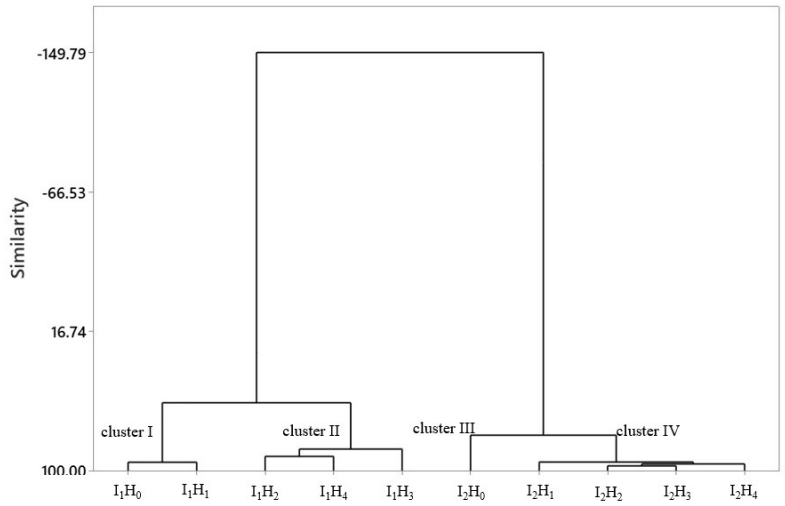


Figure 5. Cluster analysis of combined treatments in terms of similarity in the influence of different evaluated traits of Camelina. I_1: well-irrigated condition, I_2: rainfed condition, H_0: no application of herbicide, H1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-p-methyl, and H_4: pinoxaden as post-emergence.

Slika 5. Klasterna analiza kombiniranih obdelav glede na podobnost vpliva različnih ocenjenih lastnosti Camelina. I_1: dobro namakano stanje, I_2: deževno stanje, H_0: brez uporabe herbicida, H_1: trifluralin kot herbicid pred vzklitjem, H_2: cikloxidim, H_3: haloksifop-p-metil in H_4: pinoxaden kot herbicid po vzklitju.

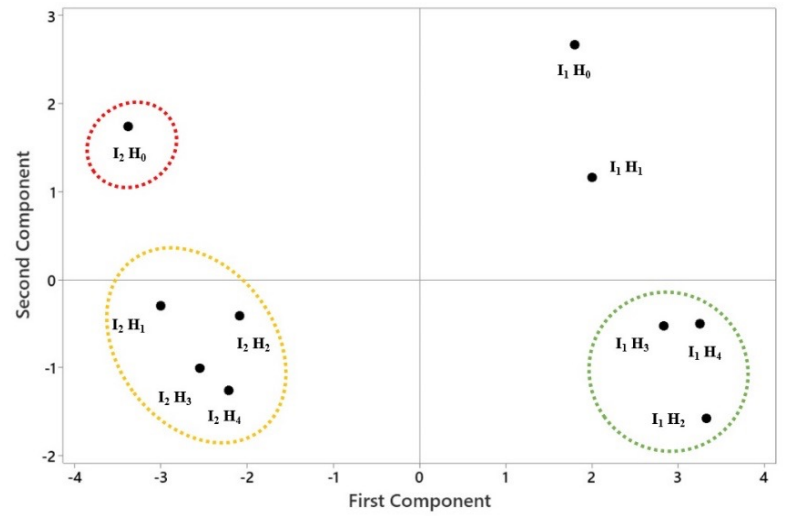


Figure 6. The plot was prepared through principal component analysis (PCA) for classification of combined treatments in terms of superiority in influencing the evaluated traits and less effective treatments in the Camelina field. I_1: well-irrigated condition, I_2: rainfed condition, H0: no application of herbicide, H_1: trifluralin as a pre-emergence herbicide, H_2: cycloxydim, H_3: haloxyfop-p-methyl, and H_4: pinoxaden as post-emergence.

Slika 6. Graf, pripravljen z analizo glavnih komponent (PCA) za razvrstitev kombiniranih obdelav glede na vpliv na ocenjene lastnosti in manj učinkovite obdelave na polju kamelije. I_1: dobro namakano stanje, I_2: deževno stanje, H_0: brez uporabe herbicida, H_1: trifluralin kot herbicid pred vzklitjem, H_2: cikloksidim, H_3: haloksifop-p-metil in H_4: pinoxaden kot herbicid po vzklitju.

Discussion

The results of the experiment indicated that the lack of soil moisture in rainfed conditions reduced biomass and weed density compared to well-irrigated conditions. At the same time, the effectiveness of herbicides also showed a significant decrease in rainfed conditions. The amount of precipitation during the growing season was about 294 mm, while the estimated amount of evaporation and transpiration during the growing season was about 485 mm (Valizadeh 2014). This indicates that if the soil is not accompanied by sufficient amounts of stored moisture in the rainfed system at the beginning of the season, the cultivated plants will face serious water stress. In such conditions, the seed yield components of Camelina can be affected significantly (Neupane et al., 2020).

The reduction of weed population under rainfed conditions is largely due to insufficient soil moisture. However, some weeds such as field bindweed (*Convolvulus arvensis*), wild lettuce (*Lactuca virosa*), wild radish (*Raphanus raphanistrum*), hedge mustard (*Sisymbrium officinale*), shepherd's purse (*Capsella bursa-pastoris*), whitetop (*Lepidium draba*), Baconweed (*Chenopodium album*), common purslane (*Portulaca oleracea*), downy brom (*Bromus tectorum*), foxtail barley (*Hordeum jubatum*), and camelthorn (*Alhagi maurorum*) were able to grow even under water shortage conditions and a major part of the biomass and density was dedicated to these weeds. On the other hand, it seems that under drought stress condition due to the closing of stomata, increased waxing of leaves, morphological changes in leaves, reduction of leaf surface, reduction of stomatal conductance, and probably the metabolism of the main herbicide molecule inside the weed plant, the effectiveness of the herbicide decreased (Peerzada et al., 2021). Also, for the pre-emergence herbicide (trifluralin), it seems that the low soil moisture content restricted the herbicide molecules' movement in the soil and reduced the possibility of reaching the weed seeds in the seed bank. The results showed that the effect of trifluralin on rainfed conditions was disappointing. The use of ACCase-inhibiting herbicides was more effective than trifluralin in both soil moisture environments. This also accords with our earlier observations, which showed that trifluralin was not more effective under well-irrigated conditions (Sabaghnia et al., 2025). Weed control and the reduction of competition for resources such as light, moisture, and food increased Camelina's vegetative growth (plant height and lateral growth) significantly. The use of ACCase-inhibiting herbicides, especially pinoxaden and cycloxydim, increased the chlorophyll content in Camelina. This can be attributed to the reduction of weed shading and the increase of light received by the shoots of the Camelina plant, as well as more access to limited water and nutrient resources in the soil (Formisano et al., 2022).

Investigating the components of vegetative growth and photosynthesising organs (source size) and seed yield components (sink size) in Camelina indicated that the use of herbicide in the current experiment also affected the source-sink relationships. It seems that the use of herbicides, especially ACCase inhibitor herbicides, has increased the production of photoassimilates or their better allocation to reproductive organs through the removal of narrow leaf weeds, reducing competition and increasing access to limited resources. However, in the investigated Camelina variety or the drought stress conditions, it seems that the limitation of the tank was one of the factors limiting the yield. This means that despite the elimination of competition and the increase in vegetative growth and possibly the increase in the supply of photoassimilates, this was not associated with an increase in the number of yield components. In this context, the results of the researchers indicate that in addition to the effect of herbicide use on reducing competition, it can affect the relationship between source and reservoir through the manipulation of carbon and nitrogen metabolism. The use of Imazethapyr herbicides (inhibitor of acetolactate synthase) in lentil genotypes caused a significant change in the synchronisation of carbon and nitrogen metabolism, and this caused a significant increase in the quantity and quality of the seed in some genotypes (Grewal et al., 2023). However, under well-irrigated conditions, both source and sink components showed a significant increase with the application of chemical herbicides. The results indicate that the crop-weed interactions can affect the yield. The presence of weeds through competition can both reduce the size and strength of sources and reduce the size of sinks (Evers and Bastiaans 2016). The obtained results showed that besides improving yield, regular irrigation also improves the efficiency of herbicides. These results confirm the previous reports of Alizadeh et al. (2020). In his research, they found that drought stress reduced the effectiveness of benzoylprop-ethyl herbicide (Suffix: Inhibitor of lipid biosynthesis) in the semi-arid region. On the other hand, it seems that weed exposure to long-term drought may lead to herbicide resistance (HR). HR can be inherited

by the next generations, and it is partially caused by the activation of some defence mechanisms and the scavenging of reactive oxygen species (Mohammad et al., 2022). Among the ACCase inhibitor herbicides, pinoxaden had a greater effect on weed control, which can be attributed to better absorption, greater penetration into the weed shoots, faster translocation and less metabolism inside the weed plant (Pacanoski and Mehmeti, 2023). The reduced effectiveness of ACCase-inhibiting herbicides under certain environmental conditions is primarily attributed to the metabolism and degradation of the herbicides or the conjugation of various compounds with herbicide molecules (Tang et al., 2020).

Conclusions

The results indicated that regular irrigation in the studied area is essential for Camelina production systems to achieve an acceptable seed yield. The Result revealed that under rainfed conditions, water shortage stress significantly decreased the seed yield (33%) in comparison with well-irrigated conditions. Also, soil water regimes affected the efficiency of herbicides. Although weed populations were lower under rainfed conditions than under full irrigation conditions, the herbicide effect was more noticeable under full irrigation conditions. However, the application of pre-planting herbicides under well-irrigated conditions was not very effective. The highest level of weed control was achieved with the use of ACCase inhibitor herbicides (especially cycloxydim and pinoxaden). However, in rainfed conditions, cycloxydim significantly reduced the biomass and population density of weeds. However, the main weed in the field was broad-leaved (field bindweed), and this may require the investigation of the application of narrow and broad-leaved herbicides for weed control in Camelina fields in the semi-arid region.

Author Contributions

Conceptualization, A.E. and M.J.; methodology, M.J. and N.S.; software, M.M. and A.E.; validation, A.E. and N.S.; formal analysis, N.S. and M.M.; investigation, M.J.; resources, A.E.; data curation, M.J and A.E.; writing—original draft preparation, M.J.; writing—review and editing, A.E. and M.M; visualization, A.E.; supervision, N.S. and A.E.; project administration, M.J.; funding acquisition, A.E and M.J. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship.

Acknowledgement

The authors would like to thank the Ministry of Science, Research and Technology for providing some of the research necessities. The cooperation, accuracy and assistance of local workers who helped during sowing, irrigation, and sampling are greatly appreciated. The authors would like to thank the Vice President for Research and Technology.

Funding

This research received no external funding.

Conflicts of Interest

The authors have no conflicts of interest to declare.

References

Alizade, S., Keshtkar, E., Mokhtasi-Bidgoli, A., Sasanfar, H., Streibig, J.C., 2020. Effect of water deficit stress on benzoylprop-ethyl performance and physiological traits of winter wild oat (*Avena sterilis* subsp. ludoviciana). Crop Protection, 137(1), 105292. https://doi.org/10.1016/j.cropro.2020.105292

Borzoo, S., Mohsenzadeh, S., Kahrizi, D., 2021. Water-deficit stress and genotype variation induced alterations in seed characteristics of *Camelina sativa*. Rhizosphere, 20(1), 100427. https://doi.org/10.1016/j.rhisph.2021.100427

Bukhari, M.A., Yousaf, M., Ahmad, Z., Rafay, M., Shah, A.N., Abbas, A., Shah, A.A., Javed, T., Afzal M., Ali S., Abdullah M.I., 2022. Enhancing drought stress tolerance in Camelina (*Camelina sativa* L.) through exogenous application of potassium. Physiologia Plantarum, 174(5), 3779. https://doi.org/10.1111/ppl.13779

Evers, J.B., Bastiaans, L., 2016. Quantifying the effect of crop spatial arrangement on weed suppression using functional-structural plant modelling. Journal of plant research, 129(3), 339-351. https://doi.org/10.1007/s10265-016-0807-2

FAOSTAT. 2024. Information on statistics provided by the Food and Agriculture Organization. http://www.fao.org/faostat/en/#country. (Accessed: 27 July 2024).

Fattahi, M., Janmohammadi, M., Abasi, A., Sabaghnia, N., 2023. The Effects of Farmyard Manure and Nitrogen Fertilizer on the Performance of Safflower. Agrotechniques in Industrial Crops 3(4), 162-169. https://doi.org/10.22126/ATIC.2023.9604.1114

Formisano, L., Miras-Moreno, B., Ciriello, M., Zhang, L., De Pascale, S., Lucini, L., Rouphael, Y., 2022. Between light and shading: morphological, biochemical, and metabolomics insights into the influence of blue photoselective shading on vegetable seedlings. Frontiers in Plant Science, 13, 890830. https://doi.org/10.3389/fpls.2022.890830

Ghidoli, M., Pesenti, M., Colombo, F., Nocito, F.F., Pilu, R., Araniti, F., 2023. *Camelina sativa* (L.) Crantz as a promising cover crop species with allelopathic potential. Agronomy, 13(8), 2187. https://doi.org/10.3390/agronomy13082187

Grewal, S.K., Gill, R.K., Virk, H.K., Bhardwaj, R.D., 2023. Effect of herbicide stress on synchronization of carbon and nitrogen metabolism in lentil (*Lens culinaris* Medik.). Plant Physiology and Biochemistry, 196, 402-414. https://doi.org/10.1016/j.plaphy.2023.01.063

Hazrati, S., Rostami, N., Mohammadi, H., Ebadi, M.T., 2024. Improving the growth parameters, yield, and oil quality of camelina in rainfed farming due to the combined use of biochar and supplementary irrigation. Journal of Agriculture and Food Research, 16, 101160. https://doi.org/10.1016/j.jafr.2024.101160

Hussain, M., Abbas, M.H., Majeed, A., Minhas, W.A., Farooq, S., Jabran, K., 2022. The influence of different row spacing and weed control intervals on weed infestation and yield-related traits of American (*Gossypium hirsutum* L.) and Desi (*Gossypium arboreum*) Cotton. Sustainability, 14(16), 9867. https://doi.org/10.3390/su14169867

Janmohammadi, M., Sabaghnia, N., 2023. Strategies to alleviate the unusual effects of climate change on crop production: a thirsty and warm future, low crop quality. A review. Biologija 69(2): 121-133. https://doi.org/10.6001/biologija.2023.69.2.1

Janmohammadi, M., Kouchakkhani, H., Sabaghnia, N., 2024. The effect of supplemental irrigation and exogenous application of glycine betaine on chickpea performance in the semi-arid region. Journal of Agriculture Faculty of Ege University, 61(2), 189-199. https://doi.org/10.20289/zfdergi.1402726

Jha, P., Stougaard, R.N., 2013. Camelina (*Camelina sativa*) tolerance to selected preemergence herbicides. Weed technology, 27(4), 712-717. https://doi.org/10.1614/WT-D-13-00061.1

Li, S., Du, P., Wu, X., He, H., Zhou, L., Dong, F., et al., 2022. Trifluralin Impacts Soil Microbial Community and Functions. Frontiers in Environmental Science, 10, 813871. https://doi.org/10.3389/fenvs.2022.813871

Martinelli, T., Galasso, I., 2011. Phenological growth stages of *Camelina sativa* according to the extended BBCH scale. Annals of applied Biology, 158(1), 87-94. https://doi.org/10.1111/j.1744-7348.2010.00444.x

Modarres, R., da Silva, V.D., 2007. Rainfall trends in arid and semi-arid regions of Iran. Journal of Arid Environments 70(2), 344-355. https://doi.org/10.1016/j.jaridenv.2006.12.024

Mohammad, V.H., Osborne, C.P., Freckleton, R.P., 2022. Drought exposure leads to rapid acquisition and inheritance of herbicide resistance in the weed *Alopecurus myosuroides*. Ecology and Evolution, 12(2), e8563. https://doi.org/10.1002/ece3.8563

Morante-Carballo, F., Montalván-Burbano, N., Quiñonez-Barzola, X., Jaya-Montalvo, M., Carrión-Mero, P., 2022. What do we know about water scarcity in semi-arid zones? A global analysis and research trends. Water, 14(17), 2685. https://doi.org/10.3390/w14172685

Neupane, D., Solomon, J.K., McLeonn, E., Davison, J., Lawry, T., 2020. Camelina production parameters response to different irrigation regimes. Industrial Crops and Products, 148, 112286. https://doi.org/10.1016/j.indcrop.2020.112286

Obour, A.K., Sintim, H.Y., Obeng, E., Jeliazkov, D.V., 2015. Oilseed camelina (*Camelina sativa* L Crantz): Production systems, prospects and challenges in the USA Great Plains. Advances in Plants & Agriculture Research, 2(2), 00043. https://doi.org/10.15406/apar.2015.02.00043

Pacanoski, Z., Mehmeti, A., 2023. Herbicides weed management in changing environmental conditions. Acta Agriculturae Slovenica, 199(4), 1-11. https://doi.org/10.14720/aas.2023.119.4.12497

Peerzada, A.M., Williams, A., O'Donnell, C., Adkins, S., 2021. Effect of soil moisture regimes on the glyphosate sensitivity and morpho-physiological traits of windmill grass (*Chloris truncata* R. Br.), common sowthistle (*Sonchus oleraceus* L.), and flaxleaf fleabane [*Conyza bonariensis* (L.) Cronq.]. Plants, 10(11), 2345. https://doi.org/10.3390/plants10112345

Rostami Ahmadvandi, H., Kahrizi, D., Ghobadi, R., Abadi, A., 2021. Camelina, a unique oilseed with high tolerance to drought and cold. Oilseed Plants, 2(2), 63-73. https://doi.org/10.22126/atic.2021.6410.1007

Sabaghnia, N., Janmohammadi, M., Nasiri, Y., Nouraein, M., 2025. Assessment of some herbicide effects on the weed control and agronomic traits of *Camelina sativa* under irrigation and rainfed conditions. Journal of Plant Protection Research, 9998.

Shimi, P., Haghigi, A., Ebtali, Y., Pourazar, R., Jamali, M., Nouralizadeh, M., 2014. Investigating combination of herbicides in canola for better control of weeds, with special reference to brassica weeds. Iranian Journal of Weed Science, 10(1), 21-31.

Sobiech, Ł., Grzanka, M., Kurasiak-Popowska, D., Radzikowska, D., 2020. Phytotoxic effect of herbicides on various camelina [*Camelina sativa* (L.) Crantz] genotypes and plant chlorophyll fluorescence. Agriculture, 10(5), 185. https://doi.org/10.3390/agriculture10050185

Tang, W., Yu, X., Chen, J., Xie, L., Lu, Y., 2020. Tolerance to some ACCase inhibitors in four common Roegneria (*Roegneria kamoji*) populations from China. Frontiers in Agronomy, 2, 587651. https://doi.org/10.3389/fagro.2020.587651

Ullah, R., Aslam, Z., Maitah, M., Zaman, Q. U., Bashir, S., Hassan, W., Chen, Z., 2020. Sustainable weed control and enhancing nutrient use efficiency in crops through Brassica (*Brassica compestris* L.) allelopathy. Sustainability, 12(14), 5763. https://doi.org/10.3390/su12145763

Valizadeh, K., 2014. Estimation of potential evapotranspiration with Estefnz method and GIS techniques in eastern Azerbaijan. Journal of Geography and Planning, 18(49), 317-334.

ABS

Original Research

Umetne vodne akumulacije za zasneževanje na vzhodu Pohorja (Slovenija): mesta razmnoževanja in ekološke pasti za dvoživke

Tina Klenovšek [1,*], Žiga Tertinek [2], Ana Skledar [2]

Izvleček

Umetni vodni habitati so lahko za dvoživke mesta za razmnoževanje kot tudi ekološke pasti. Vodne akumulacije za umetno zasneževanje niso optimalni habitati za dvoživke in o njihovem razmnoževanju v teh vodnih telesih je malo znanega. V raziskavi smo dvoživke vzorčili v dveh akumulacijah za umetno zasneževanje na območju Mariborskega Pohorja. Ena se nahaja ob vznožju Pohorja (312 m nmv) v urbanem okolju, druga pa na pobočju Pohorja (560 m nmv), obdana z gozdom, ki je del Natura 2000 območja Pohorje. V akumulacijah smo skupno potrdili razmnoževanje sedmih vrst dvoživk: velikega pupka (*Triturus carnifex*), planinskega pupka (*Ichthyosaura alpestris*), navadnega pupka (*Lissotriton vulgaris*), hribskega urha (*Bombina variegata*), navadne krastače (*Bufo bufo*), sekulje (*Rana temporaria*) in rosnice (*Rana dalmatina*). Hribski urh in veliki pupek spadata med kvalifikacijske vrste za Natura 2000 območje Pohorje. V akumulaciji ob vznožju Pohorja, v kateri so bile tudi ribe, razmnoževanje dvoživk ni bilo uspešno. V akumulaciji na pobočju Pohorja, kjer rib ni bilo, sta strma brežina (30–60°) iz nepokrite geomembrane in nizka gladina vode povzročili pogin odraslih hribskih urhov in številnih (> 500) mladih osebkov rjavih žab. Upad gladine vode je povzročil tudi propad jajc in zarodkov velikega pupka. Akumulaciji za zasneževanje sta iz več vidikov neprimerni za dvoživke, vendar jih te kljub temu uporabljajo za razmnoževanje. Za zmanjšanje poginov dvoživk bi bili potrebni naslednji ukrepi: ohranjanje maksimalnega vodostaja v času razmnoževanja (marec–julij), prekritje geomembrane z nedrsečim materialom in zmanjšanje naklona brežine akumulacije, kar bi dvoživkam olajšalo izhod iz akumulacije, odstranitev rib z jesenskim praznjenjem akumulacije ter preprečevanje povozov na cestah ob akumulaciji.

Keywords

Bombina variegata, dvoživke, ekološka past, Natura 2000, *Triturus carnifex*, umetni habitat, vodna zajetja, zadrževalniki za umetno zasneževanje

1 Fakulteta za naravoslovje in matematiko, Univerza v Mariboru, Koroška cesta 160, 2000 Maribor, Slovenija

2 Naravovarstveni inštitut Vivus, Čretniška ulica 18a, 2319 Poljčane

*** Corresponding author:**
E-mail address: tina.klenovsek@um.si

Citation: Klenovšek, T., Tertinek, Ž., Skledar, A., (2025). Umetne vodne akumulacije za zasneževanje na vzhodu Pohorja (Slovenija): mesta razmnoževanja in ekološke pasti za dvoživke. Acta Biologica Slovenica 68 (4)

Received: 03.03.2025 / **Accepted:** 14.07.2025 / **Published:** 21.07.2025

https://doi.org/10.14720/abs.68.4.21999

Artificial Water Accumulations for Snowmaking on Eastern Pohorje (Slovenia): Breeding Sites and Ecological Traps for Amphibians

Abstract

Artificial water habitats can be breeding sites for amphibians, but may also function as ecological traps. Water accumulations for artificial snowmaking are not optimal habitats for amphibians, and little is known about their reproduction in these water bodies. In this study, we sampled amphibians in two accumulations for snowmaking in the Mariborsko Pohorje area. One is located at the foothills of Pohorje (312 m a.s.l.) in an urban environment, and the other on the slopes of Pohorje (560 m a.s.l.), surrounded by forest that is part of the Natura 2000 Pohorje. In total, we confirmed the reproduction of seven amphibian species in these accumulations: Italian Crested Newt (*Triturus carnifex*), Alpine Newt (*Ichthyosaura alpestris*), Smooth Newt (*Lissotriton vulgaris*), Yellow-bellied Toad (*Bombina variegata*), Common Toad (*Bufo bufo*), Common Frog (*Rana temporaria*), and Agile Frog (*Rana dalmatina*). The Yellow-bellied Toad and the Italian Crested Newt are qualifying species for the Natura 2000 Pohorje site. In the accumulation at the foothill of Pohorje, where fish were present, amphibian reproduction was unsuccessful. In the accumulation on the slope of Pohorje, where there were no fish, the steep banks (30–60°) with uncovered geomembrane and the low water level led to the mortality of adult Yellow-bellied Toads and numerous (>500) juvenile *Rana* sp. frogs. The drop in water level also resulted in the stranding of eggs and embryos of the Italian Crested Newt. Snowmaking reservoirs are unsuitable for amphibians in several respects, yet they still use them for breeding. To reduce amphibian mortality, the following measures should be implemented: maintaining maximum water levels during the breeding season (March–July), covering the geomembrane with non-slip material and reducing the bank steepness to facilitate amphibian exit, removing fish through autumn reservoir drainage, and preventing roadkill on roads adjacent to the reservoirs.

Keywords

Amphibians, artificial habitat, *Bombina variegata*, ecological trap, Natura 2000, snowmaking ponds, *Triturus carnifex*, water reservoirs

Uvod

Dvoživke za svoj življenjski cikel potrebujejo tako kopenske kot vodne habitate. Prav habitati celinskih voda so med najbolj ogroženimi v Evropi (Janssen in sod., 2016). Degradacija in izguba habitatov sta med glavnimi razlogi za upad populacij dvoživk, tako je po podatkih IUCN v Evropi ogrožena skoraj četrtina dvoživk (Temple in Cox, 2009; IUCN, 2025). V Sloveniji živi 21 vrst dvoživk (Stanković in sod., 2015; Strah in sod., 2024), kar jo uvršča med bolj pestra območja Evrope glede na število vrst dvoživk (Temple in Cox, 2009). Dvoživke v Sloveniji ogrožajo predvsem izguba kakovostnih habitatov, ki jo povzročajo nenadzorovana in razpršena urbanizacije, regulacija in hidromorfološke spremembe vodotokov in obvodnih zemljišč, onesnaževanje z organskimi snovmi in gnojili, vnos tujerodnih rib, izginjanje mlak ter izsuševanja mokrišč (Stanković in sod., 2015).

Dvoživke za razmnoževanje uporabljajo tudi vodna telesa umetnega nastanka (Clevenot in sod., 2018; Valdez in sod., 2021), tj. telesa površinskih vod, ki so nastala zaradi posega v prostor (Ur. l. RS., 2002). Takšna vodna telesa so za dvoživke sekundarni vodni habitati, kjer je večinoma manjša biotska pestrost kot v primerljivih naravnih vodnih habitatih (Zamora- Marín in sod. 2021). Kljub temu lahko na zelo prepustnih tleh, sušnih ali močno spremenjenih območjih prevladujejo in imajo ključno vlogo za razmnoževanje ter obstoj populacij dvoživk (Brand in Snodgrass, 2010; Buono in sod., 2019; Lešnik, 2021; Zamora- Marín in sod., 2021; Caballero-Díaz in sod., 2022). Umetni vodni habitati lahko zaradi svojih lastnosti in namenske rabe delujejo tudi kot ekološke pasti (Dwernychuk in Boag, 1972), tj. kadar jih živali na podlagi okoljskih signalov ocenijo kot ustrezne in prednostno izberejo, čeprav imajo v primerjavi z drugimi razpoložljivimi habitati v njih manjše možnosti

za preživetje ter uspešno razmnoževanje (Robertson in Hutto, 2006; Clevenot in sod., 2018). Umetni vodni habitat lahko predstavlja ekološko past tudi, kadar povzroča neposredno smrtnost osebkov ali če kot razmnoževalno mesto ne omogoča zadostnega razmnoževalnega uspeha za ohranjanje stabilne ali rastoče populacije brez priseljevanja (Battin, 2004).

Primer umetnih vodnih habitatov za razmnoževanje dvoživk so vodne akumulacije namenjene umetnemu zasneževanju smučišč. Zaradi namenske rabe imajo te akumulacije podobne značilnosti. Med drugim, da so umetnega nastanka, značilne oblike, s strmimi in ravnimi brežinami prekritimi s hidroizolacijsko folijo, povsem brez vodne vegetacije, raven vode pa v njih hitro in močno niha (Fait in sod., 2020; Gerfand in sod., 2024). Zadrževalniki voda niso optimalni umetni vodni habitati za dvoživke, a so lahko edini vodni habitati, ki zadržujejo vodo dovolj dolgo, da ličinke zaključijo razvoj (Brand in Snodgrass, 2010). Redno vzdrževanje, tj. letno praznjenje in čiščenje akumulacij ob ustreznem času, preprečuje obstoj rib, kar je prav tako ugodno za dvoživke (McCarthy in Lathrop, 2011; Zamora- Marín in sod., 2021). Za razliko od zadrževalnikov meteornih voda v urbanih okoljih ali ob avtocestah (pregled v Clevenot in sod., 2018), je podatkov o dvoživkah v akumulacijah za zasneževanje zelo malo, četudi število takšnih akumulacij raste (Gerfand in sod., 2024). Gerfand in sod. (2024) so ocenili ekološko kakovost 28 akumulacij za umetno zasneževanje v francoskih Alpah. Dvoživke so našli v 68 % vseh akumulacij in akumulacije zaradi zasnove ter načina uporabe ocenili kot potencialne ekološke pasti.

Akumulacije za umetno zasneževanje so tudi na delih Pohorja, kjer je razvit smučarski turizem. Pohorje je predalpsko hribovje, ki se razteza 50 km v dolžino v smeri vzhod-zahod. Na vzhodu prehaja v Dravsko dolino in mesto Maribor. Na Mariborskem Pohorju sta dve vodni zajetji za zasneževanje smučišč. Akumulacija Arena je ob vznožju in akumulacija Trikotna jasa na pobočju Pohorja. Za namen predvidene razširitve in posodobitve zimsko športnega centra Pohorje so na vplivnem območju Govedič in sod. (2009) popisali 130 lokacij in potrdili 5 vrst dvoživk, od do takrat znanih 10 vrst dvoživk za Pohorje in vznožje Mariborskega Pohorja med Radvanjem in Razvanjem (Vogrin, 1998; Poboljšaj, 1998; Poboljšaj in sod., 2001; Cipot, 2007). V akumulaciji Trikotna jasa so bili takrat najdeni samo paglavci sekulje (*Rana temporaria*), medtem ko v akumulaciji Arena dvoživk niso zabeležili. V akumulaciji Arena je bilo kasneje potrjeno razmnoževanje navadne krastače (*Bufo bufo*) in sekulje (Tertinek in Skledar, 2022; Tertinek, 2024). Akumulacija Trikotna jasa je na ekološko pomembnem območju Pohorje (ID 41200). Strnjen gozd južno in vzhodno od akumulacije pa je del Natura 2000 območja Pohorje (ID 3000270), v katerem sta med kvalifikacijskimi vrstami dvoživk hribski urh (*Bombina variegata*) in veliki pupek (*Triturus carnifex*) (Ur. l. RS 49/2004, Ur. l. RS 110/2004, Ur. l. RS 47/18). Vrsti sta zaradi svojega pomena relativno dobro raziskani, monitoring se je izvajal tudi na Pohorju (Poboljšaj in sod., 2011; Cipot in sod., 2011; Grudnik in Triglav Brežnik, 2015).

Na območju akumulacij za zasneževanje na Mariborskem Pohorju se stikajo strnjen gozd, spremenjeno površje za namen športa in turizma ter urbano naselje. Na pobočjih Pohorja so naravne stoječe vode redke, potoki pa imajo hudourniški značaj, zato so umetne stoječe vode, kot so akumulacije za zasneževanje, lahko pomemben sekundarni habitati za razmnoževanje dvoživk. Vendar pa lahko akumulacije za zasneževanje, zaradi svojih značilnosti in rabe, dvoživkam hkrati predstavljajo grožnjo za preživetje in razmnoževalni uspeh. V letu 2024 je bil na območju akumulacije Arena izveden monitoring pomladanskih selitev dvoživk po naročilu Mestne občine Maribor (Tertinek, 2024). Neodvisno so bili istega leta izvedeni tudi popisi dvoživk v akumulaciji na Trikotni jasi. Namen raziskave akumulacij za zasneževanje na Mariborskem Pohorju je bil (1) ugotoviti, katere vrste dvoživke so prisotne v akumulacijah in ali se tam tudi razmnožujejo, (2) opisati značilnosti akumulacij, ki lahko vplivajo na preživetje in razmnoževanje dvoživk v vodnih telesih, (3) oceniti vpliv teh značilnosti akumulacij na dvoživke in njihov potencial, da delujejo kot ekološke pasti, ter (4) predlagati ukrepe za izboljšanje značilnosti akumulacij z vidika vpliva na preživetje in razmnoževanje dvoživk. Pričakujemo, da bomo v akumulacijah našli več vrst dvoživk, kot jih je bilo zabeleženih do sedaj (do sedaj sta bili zabeleženi le navadna krastača in sekulja), ter da bomo potrdili njihovo razmnoževanje. Ker pa akumulacije niso optimalni habitati za dvoživke in globoke stoječe vode brez vegetacije ne ustrezajo vsem vrstam, pričakujemo, da bo število vrst kljub temu majhno. Glede na specifične značilnosti in namensko rabo, predvidevamo, da so akumulacije potencialne ekološke pasti za dvoživke, a lahko z ukrepi za izboljšanje postanejo ustrezni sekundarni habitati za razmnoževanje dvoživk. Raziskava je tudi prispevek k boljšemu razumevanju vloge umetnih vodnih teles pri ohranjanju dvoživk v preoblikovanih gozdnih ekosistemih.

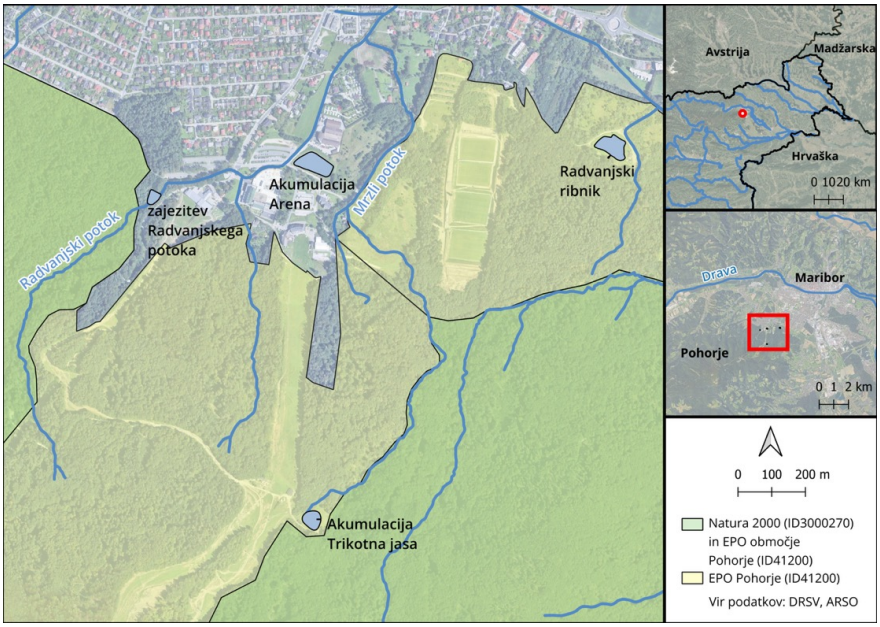
Material in metode

Akumulaciji za zasneževanje na Mariborskem Pohorju

Akumulacija Arena in Trikotna jasa (Sl. 1) sta nastali s posegom v prostor z namenom zajemanja vode za umetno zasneževanje smučišč na Mariborskem Pohorju. Akumulacija Arena je umeščena ob vznožju Pohorja v Zgornjem Radvanju, na nadmorski višini 312 m, medtem ko se akumulacija ob Trikotni jasi nahaja na pobočju Pohorja na nadmorski višini 560 m. Med akumulacijama je 1000 m zračne razdalje. Večji stalni stoječi vodi v bližini akumulacij sta Radvanjski ribnik in zajezitev Radvanjskega potoka, obe na podobni nadmorski višini kot akumulacija Arena (Sl. 1). Radvanjski ribnik je od akumulacije Arena oddaljen 780 m in od akumulacije Trikotna jasa 1350 m zračne razdalje, zajezitev Radvanjskega potoka pa 420 m oziroma 1000 m zračne razdalje.

Na podlagi podatkov o povprečni letni količini padavin za obdobje 1981–2010 leži akumulacija Arena na območju s 1100–1200 mm padavin letno, akumulacija Trikotna jasa pa na območju s 1200–1300 mm padavin letno. Obe vodni telesi pripadata območju s povprečno letno temperaturo 8–10 °C, prav tako merjeno za obdobje 1981–2010 (ARSO, 2021).

V gradbeni dokumentaciji (Akumulacija za zasneževanje Arena (št.2998/07), 2007) je kot leto izgradnje akumulacije Arena navedeno leto 1989. Od takrat je bila akumulacija vsaj štirikrat povečana in obnovljena. Njena globina znaša 5,9 m, površina ojezeritve 5400 m^2, prostornina pa 21.455 m^3. Vtok v akumulacijo Arena je iz Radvanjskega potoka. Naklon brežine je 1:2 ali 26,6°. Iz te akumulacije se voda prek cevovoda prečrpava v akumulacijo Trikotna jasa ali se iz akumulacije direktno zasnežuje območje snežnega stadiona. Gradnja akumulacije ob Trikotni jasi se je začela leta 1992 in zaključila 1994 (Sanacija zadrževalnika ob Trikotni jasi (št. 18/95), 1995). Njena globina znaša 5,1 m,



Slika 1. Lokacija akumulacij za umetno zasneževanje Arena in Trikotna jasa na Mariborskem Pohorju z bližnjimi vodnimi telesi ter naravovarstvenimi območji (levo). Desno: Lega akumulacij v severovzhodni Sloveniji (rdeča točka) in na območju vzhodnega Pohorja z mestom Maribor (rdeč okvir). (Vir: ARSO, 2021; DRSV, 2024. Oblikovanje in kartografija: Žiga Tertinek)

Figure 1. Location of snowmaking accumulations, Arena and Trikotna jasa on Mariborsko Pohorje with proximal water bodies and nature conservation areas (left). Right: The location of the accumulations in northeastern Slovenia (red dot) and within the area of eastern Pohorje with the city of Maribor (red frame). (Source: ARSO, 2021; DRSV, 2024. Design and cartography: Žiga Tertinek)

površina ojezeritve 2450 m^2 (ARSO, 2021), prostornina pa 5643 m^3. Vtok v akumulacijo je iz Mrzlega potoka na zahodni in potoka na vzhodni strani. Naklon brežine je od 1,9 do 1:1,5 ali 27,7°-33,7°. Akumulacijski bazen obeh vodnih teles je tesnjen s hrapavo polietilensko hidroizolacijsko folijo (geomembrano) iz PEHD, ki je položena na geotekstil in popolnoma neprepustna za vodo (Sanacija zadrževalnika ob Trikotni jasi (št. 18/95), 1995; Akumulacija za zasneževanje Arena (št. 2998/07), 2007). Geomembrana je črne barve in ni prekrita (z npr. kamni, gramozom, peskom, rastlinami). Ker je geomembrana drseča, so vzdolž brežin obeh akumulacijskih bazenov nameščene lestve iz gumijastih obročev, namenjene izhodu divjadi. V akumulaciji Arena sta nameščeni dve lestvi in v akumulaciji Trikotna jasa pet. Splošne značilnosti akumulacij so povzete v Tab. 2.

Akumulacija Trikotna jasa z okolico je del ekološko pomembnega območja Pohorje (EPO Pohorje ID 41200). Strnjen gozd južno in vzhodno je Natura 2000 območje Pohorje (ID 3000270) (Sl. 1). Zaradi bližine Natura 2000 (18 m) je akumulacija del vplivnega območja za kvalifikacijski vrsti hribski urh in veliki pupek (ZRSVN, 2021; Ur. l. RS 49/2004 in 110/2004).

Popisi dvoživk

Terensko delo je vključevalo popis dvoživk v akumulacijah Arena in Trikotna jasa. V akumulaciji Arena so bili marca in maja 2024 izvedeni štirje popisi dvoživk v okviru monitoringa pomladanske selitve dvoživk po naročilu Mestne občine Maribor (Tertinek, 2024). V akumulaciji Trikotna jasa je bilo med marcem in oktobrom 2024 izvedenih devet popisov. Datumi in ure terenskih obiskov so navedeni v Tab. S1. Dvoživke smo v akumulacijah iskali predvsem z opazovanjem med počasno hojo po nasipu zajetja. Zaradi strmih in gladkih brežin, prekritih z geomembrano, je bil dostop do vode mogoč le na mestih, kjer so bile vzdolž brežine nameščene lestve iz gum ali pa je gladina segala do travnatega dela brežine. Na teh mestih smo lovili z vodnimi mrežami ali z roko. Urhe smo beležili tudi s poslušanjem oglašajočih se samcev. S predvajanjem oglašanja smo izzivali samce zelene rege. Dokumentirali smo prisotnost posameznih vrst ali taksonov. Za določanje smo uporabili dihotomni ključ Veenvliet in Kus Veenvliet (2008) ter slikovni ključ Speybroeck in sod. (2016). Ujetim osebkom smo določili spol in jih razvrstili v starostne skupine: odrasli oz. adultni osebki, spolno nezreli oz. subadultni osebki (starejši od enega leta), mladi oz. juvenilni osebki (v letu po preobrazbi) in ličinke. Paglavce in ličinke smo določali s pomočjo ročnih lup že na terenu. Mreste žab in jajca pupkov smo določili do najnižjega možnega taksona. Število osebkov ali jajc posameznih vrst oziroma taksonov dvoživk smo na dan popisa ocenili z vizualnim pregledom in štetjem med obhodom akumulacije. Zaradi odsotnosti vegetacije je bilo mreste in dvoživke ob dobri vidljivosti razmeroma enostavno opaziti. Razmnoževanje v vodnih akumulacijah smo za posamezne vrste potrdili s prisotnostjo ampleksusa, jajc, mrestov, ličink ali pravkar preobraženih osebkov. Načrtno nismo ugotavljali uspešnosti razmnoževanja z iskanjem juvenilnih in subadultnih osebkov na kopnem, zabeležili smo le preobražene osebke v vodi ali neposredno ob vodi, tj. na brežini akumulacije.

Po opravljenih meritvah in določitvi vrst smo vse ujete osebke, brez poškodb, vrnili na prvotno mesto najdbe. Vse ugotovitve smo dokumentirali, vključno s fotografijami ali video posnetki dvoživk kot tudi obeh akumulacij. Terensko delo je potekalo z minimalno invazivnimi postopki in v skladu z dovoljenjem št. 35606-5/2023-2550-5 z dne 28. 3. 2023 izdanim s strani Ministrstva za naravne vire in prostor.

Kopnega izven nasipa akumulacije nismo načrtno pregledovali. Zabeležili smo prisotnost drugih vretenčarjev v akumulaciji. Vodnih nevretenčarjev nismo vzorčili, a smo zabeležili prisotnost vrst, ki smo jih opazili s prostim očesom.

Značilnosti akumulacij za zasneževanje, pomembne za dvoživke

V letu 2024 smo za obe akumulaciji popisali dejavnike oziroma značilnosti (Tab. 2), ki lahko vplivajo na preživetje in razmnoževanje dvoživk v vodnih telesih. Te smo povzeli iz splošne literature o dvoživkah (Veenvliet in Kus Veenvliet, 2008; Speybroeck in sod., 2016), iz raziskav o dvoživkah v umetnih akumulacijah, predvsem v zadrževalnikih meteornih vod in akumulacijah za zasneževanje (Brand in Snodgrass, 2010; McCarthy in Lathrop, 2011; Clevenot in sod., 2018; Fait in sod., 2020; Mathwin in sod., 2021; Gerfand in sod., 2024) ter druge literature o dejavnikih ogrožanja dvoživk (Stanković in sod., 2015; Poboljšaj in sod., 2018). Pri popisu dejavnikov se nismo osredotočili na specifične vrste dvoživk, četudi lahko isti dejavniki na različne vrste vplivajo različno (Clevenot in sod., 2018). Podatke o akumulacijah, upravljanju in okolici smo pridobili iz gradbene dokumentacije akumulacij, od upravljalca (Marprom, ustno), iz Naravovarstvenega atlasa (ZRSVN, 2021), Atlasa voda (DRSV, 2024) in meritev oziroma opazovanj na terenu med

popisi dvoživk. Med terenskimi obiski smo v akumulaciji preverjali prisotnost/odsotnost: vode, plitvin (delov z globino vode do 30 cm), vodne vegetacije in drugih naravnih (npr. kamni, veje) ali umetnih (npr. cevi, večji odpadki) skrivališč v vodi, osončenosti gladine ter drugih vretenčarjev. Pregledali smo brežino in nasip akumulacije za prisotnost lesne vegetacije. Pregledali smo neposredno okolico akumulacije (do 50 m oddaljenosti) in zabeležili vodne habitate (stoječe vode, tekoče vode), kopenske habitate (travnik, gozd) ter ceste. Ob maksimalni napolnjenosti bazena, ki je določena z iztokom iz akumulacije, smo določili maksimalno višino vode. Na dneve popisov, ko so bile v akumulaciji prisotne dvoživke, smo z merilnim trakom (± 1 cm) izmerili morebiten upad vode od maksimalne višine vode in tako dobili oceno o nihanju gladine. Vsakič smo pregledali tudi celotno brežino akumulacije in preverili morebitno prisotnost suhega pasu geomembrane, tj. geomembrane, ki je nad gladino vode. Morebiten suh pas geomembrane smo na več mestih izmerili z merilnim trakom (± 1 cm) in tako dobili oceno širine suhega pasu geomembrane, t. razdaljo med gladino vode v akumulaciji in med travnatim delom brežine. Vsakič smo s kotomerom na več mestih izmerili tudi naklon brežine v območju suhega pasu geomembrane. Podatke o letnem praznjenju in čiščenju akumulacije smo dobili od upravljalca. Najkrajšo zračno oddaljenost cest, gozda in drugih naravnih habitatov od najbližjega roba akumulacije smo izmerili na zemljevidu območja (ZRSVN, 2021).

Rezultati

Dvoživke v akumulacijah

V letu 2024 smo v dveh akumulacijah na Mariborskem Pohorju popisali osem vrst dvoživk: navadnega močerada (Salamandra salamandra), velikega pupka (Triturus carnifex), planinskega pupka (Ichthyosaura alpestris), navadnega pupka (Lissotriton vulgaris), hribskega urha (Bombina variegata), navadno krastačo (Bufo bufo), sekuljo (Rana temporaria) in rosnico (Rana dalmatina) (Tab. 1). V akumulaciji Arena so bile dvoživke zabeležene samo na enem od štirih izvedenih popisov (Tab. S1). Najdeni so bili mresti sekulje in mresti ter paglavci navadne krastače. V maju v akumulaciji ni bilo ličink ali v neposredni bližini preobraženih osebkov, zato uspešnega razmnoževanja nismo potrdili. V akumulaciji Trikotna jasa so bile dvoživke zabeležene na vseh popisih od marca do oktobra (Tab. S1). Razmnoževanje je bilo potrjeno za velikega pupka, planinskega pupka, navadnega pupka, hribskega urha, sekuljo in rosnico.

Močerad in pupki so bili najdeni zgolj v akumulaciji Trikotna jasa (Tab. 1, Tab. S1). Navadni močerad je bil zaznan le kot kadaver v oktobrskem popisu. Odrasle pupke smo opazili med dviganjem proti gladini po zrak in ležanjem na podlagi v plitvi vodi. Zaradi omejene dostopnosti in slabše vidljivosti, večine nismo identificirali do vrste oziroma spola. Potrdili smo samo oba spola velikega pupka. Od jajc

Tabela 1. Dvoživke v akumulacijah Arena in Trikotna jasa na Mariborskem Pohorju. Največje število primerkov (glede na takson in razvojni stadij) na posamezen popis: * < 5 primerkov, ** 5–10 primerkov, *** > 10 in < 30 primerkov, **** > 30 primerkov

Table 1. Amphibians in the snowmaking accumulations, Arena and Trikotna jasa at Mariborsko Pohorje. Maximum number of specimens (by taxon and developmental stage) per visit: * < 5 specimens, ** 5–10 specimens, *** > 10 and < 30 specimens, **** > 30 specimens

	Akumulacija Arena		Akumulacija Trikotna jasa			
Name of the Bacterial species	**mrest**	**ličinke**	**jajca/mrest**	**ličinke**	**mladi**	**odrasli**
navadni močerad, *Salamandra salamandra*						*
veliki pupek, *Triturus carnifex*			***			**
planinski pupek, *Ichthyosaura alpestris*				****		
navadni pupek, *Lissotriton vulgaris*				*		
pupki nedoločeni				****		***
hribski urh, *Bombina variegata*						*
navadna krastača, *Bufo bufo*	****	****				
sekulja, *Rana temporaria*	***		****	****		****
rosnica, *Rana dalmatina*			**			*
Rana sp. nedoločeni			****	****	****	****

pupkov smo opazili samo jajca in nekaj dni stare zarodke velikega pupka, ki so bili prilepljeni ter zaviti v odpadnem kosu papirja, ki je ležal v vodi med gumami (Sl. 2). Do naslednjega popisa je gladina vode toliko upadla, da je papir z jajci ostal na suhem. Ličink velikega pupka nismo našli. Ličinke planinskega pupka so bile številčne in v juliju potrjene na vseh popisih, medtem ko so bile ličinke navadnega pupka najdene samo na enem popisu (Tab. 1, Tab. S1). Navadna krastača je bila najdena samo v akumulaciji Arena, kjer smo marca zabeležili mreste in ličinke. Hribski urh je bil najden samo v akumulaciji Trikotna jasa. Najdeni so bili samo odrasli osebki in to v juniju ter juliju (Tab. S1). Opaženi so bili samci, ki so lebdeli na sredini akumulacije in se oglašali (največ 2 na posamezen popis). V začetku julija je bil, razen posameznih samcev, pod gumami v vodi najden tudi ampleksus. Teden kasneje so bili v vodi tik ob brežini, na treh različnih mestih, hkrati najdeni 3 mrtvi odrasli hribski urhi (Sl. 3). Med popisi v bližini akumulacije nismo opazili manjših začasnih stoječih vod (v jarkih, kolesnicah) ali luž, v katerih bi se lahko zadrževali. Marca so bili v akumulaciji Arena mresti sekulje (Tab. S1), vendar paglavcev ali preobraženih osebkov ni bilo najdenih v nobenem od popisov. V akumulaciji Trikotna jasa so bili marca prisotni mresti sekulje in rosnice kot tudi odrasli osebki obeh vrst, pri čemer so mresti sekulje prevladovali. Konec junija so bili najdeni tako paglavci kot pravkar preobraženi osebki rjavih žab (Tab. S1). V začetku julija so množično migrirali na kopno, pri tem pa se prilepili na geomembrano in poginili. Pojav smo opazovali kljub dopoldanski uri vzorčenja (8–10h). Na dan vzorčenja 5. 7. 2024 smo na geomembrani brežine prešteli okoli 500 mrtvih pravkar preobraženih osebkov (Sl. 3). Nekaj kadavrov mladih osebkov je bilo tudi v vodi (< 5). Marca in oktobra so bili ob robu v vodi tudi posamezni kadavri odraslih rjavih žab.

Značilnosti akumulacij za zasneževanje, pomembne za dvoživke

Za akumulaciji Arena in Trikotna jasa smo v letu 2024 popisali dejavnike oziroma značilnosti vodnih teles (Tab. 2), ki so glede na literaturo pomembne za preživetje in razmnoževanje dvoživk v vodnih telesih.

Slika 2. Odpadni papir v gumah v akumulaciji Trikotna jasa na Mariborskem Pohorju (levo), v katerega so bila zavita jajca velikega pupka (*Triturus carnifex*) (desno). (Fotografiji: Tina Klenovšek (levo) in Ana Skledar (desno)).

Figure 2. Waste paper within tires in the Trikotna jasa accumulation at Mariborsko Pohorje (left), in which the eggs of the Italian Crested Newt (*Triturus carnifex*) were wrapped (right). (Photos: Tina Klenovšek (left), Ana Skledar (right))

Tabela 2. Značilnosti akumulacij za umetno zasneževanje Arena in Trikotna jasa na Mariborskem Pohorju.

Table 2. Characteristics of the snowmaking accumulations Arena and Trikotna jasa at Mariborsko Pohorje.

Splošne značilnosti akumulacij	**Akumulacija Arena**	**Akumulacija Trikotna jasa**
lokacija	46.53396, 15.60361	46.52448, 15.60344
nadmorska višina	312 m	560 m
leto izgradnje	1989	1994
globina	5,9 m	5,1 m
površina ojezeritve	5400 m^2	2450 m^2
prostornina	21.455 m^3	5643 m^3
naklon brežine	1:2 ali 26,6˚	1:1,9–1:1,5 ali 27,7˚–33,7˚
material obloge ojezeritve	geomembrana PEHD	geomembrana PEHD
Značilnosti akumulacij pomembnih za dvoživke		
stalna voda v času razmnoževanja	da	da
nihanje gladine vode	do 30 cm	do 100 cm
plitvine (< 30 cm globine)	da	da
vodna vegetacija	ne	ne
druga naravna skrivališča v vodi	ne	ne
umetna skrivališča v vodi	lestvi iz gum	lestve iz gum
osončenost vode (senčenje)	da (brez senčenja)	da (senčenje bližnjega gozda)
ribe	sončni ostriži, krapovci	ne
drugi plenilci (vretenčarji) v/na vodi	želve	belouške, mlakarice
letno praznjenje in čiščenje akumulacije	ne	da (jeseni)
suh del brežine ob maksimalni gladini vode	travnat	travnat, pas geomembrane
širina suhega pasu geomembrane ob maksimalni gladini vode	0 cm	20-170 cm
širina suhega pasu geomembrane ob minimalni izmerjeni gladini vode	do 50 cm	200-450 cm
naklon brežine iz suhe geomembrane (izmerjen)	do 30˚	30˚-60˚
lesna vegetacija na brežini/nasipu akumulacije	ne	ne
druge stalne stoječe vode do 1000 m oddaljenosti (število in zračna razdalja)	3 (420 m, 780 m, 1000 m)	2 (obe 1000 m)
neposredna bližina (< 50 m) tekočih voda	1 kanaliziran potok	2 naravna potoka
neposredna bližina (< 50 m) manjših nestalnih stoječih voda	da	zelo redko, občasno
oddaljenost strnjenega gozda	> 250 m	< 50 m
neposredna bližina (< 50 m) travnikov	da	da
lega znotraj naravovarstvenega območja	ne	da (EPO Pohorje)
neposredna bližina (< 50 m) ceste	2 asfaltni cesti	1 gozdna cesta
urbano okolje	da	ne

Akumulacija Arena

Ob akumulaciji Arena se nahajajo naselje, smučarski center in v manjši meri naravni habitati (Sl. 1). V neposredni bližini akumulacije so športni objekt, parkirišče in dve povezovalni cesti. Vzhodno od akumulacije so raznoliki habitati, med njimi nižinski travniki, mejice, zelenice, fragmenti gozda in mokrotni travniki z majhnimi nestalnimi vodami. V bližini je kanaliziran Radvanjski potok, ki napaja akumulacijo, vendar je med njima cesta. Večji strnjen gozd je oddaljen več kot 250 m, najkrajša migracijska pot do gozda pa je prekinjana z objekti in cestami. Nasip okoli akumulacije je bil v letu 2024 poraščen s travo, košen in brez lesne vegetacije. Ob maksimalni napolnjenosti akumulacije je gladina vode segala do roba trave. Na prehodu med travnatim delom nasipa in akumulacijskim bazenom, ki je tesnjen z geomembrano, je bil približno 1 m širok pas iz neprekrite geomembrane, ki je bil ob maksimalni napolnjenosti akumulacije zalit z vodo

in tvoril plitvino. Akumulacija je bila povsem brez vodne vegetacije. Na nasipu ali v vodi ni bilo naravnih skrivališč za dvoživke. Edina nedrseča podlaga za odlaganje mrestov sta bili lestvi iz gumijastih obročev, na katerih so bili najdeni mresti sekulje in navadne krastače. V vodi so bili sončni ostriži (*Lepomis* sp.), krapovci (Cyprinidae) in invazivna tujerodna vrsta želve (*Trachemys* sp.). Akumulacije se trenutno ne čisti. Med vsemi popisi je bila v akumulaciji voda. Na popisu v marcu, ko so bili v akumulaciji mresti in paglavci, je bila gladina 30 cm nižja od maksimalne. Med travnatim delom brežine in vodo je bil zato suh pas geomembrane, širine do 50 cm.

Akumulacija Trikotna jasa

Akumulacijo Trikotna jasa obdaja 5–20 m širok košen travnat pas, v katerem se nahajata nasip akumulacije in gozdna cesta. Pas obdaja obsežno območje strnjenega gozda (kolinska kisloljubna bukovja), ki ga na zahodni strani prekinja smučišče s suhimi travišči na silikatih in ruderalnimi združbami. Ob akumulaciji sta dva potoka z manjšimi tolmuni (do 1 m^2), v bližini katerih so bili med popisi posamezni spolno nezreli osebki rjavih žab. Na vseh popisih je bila v akumulaciji voda, a je gladina nihala do 100 cm (Sl. 3). Voda v akumulaciji se je poleti uporabljala za zalivanje površin v dolini. Za razliko od akumulacije Arena, voda, niti ob maksimalni napolnjenosti akumulacije, na nobenem delu ni segala do travnatega dela nasipa. Pas nepokrite geomembrane, ki je bil tudi ob maksimalnem vodostaju suh, je bil okrog in okrog akumulacije neprekinjen ter širok od 20 cm na najožjem ter do 170 cm na najširšem delu. Ob najnižjem izmerjenem vodostaju (100 cm pod maksimalno gladino) je bil pas suhe geomembrane na najožjem delu širok 200 cm in na najširšem 450 cm (Sl. 3). Tudi ob najnižjem vodostaju so lestve iz gum segale do vode. Naklon brežine iz suhe geomembrane je bil povsod strm, od 30° do 60°. Pas iz geomembrane je bil ovira na migracijski poti dvoživk iz vode na kopno. Na suhi geomembrani okrog akumulacije smo v enem popisu prešteli več kot 500 mrtvih mladih osebkov rjavih žab. Ob najnižjem vodostaju

Slika 3. Pogini dvoživk v akumulaciji za umetno zasneževanje Trikotna jasa na Mariborskem Pohorju. Levo: Gladina vode v akumulaciji je bila med popisom v juliju 75 cm nižja od maksimalne. Zaradi nižje gladine je bil pas iz suhe geomembrane širok od 170 do 360 cm. Suha, gladka in strma brežina iz geomembrane je bila za dvoživke ovira med migracijo iz vode na travnat nasip. Desno zgoraj: Mrtev hribski urh v vodi ob robu brežine. Desno spodaj: Mrtvi, pravkar preobraženi mladi osebki rjavih žab, ki so se med migracijo iz vode prilepili na geomembrano in posušili. (Fotografije: Tina Klenovšek)

Figure 3. Amphibian mortalities in the Trikotna jasa snowmaking accumulation. Left: The water level in the accumulation was 75 cm below the maximum during sampling in July. Due to the lower water level, the strip of dry geomembrane was 170 to 360 cm wide. The dry, smooth, and steep geomembrane bank was an obstacle for amphibians migrating from the water to the grassy embankment. Top right: Dead Yellow-bellied Toad in the water at the edge of the bank. Bottom right: Dead, newly metamorphosed juvenile brown frogs that adhered to the geomembrane and dried out during migration from the water. (Photos: Tina Klenovšek)

so bili v vodi akumulacije tik ob brežini najdeni 3 kadavri odraslih hribskih urhov (Sl. 3). Podobno so bili ob različnih popisih najdeni tudi kadavri posameznih odraslih rjavih žab, navadnega močerada, kuščaric in kopenskih žuželk. Zaradi upada gladine vode so bili na suho naplavljeni jajca in zarodki velikega pupka.

V akumulaciji ni bilo vodne vegetacije, obvodne lesne vegetacije ali drugih naravnih skrivališč. Dno in stranice bazena akumulacije so bile iz neprekrite geomembrane in zato zelo gladke. Edina skrivališča in podlaga za odlaganje mrestov so bile v vodo spuščene lestve iz trdih gumijastih obročev oziroma avtomobilskih gum. Tam so rjave žabe odlagale mreste. Med popisom v oktobru so bile žabe in pupki opaženi izključno v in pod avtomobilskimi gumami. Rib v akumulaciji nismo zaznali. Akumulacijo se vsako leto izprazni in očisti. Na vseh poletnih popisih so bile v vodi ali na travnatem nabrežju akumulacije opažene belouške (*Natrix natrix*), največ 3 hkrati. Opazili smo, da na geomembrani nimajo dovolj oprijema in lahko iz akumulacije, ob nizki vodi, splezajo samo po ali tik ob lestvi iz gum. Na travnatem obrežju je bil tudi slepec (*Anguis fragilis*) in v vodi en par rac mlakaric (*Anas platyrhinchos*). Na vseh popisih so bile v vodi številne hrbtoplovke (*Notonectidae*), medtem ko drugih večjih nevretenčarjev nismo opazili.

Diskusija

Raziskava prisotnosti dvoživk v akumulacijah za zasneževanje na Mariborskem Pohorju je potrdila, da so lahko umetni vodni habitati za dvoživke mesta za razmnoževanje kot tudi potencialne ekološke pasti. V eni sezoni smo v dveh akumulacijah skupno popisali osem vrst dvoživk, od tega sedem v akumulaciji Trikotna jasa in dve v akumulaciji Arena. Pri vseh, razen navadnem močeradu, smo potrdili razmnoževanje v akumulacijah. Zabeležili smo tudi propad jajc in pogine dvoživk, ki so bili neposredno povezani s specifičnimi značilnostmi in namensko rabo akumulacij za zasneževanje.

V akumulaciji Arena smo zabeležili le sekulje in navadne krastače, četudi smo glede na nizko nadmorsko višino in število zabeleženih vrst dvoživk v okolici akumulacije (Poboljšaj in sod., 2001; Tertinek, 2024) pričakovali več vrst. Glavni razlog za odsotnost drugih vrst je verjetno prisotnost rib, saj so akumulacijo Arena prav zaradi velikega števila rib Govedič in sodelavci že leta 2009 označili kot neprimerno za dvoživke. Druge vrste dvoživk lahko v bližini najdejo tudi druga ustreznejša vodna telesa, saj akumulacija leži na ravnem terenu, kjer ilovnata tla dobro zadržujejo vodo. Četudi so mokriščni habitati v večji meri degradirani, so v bližini manjša vodna telesa, v katerih so bile najdene dvoživke (Tertinek, 2024). Navadni krastači in sekulji akumulacije za zasneževanje ustrezajo, saj so v študiji iz francoskih Alp v kar 68 % od skupno 28 akumulacij za zasneževanje potrdili razmnoževanje prav teh dveh vrst (Gerfand in sod., 2024). Na razmnoževanje sekulje in navadne krastače v akumulaciji Arena je, razen rib, močno vplivalo tudi nihanje gladine vode, saj so mreste v glavnem odložile na lestve iz gum ali v plitvine, kjer so se le-ti ob spustu gladine ujeli ali prilepili na geomembrano ter posušili. Podoben množičen propad mrestov je bil v akumulaciji Arena zabeležen že v letu 2022 (Tertinek in Skledar, 2022). Negativno na preživetje odraslih dvoživk med selitvijo na mrestišče vpliva tudi lega akumulacije Arena. Predvsem navadne krastače se v akumulacijo selijo iz strnjenega gozda na pobočju Pohorja, pri čemer morajo prečkati vsaj dve cesti in jih je veliko povoženih (Tertinek in Skledar, 2022, druga lastna opažanja). Navadna krastača je vrsta, ki za razmnoževanje izbira velike, globoke stoječe vode (Veenvliet in Kus Veenvliet, 2008), kar so tudi glavne značilnost vodnih akumulaciji. Če živali takšno vodo ocenijo kot ustrezno in jo prednostno izberejo, kljub temu da imajo v njej manjše možnosti za preživetje ter razmnoževanje kot v drugih razpoložljivih habitatih, to ustreza definiciji ekološke pasti (Robertson in Hutto, 2006). V letu 2024 v bližnjih stoječih vodah (pregledana ni bila edino zajezitev Radvanjskega potoka), ni bilo najdenih mrestov navadne krastače (ta raziskava, Tertinek 2024). Navadna krastača je torej, kljub drugim razpoložljivim habitatom, za razmnoževanje izbrala akumulacijo Arena, četudi je izbira vodila v propad mrestov in paglavcev. Ocenjujemo, da je akumulacija Arena za navadno krastačo potencialna ekološka past, saj menimo, da trenutno ne omogoča zadostnega razmnoževalnega uspeha za vzdrževanje stabilne ali rastoče populacije brez priseljevanja (Battin 2004). Za potrditev akumulacije Arena kot ekološke pasti za navadno krastačo bi bilo potrebno bolj sistematično spremljanje razmnoževanja in razvoja osebkov tako v akumulaciji kot drugih razpoložljivih vodnih habitatih skozi celo sezono.

V akumulaciji Trikotna jasa smo popisali sedem vrst dvoživk in od tega za šest potrdili razmnoževanje. Največ je bilo rjavih žab, predvsem sekulj, ki so tudi sicer najpogostejša in splošno razširjena vrsta dvoživk na Mariborskem Pohorju (Govedič in sod., 2009). Z naravovarstvenega

vidika sta bili najpomembnejši najdbi hribskega urha in velikega pupka. Hribske urhe smo v akumulaciji zabeležili na več zaporednih popisih, dvakrat po tri odrasle osebke hkrati. Samostojne stoječe vode, kot je akumulacija, štejejo kot vode z veliko gostoto hribskih urhov, če v njih ob enkratnem ogledu opazimo več kot štiri odrasle osebke (Poboljšaj in sod., 2011). Na enem od ogledov smo našli tudi tri mrtve odrasle osebke. Gladina vode je bila takrat nizka, suha brežina iz geomembrane pa široka. Geomembrana se je čez dan tudi močno segrela. Lahko so poginili od izčrpanosti, ko so poskušali zapustiti akumulacijo, saj običajno naseljujejo manjše plitke vode in niso dobri plezalci. V sezoni razmnoževanja se selijo med vodnimi in kopenskimi habitati. Daljše zadrževanje v akumulaciji pa je lahko neugodno zaradi pomanjkanja hrane, saj v vodi razen hrbtoplovk nismo opazili vodnih nevretenčarjev ali njihovih ličink. Hribski urh je na Mariborskem Pohorju zaradi pomanjkanja primernih razmnoževalnih habitatov redek (Govedič in sod., 2024; Poboljšaj in sod., 2011). V bližini akumulacije Trikotna jasa je bil v preteklosti že zabeležen v manjši stoječi vodi v kolovozu (Govedič in sod. 2009), kar je sicer zanj značilen sekundarni habitat. Med popisi v letu 2024 v bližini akumulacije nismo opazili podobnih manjših stoječih vod.

Druga pomembna vrsta je bil veliki pupek. V akumulaciji Trikotna jasa smo zabeležili odrasle osebke in jajca kot tudi propad jajc in zarodkov, vendar za razliko od rjavih žab in hribskih urhov, v vodi na nobenem od popisov nismo našli mrtvih odraslih pupkov. V letu 2009 Govedič in sodelavci, kljub intenzivnemu vzorčenju na Mariborskem Pohorju, velikega pupka niso našli. Akumulaciji Arena in Trikotna jasa so, zaradi stalnosti in velikosti vode, prepoznali kot najbolj primerni mrestišči za velikega pupka. Predvidevali so, da ga v akumulaciji Trikotna jasa ni zaradi popolne odsotnosti vodne vegetacije. Veliki pupek je v Sloveniji splošno razširjen, a razmeroma redek (Veenvliet in Kus Veenvliet, 2008). Za ohranitev viabilne populacije je potrebna mreža ustreznih mrestišč, tj. stalnih ali začasnih stoječih vod, lahko tudi antropogenih vodnih habitatov, kjer je pogosto najden (Cipot in sod., 2011; Lešnik, 2021). Akumulacija Trikotna jasa je lahko eno od mrestišč v mreži vodnih habitatov, ki vključuje tudi območja v Radvanju in Razvanju, oddaljena približno 1300 m, s katerimi jo povezuje strnjen gozd, ki je del Natura 2000 območja. Takšna mreža potencialnih mrestišč je pomembna tako za velikega pupka kot tudi za druge dvoživke.

V akumulaciji Trikotna jasa je prišlo tudi do pogina velikega števila (> 500) mladih, pravkar preobraženih rjavih žab. Pogin je bil v celoti posledica suhega pasu geomembrane, na katero so se osebki prilepili med migracijo iz vode ali nazaj. Najdeni so bili na celotnem pasu suhe geomembrane, kljub različnim naklonom in širini pasu, in niso izbrali določene smeri ali mesta migracije. Manjši naklon je sicer za dvoživke bolj ugoden, a je bila pot v tem primeru iz vode do trave daljša (do 450 cm; Tab. 2). Glavna razloga za pogin juvenilnih osebkov rjavih žab sta bila nepokritost suhega pasu geomembrane in strm naklon brežine (30° in 60°, Tab. 2). Za primerjavo, Gerfand in sod. (2024) so ugotovili, da se smrtnost dvoživk in drugih živali v akumulacijah za zasneževanje sistematično pojavlja z nakloni brežine večjimi od 16,5°, pri čemer je bilo 73 % opaženih mrtvih živali najdenih v akumulacijah brez ali s samo delno prekritostjo geomembrane (npr. s kamni, gramozom).

Pestrost dvoživk, tako po številu vrst kot številu osebkov, je bila v akumulaciji Trikotna jasa nepričakovano visoka. Manjšo pestrost smo pričakovali iz več razlogov. Prvič, v tej akumulaciji je bila do sedaj zabeležena samo sekulja (Govedič in sod., 2009). Drugič, akumulacije za zasneževanje so za dvoživke z več vidikov neugodni sekundarni habitati, ki lahko delujejo kot ekološke pasti in zmanjšujejo njihovo številčnost ali celo povzročijo lokalno izumrtje populacij (Battin, 2004). In tretjič, globoke stoječe vode, ali vode popolnoma brez vegetacije, niso značilen vodni habitat za večino od najdenih vrst (Veenvliet in Kus Veenvliet, 2008), zato ne bi pričakovali, da jih bodo izbrale za razmnoževanje. Značilnosti akumulacije Trikotna jasa, ki verjetno vplivajo na visoko pestrost dvoživk so odsotnost rib, pomanjkanje ali oddaljenost alternativnih vodnih habitatov in bližina ustreznih kopenskih habitatov. Razpoložljivost in povezanost s kopenskimi habitati pa je pomemben napovednik pestrosti dvoživk v akumulacijah (McCarthy in Lathrop, 2011). V akumulaciji smo pričakovali še navadno krastačo. Za vznožje Pohorja so znane še zelena rega (*Hyla arborea*) in zelene žabe (*Pelophylax* kl. *esculentus*), a jih, verjetno zaradi višje nadmorske višine in popolne odsotnosti vodne vegetacije, v akumulaciji Trikotna jasa nismo potrdili.

Akumulacija Trikotna jasa ima zaradi strme brežine iz nepokrite suhe geomembrane, ki je drseča in se na soncu močno segreje, elemente ekološke pasti. Nekaterim dvoživkam namreč onemogoča izhod iz akumulacije, zaradi česar te v vodi ali na brežini poginejo. Učinek je še posebej izrazit ob nizkem vodostaju, ko je brežina iz suhe geomembrane široka več metrov. Vendar učinek ni za vse vrste ali razvojne stadije dvoživk enak. Najbolj so ogroženi hribski urh in mladi osebki rjavih žab, medtem ko vpliva na

odrasle pupke nismo opazili. Za natančno oceno in potrditev učinka ekološke pasti bi morali za vsako vrsto dvoživke posebej, podobno kot za navadno krastačo v akumulaciji Arena, raziskati razmnoževalni uspeh tako v akumulaciji kot v bližnjih alternativnih habitatih. Druge omejitve te raziskave so, da vzorčenje ni bilo kontinuirano skozi celotno sezono raziskave, zato smo lahko katero od vrst dvoživk spregledali. Prav tako smo lahko spregledali vplive, ki jih imajo posamezne značilnosti akumulacij na dvoživke. Dodatno bi lahko popisali še povezljivost akumulacije z drugimi vodnimi habitati, temperaturo vode in morebitno onesnaženje vode. Zaradi razlik med trajanjem vzorčenja, rezultati med akumulacijama tudi niso neposredno primerljivi.

Iz rezultatov povzemamo, da akumulacije za zasneževanje niso optimalni vodni habitati za dvoživke, a jih te kljub temu uporabljajo za razmnoževanje. Skupne značilnosti akumulacij za zasneževanje so predvsem odsotnost vodne vegetacije, stalna prisotnost vode v času sezone razmnoževanja dvoživk in strme brežine. Med akumulacijami so lahko precejšnje razlike v vrstni sestavi dvoživk, ki akumulacijo uporabljajo za razmnoževanje. Na to med drugim vplivajo prisotnost rib, lega in okolica akumulacij. Dvoživke v akumulacijah najbolj ogrožajo strme brežine iz nepokrite suhe geomembrane in nihanje gladine vode, vendar ti dejavniki na različne vrste dvoživk vplivajo različno. Naša raziskava dopolnjuje ugotovitve, da lahko umetni vodni habitati na dvoživke delujejo kot ekološke pasti (Brand in Snodgrass, 2010; Clevenot in sod., 2018; Lešnik, 2021; Caballero-Díaz in sod., 2022; Gerfand in sod., 2024).

S primernim upravljanjem in načrtovanjem akumulacij za zasneževanje lahko preprečimo, da bi te zaradi svojih lastnosti in namenske rabe za dvoživke učinkovale kot pasti. Osnovni ukrep za uspešnost razmnoževanja v akumulaciji Arena bi bilo letno pozno jesensko praznjenje akumulacije in odstranjevane usedlin, kar bi preprečilo obstoj rib (Mathwin in sod., 2021). V obeh akumulacija bi bilo potrebno geomembrano prekriti z materialom, ki bi dvoživkam omogočil, da v vseh vodostajih varno zapustijo akumulacijo. V času razmnoževanja dvoživk (marec–julij) se iz akumulacij ne bi smela spuščati voda, saj nihanje gladine na suho naplavi jajca oziroma mreste. Nove akumulacije bi morale razen naštetega imeti položne brežine (do 16,5°), predele z vodno vegetacijo in morebitne cestne podhode za dvoživke.

Supplementary Material

Dod. Mat. Tabela S1. Prisotnost dvoživk (z razvojnim stadijem in spolom) v akumulacijah Arena in Trikotna jasa na Mariborskem Pohorju glede na datum popisa.
Supp. Mat. Table S1. Presence of amphibians (with developmental stage and sex) in the Arena and Trikotna jasa accumulations on Mariborsko Pohorje according to sampling date.

Author Contributions

Koncept, metodologija, validacija, urejanje podatkov in pisanje prispevka T.K., Ž.T. in A.S. Vsi avtorji so prebrali objavljeno različico rokopisa in se z njo strinjali.

Acknowledgement

Zahvaljujemo se Skupni občinski upravi Maribor, Javnemu podjetju Marprom in Društvu študentov naravoslovja (DŠN) za sodelovanje in podporo pri raziskavi. Za pomoč na terenu se zahvaljujemo Primožu Kovačiču, Lari Kovačič in Timu Skudniku. Za strokovno pomoč se zahvaljujemo Anji Bolčina in Pii Golob.

Funding

Zbiranje podatkov v akumulaciji Arena v Zgornjem Radvanju je bilo izvedeno v okviru monitoringa pomladanskih selitev dvoživk v Radvanju po naročilu Skupne občinske uprave Maribor. Delo je nastalo tudi s podporo P1-0403 Računsko intenzivni kompleksni sistemi (financer: ARIS).

Conflicts of Interest

Avtorji ne navajajo navzkrižja interesov.

Viri

Akumulacija za zasneževanje Arena (št.2998/07), 2007. Vodnogospodarski biro Maribor d.d.

ARSO, 2021. Atlas okolja. Ljubljana, Ministrstvo za naravne vire in prostor. Agencija RS za okolje.

Battin, J., 2004. When Good Animals Love Bad Habitats: Ecological Traps and the Conservation of Animal Populations. Conservation Biology, 18, 1482–1491. https://doi.org/10.1111/j.1523-1739.2004.00417.x

Brand, A.B., Snodgrass, J.W., 2010. Value of artificial habitats for amphibian reproduction in altered landscapes. Conservation Biology, 24, 295–301. DOI: 10.1111/j.1523-1739.2009.01301.x

Buono, V., Bissattini, A.M., Vignoli, L., 2019. Can a cow save a newt? The role of cattle drinking troughs in amphibian conservation. Aquat. Conserv. Mar. Freshw. Ecosyst., 29, 964–975. DOI:10.1002/aqc.3126

Caballero-Díaz, C., Sánchez-Montes, G., Gómez, I., Díaz, A., Martinez-Solano, I., 2022. Artificial water bodies as amphibian breeding sites: the case of the common midwife toad (*Alytes obstetricans*) in central Spain. Amphibia-Reptilia, 43, 395-406. 10.1163/15685381-bja10115.

Cipot, M., 2007. Poročilo o delu skupine za dvoživke. V: Polajnar, J. (ur.) Raziskovalni tabor študentov biologije Lovrenc na Pohorju 2005, Društvo študentov biologije. Ljubljana, str. 73-80.

Cipot, M., Govedič, M., Lešnik, A., Poboljšaj, K., Skaberne, B., Sopotnik M., Stanković, D., 2011. Vzpostavitev monitoringa velikega pupka (*Triturus carnifex*). Center za kartografijo favne in flore, Miklavž na Dravskem polju.

Clevenot, L., Carré, C., Pech, P., 2018. A Review of the Factors That Determine Whether Stormwater Ponds Are Ecological Traps And/or High-Quality Breeding Sites for Amphibians, Front. Ecol. Evol., 6, 40. doi: 10.3389/fevo.2018.00040

DRSV, 2024. Atlas voda. Direkcija RS za vode. ATLAS VODA

Dwernychuk, L.W., Boag, D.A., 1972. Ducks nesting in association with gulls: an ecological trap? Can. J. Zool., 50, 559–563. doi: 10.1139/z72-076

Fait, P., Demierre, E., Ilg, C., Oertli, B., 2020. Small mountain reservoirs in the Alps: New habitats for alpine freshwater biodiversity? Aquatic Conserv: Mar Freshw Ecosyst, 30, 617–630. https://doi.org/10.1002/aqc.3306

Gerfand, B., Arthaud, F., Evette, A., Testi, B., Peyras, L., Gaucherand, S., 2024. Ecological quality of snowmaking reservoirs in the Alps and management perspectives. Aquat Sci, 87, 9. https://doi.org/10.1007/s00027-024-01136-0

Govedič, M., Ambrožič, Š., Cipot, M., Kapla, A., Lešnik, A., Rebeušek, F., Šalamun, A., Vrezec, A., 2009. Inventarizacija izbranih živalskih skupin na vplivnem območju predvidene razširitve in posodobitve zimsko športnega centra Pohorje. Center za kartografijo favne in flore, Miklavž na Dravskem polju.

Grudnik, Z.M., Triglav Brežnik, G., 2015. Vzpostavitev in izvajanje monitoringa izbranih ciljnih vrst dvoživk v letih 2014 in 2015, Zvezek 3: Vzpostavitev in izvajanje monitoringa velikega pupka (*Triturus carnifex*) v letih 2014 in 2015. Končno poročilo. ERICo Velenje, Inštitut za ekološke raziskave d.o.o., Velenje.

IUCN 2025. The IUCN Red List of Threatened Species. Version 2025-1. https://www.iucnredlist.org

Janssen, J.A.M., Rodwell, J.S., Garcia Criado, M., Arts, G.H.P., Bijlsma, R.J., Schaminee, J.H.J., 2016. European Red List of Habitats: Part 2. Terrestrial and freshwater habitats. European Union. https://doi.org/10.2779/091372

Lešnik, A., 2021. Veliki pupek (*Triturus carnifex*) v območju Natura 2000 Slovenska Istra (SI3000212). Končno poročilo. Center za kartografijo favne in flore, Miklavž na Dravskem polju.

Mathwin, R., Wassens, S., Young, J., Ye, Q. Bradshaw, C.J.A., 2021. Manipulating water for amphibian conservation. Conservation Biology, 35, 24-34. https://doi.org/10.1111/cobi.13501

McCarthy, K., Lathrop, R.G., 2011. Stormwater basins of the New Jersey coastal plain: Subsidies or sinks for frogs and toads?. Urban Ecosyst, 14, 395–413. https://doi.org/10.1007/s11252-011-0161-z

Poboljšaj, K., 1998. Dvoživke (Amphibia) Pohorja. Prirodoslovni muzej Slovenije, Ljubljana.

Poboljšaj, K., Cipot, M., Govedič, M., Grobelnik, V., Lešnik, A., Skaberne, B., Sopotnik, M., 2011. Vzpostavitev monitoringa hribskega (*Bombina variegata*) in nižinskega urha (*Bombina bombina*). Končno poročilo. Center za kartografijo favne in flore, Miklavž na Dravskem polju.

Poboljšaj, K., Janžekovič, F., Kotarac, M., Rebeušek, F., Šalamun, A., 2001. Inventarizacija in naravovarstveno ovrednotenje območja med Razvanjem in Radvanjem za izbrane živalske skupine: kačji pastirji (Odonata), dnevni metulji (Rhopalocera), dvoživke (Amphibia), ptiči (Aves), mali sesalci (Micromammalia), prostoživeče živali (divjad). Center za kartografijo favne in flore, Miklavž na Dravskem polju.

Poboljšaj, K., Lešnik, A., Grobelnik, V., Šalamun, A., Kotarac, M., 2018. Predlog ukrepov za zaščito dvoživk na cestah v upravljanju DRSI. Končno poročilo. Center za kartografijo favne in flore, Miklavž na Dravskem polju.

Robertson, B.A., Hutto, R.L., 2006. A Framework for Understanding Ecological Traps and an Evaluation of Existing Evidence. Biological Sciences Faculty Publications. University of Montana.

Sanacija zadrževalnika ob Trikotni jasi (št. 18/95), 1995. Vodnogospodarsko podjetje Drava Ptuj.

Stanković, D., Lužnik, M., Poboljšaj, K., 2015. Conservation and declines of amphibians in Slovenia. In: Heatwole, H., Wilkinson, J.W. (ur) Amphibian Biology. Status of Conservation and Decline of Amphibians: Eastern Hemisphere: South Europe and Turkey. Pelagic Publishing.

Strah, S., Vek, M., Carreira Santos, M., Lah, F., Stanković, D., 2024. Distribution of Po's Tree Frog (*Hyla perrini*) in Slovenia, Preliminary Results. 2nd Life Amphicon International Conference. Book of Abstracts.

Speybroeck, J., Beukema, W., Bok, B., Voort, V.D.J., 2016. Field Guide to the Amphibians and Reptiles of Britain and Europe. Bloomsbury Publishing, London.

Temple, H.J., Cox, N.A., 2009. European Red List of Amphibians. Office for Official Publications of the European Communities, Luxembourg.

Tertinek Ž. 2024. Monitoring pomladanskih selitev dvoživk v Radvanju. Končno poročilo. Društvo študentov naravoslovja, Maribor.

Tertinek, Ž., Skledar, A. 2022. Monitoring dvoživk na izbranih lokacijah na območju Mestne občine Maribor. Poročilo. Društvo študentov naravoslovja, Maribor.

Ur. l. RS., 2002. Zakon o vodah. Uradni list RS št. 67/02 z vsemi nadaljnjimi spremembami).

Ur. l. RS., 2004. Uredba o posebnih varstvenih območjih (Natura 2000). Ur. l. RS, št. 49/2004 in 110/2004.

Ur. l. RS.. 2018. Uredba o spremembah in dopolnitvah Uredbe o posebnih varstvenih območjih (Natura 2000). Ur. l. RS, št. 47/2018.

Valdez, J.W., Gould, J., Garnham, J.I., 2021. Global assessment of artificial habitat use by amphibian species. Biol. Conserv, 257, 109129. https://doi.org/10.1016/j.biocon.2021.109129

Veenvliet, P., Kus Veenvliet, J., 2008. Dvoživke Slovenije - priročnik za določanje. Symbiosis - Zavod za naravovarstveno raziskovanje in izobraževanje, Grahovo.

Vogrin, N., 1998. Populacijska ekologija sintopnih populacij navadnega pupka (*Trituris v. vulgaris*), planinskega pupka (*T. a. alpestris*) in alpskega velikega pupka (*T. carnifex*) v mlaki na Arehu (Pohorje). Diplomsko delo. Oddelek za biologijo, Pedagoška fakulteta, Univerza v Mariboru.

Zamora-Marín, J.M., Ilg, C., Demierre, E., Bonnet, N., Wezel, A., Robin, J., ... Oertli, B., 2021. Contribution of artificial waterbodies to biodiversity: A glass half empty or half full? Science of The Total Environment 753: 141987. https://doi.org/10.1016/j.scitotenv.2020.141987

ZRSVN, 2021. Naravovarstveni atlas. Zavod Republike Slovenije za varstvo narave.

ABS

Original Research

Recognising the benefits of large carnivore presence in Slovenia: A focus group study

Irena Kavčič [1,*], Manca Dremel [2], Aleksandra Majić Skrbinšek [1,3]

Abstract

In recent decades, populations of the brown bear, grey wolf, and Eurasian lynx in Europe have made a significant recovery. This success is largely attributable to effective legislation, an increase in prey species, and the expansion of forested areas. As a result, large carnivores are gradually returning to regions from which they had long been absent. However, the return of these predators also brings challenges for local communities. While much research emphasises conflicts and damages, this study focuses on the often-overlooked positive perspectives held by key stakeholder groups in Slovenia. Through focus group interviews with hunters, farmers, tourism professionals, and artists, the study explores the benefits of large carnivore presence using the framework of Cultural Ecosystem Services (CES), based on the CICES classification. Present research has found that tourism professionals perceive the presence of large carnivores as a key advantage for the development of tourism. For hunters, encounters with these animals provide opportunities to deepen their ecological knowledge, develop tracking skills, and share this expertise with pride within their communities. Some farmers, despite experiencing livestock losses, acknowledge the role of predators in regulating populations of wild herbivores. Artists experience large carnivores as a source of inspiration and as an opportunity to foster better interpersonal relationships and social dialogue. Recognising the benefits and values that different stakeholder groups attribute to large carnivores can support the development of more inclusive conservation strategies, strengthen dialogue with stakeholders, and shift the focus from conflict mitigation toward fostering coexistence.

Keywords

Large carnivores; coexistence; cultural ecosystem services; benefits.

1 Biotehnical Faculty, University of Ljubljana, Jamnikarjeva ulica 101, 1000 Ljubljana, Slovenia

2 Urban Planning Institute of the Republic of Slovenia, Trnovski pristan 2, 1000 Ljubljana

3 DivjaLabs d.o.o., Stegne 21c, 1000 Ljubljana, Slovenia

*** Corresponding author:**
E-mail address: irena.kavcic@bf.uni-lj.si

Citation: Kavčič, I., Dremel, M., Majić Skrbinšek, A., (2025). Recognising the benefits of large carnivore presence in Slovenia: A focus group study. Acta Biologica Slovenica 68 (4)

Received: 05.05.2025 / **Accepted:** 25.07.2025 / **Published:** 01.08.2025

https://doi.org/10.14720/abs.68.4.22661

Prepoznavanje koristi prisotnosti velikih zveri v Sloveniji z metodo fokusnih skupin

Izvleček

V zadnjih desetletjih so si populacije rjavega medveda, volka in evrazijskega risa v Evropi občutno opomogle. Ta uspeh gre pripisati učinkoviti zakonodaji, porastu številčnosti plenskih vrst in povečanju gozdnih površin. Velike zveri se tako ponovno vračajo na območja, kjer so bile dolgo časa odsotne. Vrnitev velikih zveri pa hkrati prinaša tudi izzive za lokalno prebivalstvo. Medtem ko večina dosedanjih raziskav poudarja predvsem konflikte med ljudmi in velikimi zvermi, se pričujoča študija osredotoča na pogosto prezrte pozitivne perspektive ključnih deležnikov v Sloveniji. S pomočjo fokusnih skupin z lovci, kmeti, turističnimi delavci in umetniki smo raziskali koristi prisotnosti velikih zveri v okviru kulturnih ekosistemskih storitev, na podlagi mednarodne klasifikacije CICES. Turistični delavci prisotnost velikih zveri vidijo kot ključno prednost za razvoj turizma. Lovcem srečanja z velikimi zvermi omogočajo pridobivanje znanja o ekologiji vrst, razvijanje veščin sledenja, svoje znanje pa pogosto delijo znotraj širše skupnosti. Nekateri kmetje, kljub izgubi pašnih živali, prepoznavajo vlogo plenilcev pri uravnavanju številčnosti prakljarjev v naravi. Umetniki velike zveri doživljajo kot vir navdiha in priložnost za izboljšanje medsebojnih odnosov in družbenega dialoga. Študija razkriva bogato paleto vrednot, ki jih različne deležniške skupine pripisujejo velikim zverem. Razumevanje teh raznolikih perspektiv lahko prispeva k oblikovanju bolj vključujočih naravovarstvenih strategij, krepitvi dialoga z deležniki ter preusmeritvi pozornosti od konflikta k iskanju trajnostnih oblik sobivanja.

Ključne besede

velike zveri; sobivanje; kulturne ekosistemske storitve, koristi.

Introduction

Large carnivores are returning to many parts of Europe from which they were previously eradicated due to human persecution. This conservation success story, driven by legal protection, rewilding efforts, and changing socio-political landscapes, is accompanied by growing societal tensions. As people hold divergent views on how large carnivore populations should be managed, their return often leads to polarisation and conflict (Redpath et al., 2017; Lute et al., 2018; Lute et al., 2020). Humans often determine where large carnivores will exist, and our ability to conserve them in the long term will ultimately depend upon our tolerance for their existence (Bruskotter and Wilson, 2014).

Despite the growing attention towards the socio-ecological aspects of human-carnivore relationships in recent decades, the existing literature is skewed and predominantly centred on conflicts rather than on ecosystem services (Rode et al., 2021; Giergiczny et al., 2022; Palacios-Pacheco et al., 2024). Conflicts related to the presence of large carnivores attract media and fuel heated debates, especially in human-dominated landscapes of Europe. Even academic literature is biased towards investigations of negative economic impacts and public safety issues, while the positive impact of large carnivore presence and broader socio-economic impacts are often underrepresented (Rode et al., 2021). Moreover, recent research highlights that people's tolerance is also shaped by their perception of the benefits these species provide (Bruskotter and Wilson, 2014).

To fill this gap, our investigation engaged with diverse stakeholder groups to reflect upon benefits and opportunities arising from the presence of large carnivores. We can expect that different groups of people will have different views on these benefits, and it is important to communicate these diverse local perspectives around the presence of large carnivores in shared landscapes (Linnell and Immerzeel, 2023). Broad, generalised messaging often fails—or even backfires—when it clashes with group-specific norms and values (Toomey et al., 2023). Therefore, we looked into benefits as seen by four stakeholder groups: farmers, hunters, tourism professionals, and artists. These groups were chosen based on their distinct relationships with large carnivores, which we expected would reveal a diverse range of practical and symbolic benefits associated with carnivore presence.

To investigate the benefits provided by large carnivore presence in Slovenia, we have used the Cultural ecosystem services (CES) framework provided by the International Classification of Ecosystem Services (CICES), which offers several categories and supports a comprehensive capture of benefits (Daněk et al., 2023). CES are primarily regarded as "the physical settings, locations or situations that give rise to changes in the physical or mental states of people, and whose character is fundamentally dependent on living processes" (Haines-Young and Potschin, 2013). They are somewhat unique as these are regarded as entirely linked to human societies and to a human' appreciation' of biodiversity (Horgan et al., 2021). Although often intangible and hard to measure (Slovák et al., 2023), there has been a growing recognition of the value of cultural services in driving actions and determining the success or failure of conservation programs (Horgan et al., 2021).

We have used the focus group method, which has proven effective for exploring CES, to gather in-depth insights from the selected stakeholders (Slovák et al., 2023). In this article, we present the benefits of large carnivore presence as perceived by each stakeholder group investigated. Our findings underscore the importance of recognising a broader set of motivations behind tolerance and conservation support. Identified benefits can help to support the development of more targeted communication strategies that foster coexistence and broaden public support for conservation.

Materials and Methods

Conceptual framing

To frame the identified benefits provided by large carnivores, we used the CICES framework (Haines-Young and Potschin, 2018) as it offers several categories and supports a comprehensive capture of benefits from large sets of qualitative data (Daněk et al., 2023). Moreover, CICES also allows translation between different ecosystem service classification systems, such as those used by the Millennium Ecosystem Assessment (MA) and The Economics of Ecosystems and Biodiversity (TEEB). 11 categories from CICES version 4.3 were used: experiential use, physical use, scientific, educational, heritage/cultural, aesthetic, entertainment, symbolic, sacred and/or religious, existence and bequest (Table 1).

Data collection

In this study, we employed the focus group methodology, augmented with a structured protocol designed to elucidate benefits in the frame of CES as a potent tool for uncovering the positive facets of large carnivore coexistence. Unlike quantitatively driven surveys, this approach allows for a nuanced exploration of the values and relationships between local communities and their ecosystems.

In the first phase, we prepared a protocol for conducting focus groups to identify and document the range of different CES provided by large carnivores (Table S1). We modelled our approach after the elicitation method for nonmaterial values described by Gould et al. (2014), which combines various qualitative data collection techniques. In the protocol, we defined themes based on the classes of the CES outline in the CICES. A set of questions was developed to encourage participants in all focus groups to express their opinions, feelings, and values, covering 11 classes of CES presented in Table 1. Based on their responses, we asked additional customised sub-questions during the interviews. We did not use the term "cultural ecosystem services" during the interviews. Instead, we addressed questions related to the topics associated with large carnivores: personal experiences with large carnivores, benefits, and disservices of large carnivores for people, ecotourism, recreation and hunting, spiritual, artistic value, heritage, local and traditional knowledge, educational value, and intergenerational value. To stimulate the sharing of thoughts and feelings, we also showed participants 14 photographs of large carnivores in different situations (in their natural habitat, causing damage, interacting with people). We designed the protocol to obtain as many diverse benefits of large carnivores and their significance for the participants themselves and the society they live in as possible. We encouraged interaction among participants and a free flow of thoughts while also allowing for real-time clarification of the researcher's understanding.

In the next phase, we defined stakeholder groups that we wanted to interview. Following Litosseliti's (2003) guideline that focus groups typically include 4 to 6 participants to ensure diverse perspectives without limiting individual contributions, our focus groups were composed of participants from the same interest groups who shared relevant experiences with the studied topic. Stakeholders associated with large carnivores are usually individuals

influenced by their presence, individuals who can influence their population or have other interests in large carnivores and their populations (Linnell, 2013; Grossmann et al., 2020). We have chosen four stakeholder groups: farmers (beekeepers, livestock breeders from large carnivore area), hunters from two hunting families active in the large carnivore area and involved in bear viewing activities, tourism professionals engaged in wildlife tourism and artists. Artists participated in a lynx-themed art residency organised by the LIFE Lynx project (LIFE16 NAT/SI/000634), where they engaged with lynx conservation topics through presentations and field trips, and created lynx-related artworks during the residency. Farmers were selected because of the impacts large carnivore presence has on their livelihoods, hunters are an important stakeholder group because they can influence large carnivore populations, while tourism professionals and artists were selected due to their interest in large carnivore populations. We initially contacted stakeholders based on our previous cooperation with them through other projects related to large carnivores in Slovenia. After that, we followed a snowballing process to identify additional relevant people for our focus group interviews.

Focus groups were conducted between October and December 2019. They lasted between 2 and 4 hours and took place in the local setting of the focus groups: for farmers at the tourist farm, for hunters at the hunting lodge, for artists at the place of their art residency and for tourism professionals at the tourism centre. Altogether, we interviewed 29 participants (five farmers, eight hunters, nine tourism workers and seven artists). Each focus group was internally homogeneous in terms of participants' professional or interest background, consisting exclusively of farmers, hunters, tourism professionals, or artists. Group sizes were intentionally kept small to facilitate open discussion and ensure that diverse individual perspectives could be expressed. The final number of participants in each group was also influenced by participants' availability and willingness to engage in the study.

Sessions were led by a facilitator who used a semi-structured interview approach that relies on participants' responses (Gould et al., 2014; Table S1). The aim for the discussions was that they were enjoyable, without participants feeling pressured to speak or seek consensus, but rather to feel encouraged to express different viewpoints. The focus groups were recorded on a voice recorder (dictaphone) with the consent of the stakeholders.

Data analysis and interpretation

Digital recordings were manually transcribed for analysis. From each transcript, we extracted statements (sentences or coherent sequences of sentences) related to the CES of large carnivores and entered them into a spreadsheet. Each statement was placed in its own row, and the speakers of the statements remained anonymous. For each recorded statement, we also noted the corresponding stakeholder group (farmers, hunters, tourism professionals, or artists). We collected all statements reflecting participants' subjective attitudes or personal experiences regarding large carnivores, whether positive or negative. We assigned nominal variables to each analysed statement: the type of large carnivore the statement referred to (bear, wolf, lynx, or all three large carnivores) and one of the 11 classes of CES according to CICES (experiential use, physical use, scientific, educational, heritage-cultural, entertainment, aesthetic, symbolic, sacred and/or religious, existence, and bequest). The category "other" was used when we were unable to fit the statement into the existing categories. Many times, statements were difficult to categorise within the CICES framework, and the categorisation process is subjective. For that reason, the statements were reviewed by two researchers to minimise the degree of subjectivity.

Next, the collected statements were categorised according to the framework proposed by Rode et al. (2021), which defines specific large carnivore-related impacts within three overarching categories: economic impacts, health and well-being impacts, and social and cultural impacts. Each of these categories includes both positive and negative impacts. Following this structure, we assigned each statement in our dataset to the appropriate positive impact category, enabling us to identify and extract statements referring specifically to the positive contributions, benefits, and cultural ecosystem services associated with the presence of large carnivores.

Results

In all focus groups combined, we identified a total of 476 statements that discuss the positive contributions or benefits of large carnivores for individuals and society. The number of statements related to a specific class of CES for each interest group is presented in Fig. 1. Most statements were classified as having experiential or educational value.

Table 1. Categories of CES from CICES version 4.3 and their descriptions were adapted to the purpose of the present research.

Tabela 1. Kategorije kulturnih ekosistemskih storitev glede na CICES različico 4.3 in njihovi opisi prilagojeni glede na namen te raziskave.

Class	Description and example of goods or benefits
Experiential use	In situ, large carnivore watching and recreation are in the area of large carnivore presence.
Physical use	Hunting of large carnivores.
Scientific	Large carnivores are a subject matter for research both on location and via other media, and knowledge about large carnivores and their habitat is important.
Educational	Large carnivores are a subject matter of education, both on location and via other media, and they provide skills and knowledge about large carnivore management and their habitat.
Heritage, cultural	Large carnivore resonance in culture or heritage, history, local identity, historic records of a place, and preserved cultural heritage.
Aesthetic	Large carnivores and their environment are a source of artistic inspiration and representation, aesthetic experiences, appreciation of the beauty of large carnivores, and a sense of place.
Entertainment	Ex situ experience large carnivores and their environment via different media.
Symbolic	Symbolic meaning of large carnivores, large carnivores as emblems, signifiers.
Sacred and/or religious	Large carnivores as a spiritual or ritual identity, holy places, and sacred animals.
Existence	Non-utilitarian qualities of the presence of large carnivores, such as the enjoyment/philosophical perspective provided by the knowledge of their existence.
Bequest	Moral/ethical perspective or belief regarding large carnivores, willingness to preserve large carnivore and their environment for the experience and use of future generations.

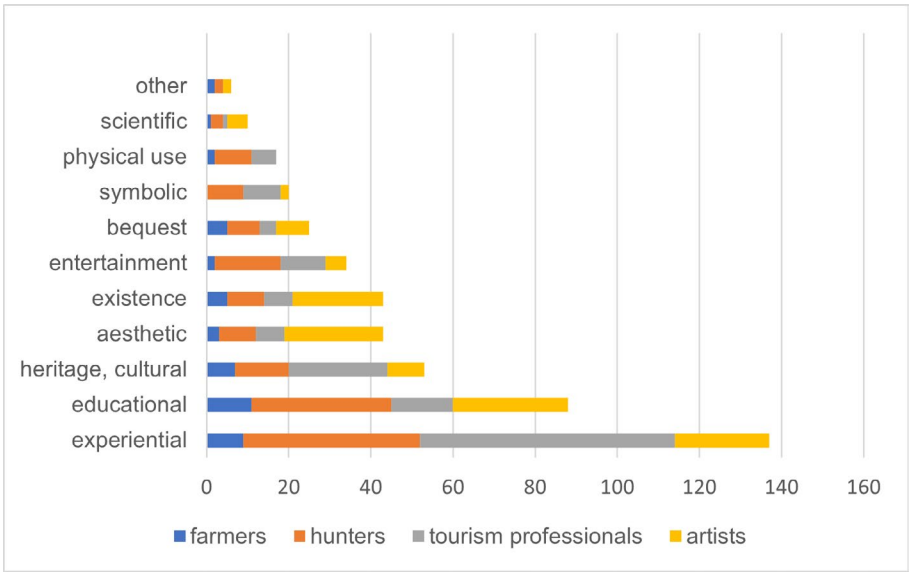


Figure 1. The number of statements related to a specific class of CES for each interest group.

Slika 1. Število izjav v posamezni kategoriji kulturnih ekosistemskih storitev za vsako interesno skupino.

Table 2 displays various keywords and terms that we identified in the statements from focus groups. We do not evaluate them by importance, as it is impossible to do so solely based on the transcript analysis. Keywords from CES classes are listed below in order to reflect the number of statements we have placed in each class. There were no statements within the sacred/religious class; therefore, only 10 CES classes are shown. Class Other was established for statements that couldn't be attributed to any of the CES classes.

Below, we present all CICES categories, listed in order based on the number of statements attributed to each category (Fig. 1). For each CICES category, we provide narrative examples drawn from stakeholder group discussions.

Experiential values

The experiential class of Cultural Ecosystem Services (CES) encompasses non-intrusive, sensory and emotional engagements with land- and seascapes, such as wildlife observation, that provide personal enrichment and enjoyment (Czúcz et al., 2018). Respondents most often mention direct observations of animals, but also express enjoyment when encountering their signs of presence, or because of the mere awareness of the existence of these animals.

Among focus group participants, especially tourism professionals and hunters, watching/encountering large carnivores and related activities are a benefit. Observing the animals in the sense of organised guiding mainly referred to bears. Participants noted that this tourism, in turn, stimulates the local economy, with ancillary benefits to hospitality, retail, and other service sectors: "Those who come to see the bears also go to restaurants, accommodation providers, shops and other tourist attractions." They recognise the value of bears and, to a lesser extent, the value of wolves and lynx in increasing the attractiveness of the local area and transforming previously overlooked areas into destinations with distinctive appeal: "What was previously 'no name' has now become 'The land of bears'". Hunters within our focus group highlighted the economic benefits of wildlife tourism, particularly bear watching, which they increasingly view as an important supplementary source of income alongside traditional hunting activities. As one participant noted: "We didn't hunt in the summer, because photographic tourism is our main source of income." Beside direct observation, the benefits of the sole presence of large carnivores in the

Table 2. Generated keywords and terms mentioned during focus groups, classified for different CES classes.

Tabela 2. Ključne besede in izrazi, omenjeni med fokusnimi skupinami, razvrščeni po različnih razredih kulturnih ekosistemskih storitev.

Class	Keywords and terms
Experiential	encounters with large carnivores, photo hunt, photo tourism, socialising, tourism, watching animals
Educational	Bear-friendly label, best practice farm, better coexistence, interpretation centre, interpretation, learning for life, learning through the media, museum, natural science centre, natural science day in school, nature trail, self-upbringing/ education, tourism, understanding nature
Heritage, cultural	cultural landscape, folk tradition, hunting tradition, interpretation of cultural heritage, literature, metaphors, monuments, proper names, toponyms, tourism, tradition of coexistence, traditional methods of protection against damage
Aesthetic	aesthetic experiences, beauty, creation, imagination, inspiration, photos, the spirit of space
Existence	autochthony, diversity, inspiration and ideas, interest, part of the culture and system, rejection to shoot, respect, tolerance, understanding nature
Bequest	economic benefit, emotional and social enrichment, ethics and morals, national interest, organisations for the conservation and growth of the population, population management, species conservation projects
Symbolic	authenticity, autochthony, coat-of-arms, logos, metaphors, personification of species, proverbs, recognisability, symbol of preserved nature
Physical use	hunting equipment, hunting tourism, hunting, population management, sale of (bear) meat and fur, stuffed animals, trophies
Scientific	better coexistence and management, lynx reintroduction, photo traps, recording tracks, research, technology development, telemetry collars
Other	agreement, balance, partnership

area was recognised as well: "An Englishman once told me that they don't need to see the bear, just show them a footprint. You must tell a story. And then you win." Others described deep emotional and even spiritual connections formed through rare, unpredictable wildlife encounters: "Even though you know they are there, you cannot predict the meeting. And that's always a fantastic moment because it's unexpected." Some statements reflect the awe-inspiring nature of large carnivores, suggesting that their presence limits human overuse of natural areas: "If it weren't for the large carnivores in our forests, people would walk all over these areas, everywhere". Finally, the bear also emerged as a socialising factor for locals and tourists alike, bringing people together through shared experiences like group hikes due to safety concerns: "The bear has become a socialising moment, because those of us who are regular hikers don't go alone, we go together."

Educational values

Educational class refers to the contributions of living systems to education, training, and the development of skills and knowledge (Potschin and Haines-Young, 2018). Large carnivores, as keystone species, offer powerful opportunities for learning. Their presence can serve as a focal point for understanding ecological relationships, the importance of biodiversity, and the interconnectedness of natural systems.

In our study, hunters most frequently highlighted the educational value of large carnivores. They described a deep observational engagement with these animals, often inspired by admiration for their behaviour and hunting abilities: "I have seen wolves chasing a deer, how they ambush it. A couple of them go ahead, some are behind... how they intercept it. I analysed how they caught a deer." Some are challenged by their secretive nature: "To see a lynx, you had to be able to move properly, know the location, its specific behaviour." Through these encounters, they learn about their basic ecology, gain tracking skills and recognise their value for obtaining a healthy forest ecosystem. They want to pass on their knowledge and respectful attitude to the younger generation by designing nature education visitor centres and trails. Additionally, they work with older members of the community through creative programs such as art workshops: "We talk while we create, we bring books about nature, and we try to eradicate stereotypes about the wolf in the minds of the older people who have formed stereotypes about the wolf."

Though more reserved in their expressions, farmers show their understanding of natural processes and predator-prey relationships. They recognised the role of large carnivores in maintaining a healthy forest ecosystem: "If large carnivores were not in our environment, we would be worse off as far as fields are concerned. The wolf and the bear are also scavengers, and they also maintain the deer."

Artists conveyed additional, more indirect educational values, as people gain awareness and interconnectedness of nature and their own behaviour and develop ethical values and empathy: "The presence of large carnivores can also build a relationship with our fellow man. Just as we behave towards animals, we can behave towards humans." Their presence provoked reflection on human actions: »We can slaughter an animal and it's OK. If a wolf eats the animal, it is horrible. The outcome is the same."

Although tourism professionals discussed educational value to a lesser extent, they recognise the opportunity of their presence for interpretation and interpretation of nature in different ways. They offer guided tours where they teach visitors about the biology of large carnivores and the nature of the area through interpretation and the search for animal tracks, often including cultural heritage in the interpretation.

Heritage, cultural values

Heritage/cultural class refers to elements of nature that hold cultural or historical significance, helping people identify with the history or culture of where they live or come from (Potschin and Haines-Young, 2018).

Among all stakeholder groups, tourism professionals most strongly emphasised the cultural importance of large carnivores. For them, these animals are integral to the region's identity: "Bears and other large carnivores are part of our culture, part of our system. If they disappeared now, we would become very poor and empty". Several participants reflected on historical practices like shepherding and protection of livestock, underscoring the effort required in preventing conflicts with large carnivores and preserving traditional ways of life: "In our youth, there were no sheep slaughtered by wolves ... No livestock was left outside, there was always a shepherd present, and there were a couple of hundred sheep in the flock." Hunters expressed sense of pride in local efforts to live harmoniously with large carnivores: "Large carnivores give a good picture of Slovenia ... In the museum in Paris, our place was presented as

an example of good practice of coexistence between bears and humans." Same sense of pride is mentioned by artists, seeing the presence of large carnivores as a conservation success: "For us, the presence of large carnivores means the conservation of the species and an opportunity to show that coexistence is possible, which is also of interest to other countries". Farmers mostly mentioned different surnames, names of villages that are derived from animal names or associated with large carnivores, reflecting a long-standing cultural imprint of large carnivores on everyday life.

Aesthetic value

Aesthetic classes are elements of nature that enable aesthetic experiences and are appreciated for their beauty, which can become a source of artistic inspiration (Potschin and Haines-Young, 2018).

In this context, artists most vividly expressed the aesthetic and inspirational power of these animals: "Large carnivores are an immense source of ideas and inspiration for creation, for enriching life, including spiritual life. They do not have a concrete impact on us, but an indirect one. It is almost impossible to live without it." Hunters described visual encounters with large carnivores as special, unique, unforgettable aesthetic experiences that have an enduring value in shaping personal memories and contributing to the overall aesthetic appreciation of nature: "I saw lynx when it was just walking along nicely in the shade. It was a full moon... It was a picture you never forget." Even farmers acknowledged their beauty, but with a sense of caution due to the potential impacts on their livelihoods:" A picture of a wolf and her cubs is beautiful, idyllic, cute, as long as they are in their natural habitat, as long as they are not under my feet, or doing harm."

Existence value

The existence value refers to the intrinsic worth of these species simply by virtue of their presence in nature—independent of any direct human use (Potschin and Haines-Young, 2018). Mostly the value was recognised by artists, who emphasised that the mere presence of large carnivores contributes to overall diversity: "Our country is more colourful, more diverse and more fulfilled because of the large carnivores". This value was also clearly articulated by hunters who emphasized the importance of large carnivores simply being part of the landscape, regardless of direct human benefit: "Every animal, every individual has its function in nature." Participants from various interest groups expressed their deep appreciation for the untamed nature of large carnivores, reminding us that some things in nature still operate beyond our influence." The wolf pack expresses something primal, wild; they have a life of their own".

Bequest value

Bequest value expresses the willingness of individuals to preserve animals and ecosystems to ensure that future generations can also enjoy and benefit from the resource (Potschin and Haines-Young, 2018). The desire to conserve large carnivore populations for present and future generations was highlighted in all focus groups.

Artists expressed their concern for the potential ecological consequences of the absence of large carnivores: "The absence of large carnivores would be the bleakest prognosis for life because if it started with the umbrella species, it could lead to the decline of bees, and that is the worst stage of the apocalypse, affecting all of us, even if we don't have a personal connection to these animals or their habitats." Participants of the focus groups advocate for the conservation of the population on ethical and moral grounds, as well as the economic benefits that large carnivores bring to the area through tourism and other activities. They oppose unplanned and inappropriate culling and stress that the long-term conservation of large carnivores also requires appropriate management in several areas. Hunters and farmers are mentioning appropriate management in terms of population size: "The aim is to conserve large carnivores, but only in certain numbers." Farmers specifically acknowledged their interconnection with nature, and the well-being of the environment: "Farmers are connected to nature, and we do not want to kill or destroy a population", they believe large carnivores belong to Slovenia: "Large carnivores are autochthonous to Slovenia and should therefore be preserved, while foreign species that lack natural predators (e.g., jackals) should not be introduced."

Entertainment

Entertainment class includes characteristics or qualities of species or ecosystems that provide material or subject matter that can be communicated to others via different media. (e.g. films or books) (Potschin and Haines-Young, 2018). Several statements recall childhood memories that

involve stories and folklore from that time, including fairy tales and folktales like Red Riding Hood and popular movies.

In modern times, social media has changed the way the public receives and transmits news, where sensationalistic reports receive a higher number of shares than reports presenting facts more objectively, which can generate unwarranted fear and prejudice against predators (Nanni et al., 2020). Some participants in our focus groups mention that large carnivores are a useful topic for sensationalist media and are used to further polarise society:" I would add that this is a media campaign. These are presentations of catastrophic situations as if politics is bloodthirsty. Before it was migrants, they needed this victim."

Symbolic

Symbolic class includes elements of nature that hold symbolic meaning, help shape a sense of place, and serve as emblems of identity, character, or meaning (Potschin and Haines-Young, 2018).

Participants' statements reflect a complex and diverse set of symbolic values associated with large carnivores, ranging from their role in local identity and authenticity to their portrayal in stories: "Large carnivores are part of the authenticity of these places." The presence of a bear in the municipal emblem signifies its significance as a symbol of the area's identity and values.

Participants also projected human-like qualities onto these species. One hunter regards bears "as another human being"; this suggests a deep connection and perhaps a sense of kinship with bears. Another statement portrayed the wolf as "professors who study and observe humans", adding depth to their symbolism, while the lynx is portrayed as "a princely figure" because of its unique behaviours, such as covering its prey with leaves: "The lynx is a prince to me because it covers its prey with leaves and returns to eat". For tourism professionals' large carnivores are not only subjects of ecological interest, but integral to their personal identity: "For me as interpreter of nature, my personal life would change a lot in the absence of large carnivores, because I don't know what I would be looking at in the puddles, whose footprints I would be following..."

Physical use

Physical use of large carnivores refers mainly to hunting and related activities; therefore, it was mostly recognised by hunters: "Deer hunting or wolf hunting is indeed hunting. It takes hours and hours. Yes, I hunt. That is why I am a hunter. I hunt."

Scientific value

The scientific class includes elements of living systems that enable scientific investigation or the creation of traditional ecological knowledge. Focus group respondents recognised the importance of scientists and experts researching these animals, especially for better coexistence and management:" When defining the acceptable number of large carnivores in Slovenia, the experts must take into account the results of technology (cameras, collars), where and how many animals are currently present."

Other

Some statements could not be classified into any of the classes, but nevertheless, they express the important opportunities offered by the presence of large carnivores, mostly related to recognising and respecting diverse perspectives and opportunities for mutual agreements and partnerships. An artist shared the following statement, reflecting the viewpoints of people directly affected by large carnivore presence: "Personally, I think there are too many large carnivores in many areas of Slovenia. Farmers say that three wolf packs would be enough for Slovenia, and around 200–500 bears at most. That's the number, according to those who live with nature, that would be sustainable for a small country like Slovenia."

Discussion

Public attitude studies in Slovenia indicate that people generally support the conservation of large carnivores (Majić Skrbinšek et al., 2019; Bele et al., 2022; Krofel et al., 2025), with the presence of bears perceived as beneficial for local communities (Kavčič and Majić Skrbinšek, 2023). However, the fact that humans were once able to significantly reduce or eliminate carnivore populations suggests that successful conservation requires a deeper understanding of the factors driving human tolerance towards these species (Bruskotter and Wilson, 2014). Previous studies have identified knowledge of species biology and behaviour as a key determinant of attitudes and conservation beliefs

(Majić Skrbinšek et al., 2019; Oražem at. al, 2019; Oražem et al., 2021). In addition, tolerance towards large carnivores is also influenced by people's perception of the benefits these species provide (Slagle et al., 2013; Bruskotter and Wilson, 2014). Bruskotter and Wilson (2014) argue that communication should not only address risks but also highlight benefits and provide guidance for mitigating potential conflicts. Marino et al. (2020) found a significant positive relationship between perceptions of intangible benefits and tolerance towards bears and wolves, suggesting that enhancing recognition of these intangible benefits could serve as an important management tool to foster tolerance. A recent review of the academic literature identified a clear research gap regarding the positive, especially non-material, impacts of large carnivores (Rode et al., 2021). Our study sought to address this gap by applying the cultural ecosystem services framework to identify and explore the benefits associated with large carnivore presence in Slovenia. Through focus groups conducted with four stakeholder groups, farmers, hunters, tourism professionals and artists, we provide insights into both the evident and less obvious benefits linked to large carnivores.

Tourism professionals emphasised both economic and cultural benefits of large carnivores. This is reflected in the way large carnivores are used to design tourism programmes, while cultural heritage arising from the long tradition of coexistence is used as part of storytelling. They also noted that the presence of these animals stimulates the local economy and supports a wide range of businesses, generating indirect but meaningful economic benefits across the community. However, the value of large carnivores extends beyond economics. For some tourism professionals, these animals have become a defining element of personal identity, contributing directly to their well-being and sense of purpose. As Buijs et al. (2020) note, happiness generated through the positive emotions induced by wildlife encounters can foster greater empathy and increase tolerance toward controversial species like large carnivores. Marino et al. (2020) similarly reported that perceptions of benefits, combined with exposure to positive meaningful experiences, like unexpected encounters that elicit positive emotions, can enhance tolerance towards bears and wolves in the Abruzzo region in Italy. They suggest that organised wildlife viewing could generate similar emotional benefits across the wider community. Likewise, Oražem et al. (2022) highlighted that direct experiences with animals serve as effective precursors of positive attitudes. While our study did not directly assess tourists' emotional responses, the insights from tourism professionals suggest that positive emotional connections with large carnivores—whether experienced by visitors engaged in wildlife tourism or by professionals themselves—represent a potential for promoting tolerance. These could be extended to the wider community through organised nature treks (Marino et al., 2020) or educational encounters in informal learning settings, like zoos (Oražem et al., 2022).

Hunters offered a different perspective grounded in personal experience, ecological knowledge, and their role in wildlife management. They expressed deep interest in the biology and behaviour of large carnivores. Particularly captivated by the animals' secretive nature, they are challenged to gain the ability to track or hunt them. They most often encounter large carnivores in the wild, and their encounters are described as unique, special, and unforgettable. These immersive experiences with wildlife may contribute to long-term well-being (Buijs et al., 2020). Slovenian hunters are often involved in wildlife monitoring; they provide hunting bag data and collect biological samples. Their voluntary engagement in wildlife management suggests that such activities may be emotionally rewarding and reinforce stewardship practices (Buijs et al., 2020). Their statements show that they are feeling proud of their contributions; similar narratives were collected in other participatory processes where hunters wanted to be respected as legitimate managers and stewards of their land (Salvatori et al., 2021). Hunters also play an active role in community education, engaging with children and the elderly, organising guided walks, and designing educational trails and visitor centres. They often express pride in local efforts to live harmoniously with large carnivores, which is recognised internationally. Some mentioned the economic benefits of bear watching, saying that a bear is worth more alive than dead. In certain instances, they adapted hunting practices to accommodate photo tourism by avoiding hunting in specific areas.

In contrast to hunters and tourism professionals, farmers offered a more cautious and critical view, shaped by the direct impacts of large carnivore presence. Farmers found it hardest to articulate the benefits associated with large carnivores, as their experiences are often shaped by livestock losses and threats to livelihoods. For farmers, large carnivores are not only an economic threat but also a challenge to values closely tied to their way of life (Larsson et al., 2022). Research among Slovenian vocational students revealed that those in agricultural programmes held

more negative attitudes and had less knowledge about large carnivores compared to their peers in environmental and veterinary programmes, highlighting the influence of early informal learning experiences on attitude formation (Oražem and Tomažič, 2018). Nevertheless, even farmers in our focus group showed their understanding of natural processes and predator-prey relations, but they believe that wolves have an important ecological role and regulate prey populations. This aligns with findings from Sweden, where farmers cited wolves' regulatory role in prey populations as a rationale for their conservation (Karlsson and Sjöström, 2008). Despite concerns, farmers in our study expressed a strong connection to nature and emphasised the need to balance conservation goals with viable farming. They supported keeping carnivore numbers at "reasonable" levels, as defined by experts, that would still allow sustainable livestock production. This perspective reflects the findings of Piscopo et al. (2021), who stressed that successful coexistence depends on collaboration between scientists, farmers, and policymakers.

Stakeholders most directly affected by large carnivores, like farmers and hunters, tend to hold the most negative attitudes (Dressel et al., 2014; Franchini et al., 2021). Considering that negative perceptions often outweigh positive ones in shaping attitudes (Kansky et al., 2014), we cannot expect communication alone to change their views. However, engagement with these groups remains essential, as it can help anticipate potential areas of social conflict over species protection (Lischka et al., 2019). It is essential to approach communication strategies with stakeholder groups like farmers and hunters with sensitivity and a recognition of the complexities involved. Acknowledging the additional efforts these stakeholders have made to protect their livelihoods (farmers) or to contribute to large carnivore research (hunters) can foster a more constructive dialogue and minimise tensions.

Together, the perspectives of tourism professionals, hunters, and farmers illustrate the wide range of tangible and intangible benefits and challenges associated with large carnivores. A group of artists offered a distinct viewpoint, describing these animals as a source of inspiration and appreciating their aesthetic value. They also conveyed additional, less obvious benefits, believing that the presence of large carnivores teaches us about respect and patience, enables us to build a better relationship with other people and promotes mutual understanding. Large-carnivore conflicts may, in fact, represent an opportunity for wider social learning and for improved stakeholder relationships (Hovardas, 2020), leading to better mutual understanding and collaboration among stakeholders. Participatory processes over large carnivore management are being used increasingly to share different views and increase knowledge of other points of view and recognition of the legitimacy of stakeholder positions (Salvatori et al., 2021).

To promote coexistence between large carnivores and humans in Europe, greater attention must be paid to understanding how specific stakeholder groups perceive not only the detrimental but also the beneficial aspects of living alongside these species (Marino et al., 2020; Palacios-Pacheco et al., 2024). Recognising and highlighting the benefits of local practices aimed at fostering coexistence may help reduce conflict (Salvatori et al., 2021; Pettersson et al., 2022). Emerging communication models should focus on creating spaces for dialogue and debate, engaging emotions, and embracing diverse perspectives (Toomey, 2023). Additionally, promoting direct experiences, such as observing animals (Oražem et al., 2021, 2022), as well as increasing positive experiences and implementing damage prevention measures (Marino et al., 2020), can help shift attitudes. Communication strategies should also place greater emphasis on the benefits large carnivores provide (Bruskotter and Wilson, 2014; Rode et al., 2021). Integrating these stakeholder perspectives into policy design could support more inclusive and adaptive management strategies, tailored to the diverse values and experiences across affected communities. Within this study, we applied the cultural ecosystem services lens to identify a wide range of benefits, as perceived by key stakeholder groups in Slovenia. Our findings contribute to the growing recognition that intangible benefits are important drivers of tolerance towards large carnivores and should be more prominently integrated into conservation strategies.

Conclusions

Our engagement with stakeholders through focused dialogue sessions facilitated the discovery of diverse benefits and a multitude of values associated with large carnivores, each varying across stakeholder groups. Recognising and understanding different social groups' values and attitudes relating to wildlife is important for fostering trust and facilitating fruitful dialogue and communication. Our study provides insights that illuminate the origins of both opposition

and agreement concerning large carnivore management, making one step forward in navigating the complex world of the plurality of values.

Supplementary Materials

Table S1. Interview protocol

Author Contributions

Conceptualisation, I.K. and A.M.S.; methodology, I.K. and A.M.S.; formal analysis, M.D. and I.K.; investigation, I.K., M.D. and A. M. S.; writing—original draft preparation, I.K.; writing—review and editing, I.K., M.D., A.M.S. All authors have read and agreed to the published version of the manuscript.

Acknowledgement

We express our sincere appreciation to all participants who generously contributed their time, experiences, and insights to this study. We thank the LIFE Lynx project for enabling access to focus group participants, including artists involved in its lynx-themed residency. The authors acknowledge the AI language model ChatGPT (OpenAI) was used in order to improve text (language). After using this tool, we reviewed and edited the content and take full responsibility for the content of the publication.

Funding

We acknowledge financial support provided by Interreg VA Slovenia-Croatia programme. The research was conducted as part of the CARNIVORA DINARICA project. The authors acknowledge the project Thinking Animals, J6 3129, financially supported by the Slovenian Research Agency for writing of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

Bele, B., Skrbinšek, T., Ambrogini, C., Berzins, R., Chioso, C., Faure, N., Gambini, I., Knauer, F., Kuralt, Ž., Majić Skrbinšek, A., Mavec, M., Minola, L., Potočnik, H., Rossi, E., Ruco, V., Simon, R. N., Trombin, J., Vettorazzo, E., Walter, T., 2022. Public attitudes toward wolves and wolf conservation in Austrian, French, Italian and Slovenian Alps, Technical report, Project LIFE 18 NAT/IT/000972 WOLFALPS EU.

Bruskotter, J., Wilson, R., 2014. Determining Where the Wild Things Will Be: Using Psychological Theory to Find Tolerance for Large Carnivores. Conservation Letters, 7(3), 158-165. https://doi.org/10.1111/conl.12072

Buijs, A., Jacobs, M., 2021. Avoiding negativity bias: Towards a positive psychology of human-wildlife relationships. Ambio, 50(2), 281-288. https://doi.org/10.1007/s13280-020-01394-w

Czúcz, B., Arany, I., Potschin-Young, M., Bereczki, K., Kertész, M., Kiss, M., Aszalós, R., Haines-Young, R., 2018. Where concepts meet the real world: A systematic review of ecosystem service indicators and their classification using CICES. Ecosystem Services, 29, 145-157. https://doi.org/10.1016/j.ecoser.2017.11.018

Daněk, J., Blättler, L., Leventon, J., Vačkářová D., 2023. Beyond nature conservation? Perceived benefits and role of the ecosystem services framework in protected landscape. Ecosystem Services, 59, 101504. https://doi.org/10.1016/j.ecoser.2022.101504

Dressel, S., Sandström, C., Ericsson, G., 2014. A meta-analysis of studies on attitudes toward bears and wolves across Europe 1976–2012. Conservation Biology, 29(2), 565-574. 10.1111/cobi.12420.

Franchini, M., Corazzin, M., Bovolenta, S., Filacorda, S., 2021. The Return of Large Carnivores and Extensive Farming Systems: A Review of Stakeholders' Perception at an EU Level. Animals: an Open Access Journal from MDPI, 11(6), 1735. https://doi.org/10.3390/ani11061735

Giergiczny, M., Swenson, J., Zedrosser, A., Selva, N., 2022. Large carnivores and naturalness affect forest recreational value. Scientific Reports, 12(1), 13692. https://doi.org/10.1038/s41598-022-17862-0

Gould, R., Klain, S., Ardoin, N., Satterfield, T., Woodside, U., Hannahs, N., Daily, G., Chan, K., 2014. A Protocol for Eliciting Nonmaterial Values Through a Cultural Ecosystem Services Frame. Conservation biology, 29(2), 575-586. https://doi.org/10.1111/cobi.12407

Grossmann, C., László, P., Ortseifen, D., Kimmig, E., Cattoen, E.M., Schraml, U., 2020. Human-Large Carnivores Co-existence in Europe – A Comparative Stakeholder Network Analysis. Frontiers in Ecology and Evolution, 8, 266. https://doi.org/10.3389/fevo.2020.00266

Haines-Young, R., Potschin, M.B., 2013. Common International Classification of Ecosystem Services (CICES), Version 4.3. Report to the European Environment Agency.

Haines-Young, R., Potschin, M. B., 2018. Common International Classification of Ecosystem Services (CICES) V5.1 and guidance on the Application of the Revised Structure. URL: https://cices.eu/content/uploads/sites/8/2018/01/Guidance-V51-01012018.pdf

Horgan, F., Mundaca, E., Crisol-Martínez, E., 2021. Emerging Patterns in Cultural Ecosystem Services as Incentives and Obstacles for Raptor Conservation. British Birds, 2(2), 185-206. https://doi.org/10.3390/birds2020014

Hovardas, T., 2020. A Social Learning Approach for Stakeholder Engagement in Large Carnivore Conservation and Management. Frontiers in Ecology and Evolution, 8, 525278. https://doi.org/10.3389/fevo.2020.525278

Kansky, R., Knight, A.T., 2014. Key factors driving attitudes towards large mammals in conflict with humans. Biological Conservation, 179, 93-105. https://doi.org/10.1016/j.biocon.2014.09.008

Karlsson, J., Sjöström, M., 2008. Direct use values and passive use values: Implications for conservation of large carnivores. Biodiversity and Conservation, 17(4), 883–891. https://doi.org/10.1007/s10531-008-9334-3

Kavčič, I., Majić Skrbinšek, A. 2023. Recognizing the benefits of large carnivore presence in Slovenia: a focus group study = Prepoznavanje koristi, ki jih prinaša prisotnost velikih zveri : metoda fokusnih skupin. In: Babič, S., Serec Hodžar, A., Golež Kaučič, M. (Eds.). Thinking animals: international conference : programme and abstracts : October 16-19 2023, Ljubljana, Slovenia = Misliti živali: mednarodna konferenca: program in povzetki : 16.-19. Ljubljana, Slovenija. 1st e-ed. Ljubljana: Založba ZRC, ZRC SAZU, 2023. p. 28-29. ISBN 978-961-05-0780-2.

Krofel, M., Fležar, U., Černe, R., Hočevar, L., Konec, M., Majić Skrbinšek, A., Skrbinšek, T., Wilson, S., Bele, B., Črtalič, J., Gomerčić, T., Hvala, T., Kubala, J., Kvapil, P., Mavec, M., Molinari-Jobin, A., Molinari, P., Pazhenkova, E., Potočnik, H., Oliveira, T., 2025. Saving the Dinaric lynx: multidisciplinary monitoring and stakeholder engagement support large carnivore restoration in human-dominated landscape, 35(3), e70052. 10.1101/2024.10.15.617164.

Larsson, S.B., Larsson, S., Bennett, J., Sjölander-Lindqvist, A., 2022. Contextualizing negative attitudes to wildlife and wildlife governance in the moral economy of Swedish farmers. Frontiers in Conservation Science, 3, 1014769. https://doi.org/10.3389/fcosc.2022.1014769

Linnell, J.D.C., Lescureux, N., Majić Skrbinšek, A., von Arx, M., Salvatori, V., 2013. From conflict to coexistence: insights from multi-disciplinary research into the relationships between people, large carnivores and institutions. Results from a stakeholder workshop on large carnivores in Brussels, 25, January, 2013. [online] Accessed 17 June, 2025.

Linnell, J.D.C., Immerzeel, B., 2023. The potential for large carnivore-based wildlife tourism in Norway: a critical review. NNA Report 2167. Norwegian Institute for Nature Research. [online] Accessed 23 October, 2023.

Lischka, S.A., Teel, T.L., Johnson, H.E., Crooks, K.R., 2019. Understanding and managing human tolerance for a large carnivore in a residential system. Biological Conservation, 238, 108189. https://doi.org/10.1016/j.biocon.2019.07.034

Litosseliti, L., 2003. Using focus groups in research. London, UK, Continuum, 104 pp.

Lute, M., Carter, N., López-Bao, J. C., Linnell, J.D., 2018. Conservation professionals agree on challenges to coexisting with large carnivores but not on solutions. Biological Conservation, 218, 223-232. https://doi.org/10.1016/j.biocon.2017.12.035

Lute, M.L., Carter, N.H., López-Bao, J.V., Linnell, J.D., 2020. Conservation professionals' views on governing for coexistence with large carnivores. Biological Conservation, 248, 108668. https://doi.org/10.1016/j.biocon.2020.108668

Majić Skrbinšek, Skrbinšek, T., Knauer, F., Reljić, S., Molinari-Jobin, A., Calderola, S., Marinko, U., Bragalanti, N., 2019. Public attitudes, perceptions, and beliefs about bears and bear management and LIFE DINALP BEAR project visibility in media. Final report of the Action D3, project LIFE DINALP BEAR. University of Ljubljana. 71 pp.

Marino, F., Kansky, R., Shivji, I., Di Croce, A., Ciucci, P., Knight, A.T., 2020. Understanding drivers of human tolerance to gray wolves and brown bears as a strategy to improve landholder–carnivore coexistence. Conservation Science and Practice, 3(3), e265. https://doi.org/10.1111/csp2.265

Millennium Ecosystem Assessment, Overview of the Milliennium Ecosystem Assessmen. 2020. URL: https://www.millenniumassessment.org/en/About.html#1

Nanni, V., Caprio, E., Bombieri, G., Schiaparelli, S., Chiorri, C., Mammola, S., Pedrini, P., Penteriani, V., 2020. Social Media and Large Carnivores: Sharing Biased News on Attacks on Humans. Frontiers in Ecology and Evolution, 8, 71. https://doi.org/10.3389/fevo.2020.00071

Oražem, Tomažič, I., 2018. The vocational upper secondary schools students' knowledge and their attitudes toward wolves. Journal of Baltic Science Education, 17(6), 918-934. 10.33225/jbse/18.17.918.

Oražem, V., Tomažič, I., Kos, I., Nagode, D., Randler, C., 2019. Wolves' Conservation through Educational Workshops: Which Method Works Best? Sustainability, 11(4), 1124. 10.3390/su11041124.

Oražem, V., Smolej, T., Tomažič, I., 2021. Students' Attitudes to and Knowledge of Brown Bears (Ursus arctos L.): Can More Knowledge Reduce Fear and Assist in Conservation Efforts? Animals, 11(7), 1958. https://doi.org/10.3390/ani11071958

Oražem, V., Majić Skrbinšek, A., Šorgo, A., Tomažič, I., 2022. Factors Affecting Zoo Visitors' Conservation Beliefs and Knowledge of Large Carnivores in 2009 and a Dozen Years Later. Sustainability, 14(2), 890. https://doi.org/10.3390/su14020890

Palacios-Pacheco, S., Martín-López, B., Expósito-Granados, M., Requena-Mullor, J., Lozano, J., Sánchez-Zapata, J.A., Morales-Reyes, Z., Castro, A., 2024. Carnivores' contributions to people in Europe. Ecology and Society, 29(3), 9. https://doi.org/10.5751/ES-15249-290309

Pettersson, H., Holmes, G., Sait, S., Quinn, C., 2022. 'They Belong Here': Understanding the conditions of human-wolf coexistence in north-western Spain. Conservation and Society, 20(2), 113-123. https://doi.org/10.4103/cs.cs_13_21

Piscopo, N., Gentile, L., Scioli, E., Eguren, V. G., Carvajal Urueña, A. M., García, T. Y., Alberti, J. P., Esposito, L. 2021. Management Models Applied to the Human-Wolf Conflict in Agro-Forestry-Pastoral Territories of Two Italian Protected Areas and One Spanish Game Area. Animals, 11(4), 1141. https://doi.org/10.3390/ani11041141

Redpath, S., Linnell, J.D., 2016. Don't forget to look down-collaborative approaches to predator conservation. Biological Reviews, 92(4), 2157-2163. https://doi.org/10.1111/brv.12326

Rode, J., Flinzberger, L., Karutz, R., Berghöfer, A., Schröter-Schlaack, C., 2021. Why so negative? Exploring the socio-economic impacts of large carnivores from a European perspective. Biological Conservation, 255, 108918. https://doi.org/10.1016/j.biocon.2020.108918

Salvatori, V., Balian, E.V., Blanco, J.C., Carbonell, X., Ciucci, P., Demeter, L., Marino, A., Panzavolta, A., Sólyom, A., von Korff, Y., Young, J.C., 2021. Are Large Carnivores the Real Issue? Solutions for Improving Conflict Management through Stakeholder Participation. Sustainability, 13(8), 4482. https://doi.org/10.3390/su13084482

Slagle, K.M., Zajac, R.M., Bruskotter, J.T., Wilson, R.S., Prange, S., 2013. Building tolerance for bears: A communications experiment. Journal of Wildlife Management, 77(4), 863-869. https://doi.org/10.1002/jwmg.515

Slovák, Ľ., Danek, J., Danek, T., 2023. The use of focus groups in cultural ecosystem services research: a systematic review. Humanities and Social Sciences Communications, 10(45), 1-13. https://doi.org/10.1057/s41599-023-01530-3

Toomey, A., 2023. Why facts don't change minds: Insights from cognitive science for the improved communication of conservation research. Biological Conservation, 278, 109886. https://doi.org/10.1016/j.biocon.2022.109886

ABS

Original Research

Distribution of two invasive alien diatom species *Achnanthidium delmontii* and *Achnanthidium druartii* in Slovenia

Aleksandra Krivograd Klemenčič [1,2,*], Tadeja Šter [1]

Abstract

The distribution of two invasive diatom species in Slovenia, *Achnanthidium delmontii* (ADMO) and *Achnanthidium druartii* (ADRU), was investigated in this study. Data from 87 rivers and 11 lakes collected between 2019 and 2024 in the frame of national monitoring of the ecological status of surface waters were included. ADMO was present in 40 rivers (46%) and was dominant (>5% relative abundance) in 27 rivers (31%), with the highest abundance reaching 77% in the Bolska River. It was rare in lakes, detected in only three lakes (27%). ADRU was detected in 12 rivers (14%) and was dominant in four rivers (5%), with the highest abundance reaching 27% in the Drava River. In contrast, ADRU was common in lakes, with a presence in eight lakes (73%), dominating Lake Slivnica and Lake Pernica. ADMO presence was associated with reduced diatom species diversity and evenness, although no direct ecological impact was observed. ADMO was present mainly in upland river sections, while ADRU was more frequent in lowland river sections and lakes. The results of this study confirmed the invasive character of ADMO, whereas ADRU did not affect species diversity and evenness of the diatom assemblages, and thus its invasive character could not be confirmed.

Keywords

Achnanthidium delmontii; *Achnanthidium druartii*; alien invasive species; diatoms; Slovenia

1 Slovenian Environment Agency, Vojkova 1b, SI-1000 Ljubljana, Slovenia

2 Faculty of Civil and Geodetic Engineering, University of Ljubljana, Hajdrihova 28, SI-1000 Ljubljana, Slovenia

* **Corresponding author:**
E-mail address: aleksandrakrivograd@gmail.com

Citation: Krivograd Klemenčič, A., Šter, T., (2025). Distribution of two invasive alien diatom species *Achnanthidium delmontii* and *Achnanthidium druartii* in Slovenia. Acta Biologica Slovenica 68 (4)

Received: 26.05.2025 / **Accepted:** 01.08.2025 / **Published:** 08.08.2025

https://doi.org/10.14720/abs.68.4.22822

Razširjenost dveh invazivnih tujerodnih vrst diatomej *Achnanthidium delmontii* in *Achnanthidium druartii* na območju Slovenije

Izvleček

V raziskavi smo proučevali razširjenost dveh invazivnih tujerodnih vrst diatomej v Sloveniji, *Achnanthidium delmontii* (ADMO) in *Achnanthidium druartii* (ADRU). V raziskavo smo vključili podatke iz 87 rek in 11 jezer zbranih med letoma 2019 in 2024 v okviru nacionalnega monitoringa ekološkega stanja površinskih voda. ADMO je bil prisoten v 40 rekah (46 %), dominantna vrsta (>5 % relativne abundance) je bil v 27 rekah (31 %), pri čemer je s 77 % v reki Bolski dosegel najvišjo zastopanost. ADMO je bil v jezerih redek, zaznan je bil le v treh jezerih (27 %). ADRU je bil prisoten v 12 rekah (14 %), dominantna vrsta je bil v štirih rekah (5 %), z največjo zastopanostjo 27 % v reki Dravi. V jezerih je bil ADRU pogost, prisoten je bil v osmih jezerih (73 %), prevladoval je v Slivniškem in Perniškem jezeru. Prisotnost ADMO je bila povezana z zmanjšano vrstno pestrostjo in enakomernostjo združb diatomej, čeprav neposrednega negativnega ekološkega vpliva nismo zaznali. ADMO se je večinoma pojavljal v povirnih delih rek, medtem ko je bil ADRU pogostejši v nižinskih delih rek in jezerih. Rezultati potrjujejo invazivni značaj ADMO, medtem ko invazivnost ADRU ni bila potrjena, saj njegova prisotnost ni pomembno vplivala na sestavo združb diatomej.

Ključne besede

Achnanthidium delmontii; *Achnanthidium druartii*; invazivne vodne vrste; diatomeje; Slovenija

Introduction

Primary producers in rivers and lakes form the basis of the food web, and as such, any change in their quantity or in the composition of their communities can result in a disturbed ecological balance in waterbodies, with a domino effect on higher trophic levels such as benthic macroinvertebrates and fish (Buczkó et al., 2022).

Among invasive benthic diatoms, the most attention in the past has been paid to one of the largest freshwater diatom species, *Didymosphenia geminata* (Lyngbye) W.M. Schmidt, which is native to Europe and North America but highly invasive and aggressive in New Zealand. It causes a lot of problems in freshwater systems, such as the formation of large and extensive mats that impact fish, aquatic plants, and insects, resulting in severe disturbances in food webs (Blanco and Ector, 2009). However, according to Taylor and Bothwell (2014), *D. geminata* blooms in New Zealand are not caused solely by the introduction of its cells in new areas, as similar, nearly synchronous blooms have occurred in areas where *D. geminata* is native, such as North America and Europe. Next to large-celled diatoms such as *D. geminata*, also small-celled diatoms (<25 µm), such as *Achnanthidium delmontii* Pérès, Le Cohu & Barthès (ADMO), and *Achnanthidium druartii* Rimet & Couté (ADRU), are considered invasive or potentially invasive species (e.g., Buczkó et al., 2022; Falasco et al., 2023; Ivanov, 2018). ADMO was discovered in 2007 and formally described in 2012, based on specimens from a French river (Pérès et al., 2012), while ADRU was first discovered in 2004 in the Rhone River in France and formally described in 2010 as a new species invading the rivers in France and Spain (Rimet et al., 2010).

Since then, ADMO has been reported from several countries in Europe (France, Germany, Hungary, Italy, Netherlands, Switzerland) and Asia (China) (Guiry and Guiry, 2022), while ADRU has been reported from several countries in Europe (Bulgaria, France, Germany, Netherlands, Serbia and Spain), North and South America, the Middle East, and Asia (Guiry and Guiry, 2022). ADMO has been reported in all Slovenian neighbouring countries, namely Hungary, Italy, Austria, and Croatia. Buczkó et al. (2022) reported the presence of ADMO in Hungary from 2015 onward and in Austria in several sections of the Danube River from 2013 onward. Falasco et al. (2023) reported on the first findings of ADMO in Italy in 2013 in the rivers of Liguria (NW-Italy). In Croatia, there are records of ADMO in the Drava River and the Danube River from 2019, which

were collected in the framework of Joint Danube Survey 4 (JDS4) (ICPDR, 2019). Information on the presence of ADRU in Slovenia's neighbouring countries is scarce. There is a record of ADRU from the Danube River in Austria from 2019, collected in the framework of JDS4 (ICPDR, 2019). However, according to our information, there is no available information on the presence of ADRU in Croatia, Hungary, or Italy.

In this research, the distribution of two potentially invasive, alien benthic diatom species, namely ADMO and ADRU, in the territory of Slovenia is presented for the first time. Moreover, for the sampling sites where ADMO and ADRU were identified, nutrient and organic matter loading, as well as species diversity and evenness, are also reported.

Materials and Methods

Phytobenthos sampling

Sampling of the phytobenthos was carried out in 2019–2024 as part of the national monitoring of surface water quality according to the Water Framework Directive (WFD) (Directive 2000/60/EC). Altogether, 247 phytobenthos samples were collected at 175 sampling sites in rivers, and 35 samples were collected at 32 sampling sites in lakes and reservoirs (hereinafter referred to as lakes) across Slovenia according to the national programmes for monitoring chemical and ecological status of surface waters (ARSO, 2017, 2022). In total, 87 rivers and 11 lakes were included in this research.

According to the Slovenian national methodologies for ecological status assessment of rivers and lakes using phytobenthos and macrophytes (MOP, 2016a, 2016b), river samples were collected once per year from June to September, up to a depth of 60 cm and at a distance of at least 1 m from the riverbanks, or in cases of smaller rivers, at least 10% of the river's width from the banks. A multi-habitat approach was used, meaning that the samples were collected from a range of habitats (e.g., gravel, mud, riffle, and pool) differing in substrate type, depth, current velocity, and shadiness. For lakes, the national methodology applies only to natural lakes (Lake Bled and Lake Bohinj). Multi-habitat sampling was also performed in these and other lakes. Phytobenthos was collected along a 50 m shoreline stretch that included various habitats characterised by varying depth, substrate, and shading conditions. In each lake, phytobenthos was sampled once per year, typically between June and September, and usually at three different sampling sites representing the dominant substrate type. An exception was Lake Vogršček, where only two sites were sampled. Sampling in Lake Pernica deviated from the recommended timeframe, as it was conducted in October, outside the June-September window defined by national methodology for natural lakes. In both rivers and lakes, phytobenthos was removed from the substrate (stones, pebbles, wood, macrophytes, etc.) using a toothbrush in a tray containing a small amount of river or lake water. The material was homogenised and poured into a wide-necked plastic bottle. Each sample was fixed with ethanol to a final concentration of 70%.

Laboratory analyses

The phytobenthos samples were transferred to the laboratory and treated with concentrated nitric acid (HNO_3) to remove cell contents and other organic matter, following the standard procedure (SIST EN 14407, 2014) and the instructions of the Slovenian national methodologies for ecological status assessment of rivers and lakes using phytobenthos and macrophytes (MOP, 2016a, 2016b). Cleaned samples were mounted in Naphrax® (Brunel Microscopes, Chippenham, Wiltshire, UK), a medium with a high refractive index, for permanent slide preparation. Permanent slides were examined using a light microscope (Leica DM RB, Germany) at 1000× magnification. Diatom identification and enumeration were performed according to the standard procedure (SIST EN 14407, 2014) and the Slovenian national methodologies (MOP, 2016a, 2016b). For each permanent slide, at least 500 valves were counted and identified to the species or lower taxonomic level. Diatom identification and nomenclature followed the identification monograph by Lange-Bertalot et al. (2017). Scanning electron microscope (SEM) images were obtained using a JEOL JSM-7500F microscope and sample preparation according to Hasle and Fryxell (1970).

Statistical analyses

The abundance of diatom species was expressed as relative counts (in %). The levels of nutrient and organic loading in the investigated rivers were evaluated using the Trophic Index (TI) (Rott et al., 1999) and Saprobic Index (SI) (Rott et al., 1997), respectively. For the investigated lakes,

nutrient loading levels were evaluated using the TI (Rott et al., 1999). Both indices were calculated solely based on diatom data, followed by an assessment of the ecological trophic status (for both rivers and lakes) and the ecological saprobic status (for rivers only), in accordance with the Slovenian national methodologies for ecological status assessment of rivers and lakes using phytobenthos and macrophytes (MOP, 2016a, 2016b).

Species diversity was assessed using the Shannon-Wiener diversity index (SW), and species evenness was assessed using the Evenness index (E). Both indices were calculated using OMNIDIA 6.0.9, a software tool for the calculation of 18 diatom water quality indices. Correlations between the abundance of target species, species diversity, and species evenness were calculated using the Pearson correlation coefficient and Microsoft Excel software.

Results and Discussion

Altogether, 403 diatom taxa were identified in river samples and 238 diatom taxa in lake samples. In Slovenia, benthic diatoms are used, together with macrophytes, phytoplankton, fish, benthic macroinvertebrates, and macroalgae, as a biological quality element for the assessment of the ecological status of surface waters, namely the quality of the structure and functioning of aquatic ecosystems, as defined by the WFD (Directive 2000/60/EC). Two diatom indices, the Trophic index (Rott et al., 1999) and the Saprobic index (Rott et al., 1997), are used in Slovenia to assess nutrient and organic matter loading in rivers, respectively. For natural lakes in Slovenia (Lake Bled and Lake Bohinj), the trophic state is assessed using benthic diatoms and the Trophic index (Rott et al., 1999), in combination with phytoplankton, in accordance with national methodology (MOP, 2016b). For other lakes, where there is no national methodology, benthic diatoms and the Trophic Index (Rott et al., 1999) may still be used as an additional or indicative assessment tool, but are not formally part of the ecological status assessment under national monitoring.

Morphology of Achnanthidium delmontii and Achnanthidium druartii

The morphology of ADMO is described in detail in the work of Pérès et al. (2012) and that of ADRU in the work of Rimet et al. (2010), in which both species were first formally described. ADMO and ADRU both belong to the group of taxa related to *Achnanthidium pyrenaicum* (Hustedt) Kobayasi based on the stria density, which is around 20 µm, and the linear-lanceolate valve shape.

According to Pérès et al. (2012), the main morphological characteristics of ADMO (Figure 1) are as follows: Valves are linear with rounded apices, becoming elliptical in smaller individuals; valve length ranges from 7.3 to 21.4 µm, and valve width from 3.3 to 5.1 µm. On the raphe valve, the axial area is narrow, and the central area is irregular, typically forming a rectangular fascia, but a shortened stria may be present on one of the margins. The raphe is filiform and straight, with distinct central pores. Striae are slightly radial, numbering 20–26 in 10 µm in the central part of the valve, and up to 35 in 10 µm near the apices. On the rapheless valve, the axial area is acicular. Striae are parallel to slightly radiate near the apices; in most cases, two striae in the central part are slightly more widely spaced. Striae are slightly radial, numbering 18–22 in 10 µm in the central part and up to 25 in 10 µm at the apices.

Under light microscopy, ADMO can be distinguished from similar taxa by a rectangular fascia on the raphe valve and a typical irregular cell shape.

The main morphological characteristics of ADRU (Figure 2) according to Rimet et al. (2010) are: Valves are lanceolate with slightly subrostrate ends, never capitate; valve length ranges from 12 to 29 µm, and valve width from 3.9 to 5.8 µm. The raphe sternum is larger in the middle of the valve than in the extremities. The rapheless valve is convex, with a narrow, straight sternum that is only slightly enlarged in the centre. Striae are very weakly radiate throughout on both valves. Occasionally, short striae are inserted near the middle of the valve. For both valves, stria density is 15-22 in 10 µm in the central part of the valve and approximately 40-50 in 10 µm near the apices.

Under light microscopy, ADRU can be distinguished from similar taxa, namely *A. convergens* (Kobayasi) Kobayasi, *A. deflexum* (Reimer) Kingston, *A. japonicum* (Kobayasi) Kobayasi, *A. latecephalum* Kobayasi, *A. pyrenaicum* (Hustedt) Kobayasi and *A. rivulare* Potapova & Ponader, by its generally wider and longer valve dimensions. However, in many cases, the size may overlap (Rimet et al., 2010). According to current knowledge, only *A. pyrenaicum* and *A. rivulare* have been recorded in Slovenia among the above-listed taxa. Another interesting characteristic that differentiates ADRU is that it shows an important stria density difference between the centre and the apices.

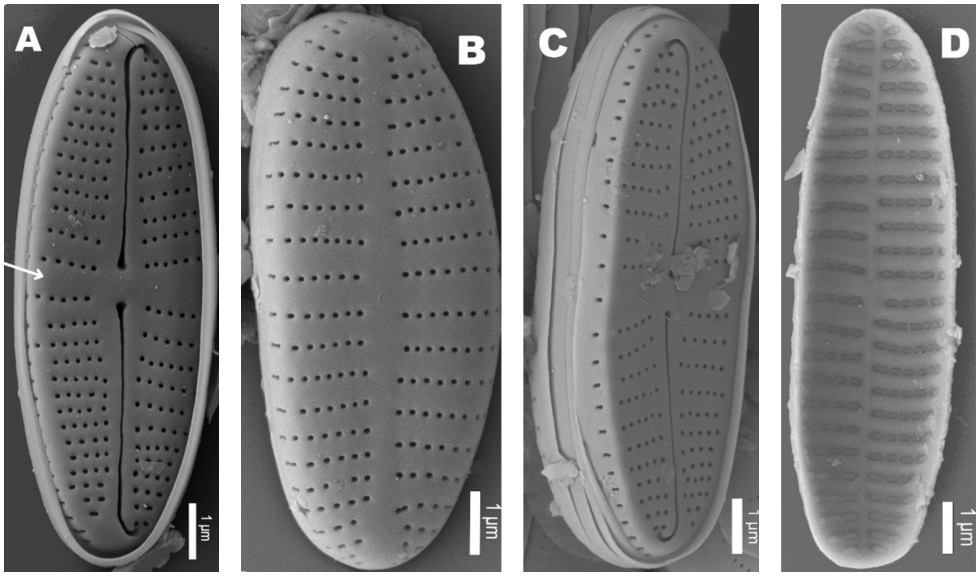


Figure 1. Scanning electron microscope (SEM) images of Achnanthidium delmontii (ADMO). SEM: external view of the raphe valve with a rectangular fascia (marked with an arrow) (A), external view of the rapheless valve (B), external girdle view of the raphe valve (C), internal view of the rapheless valve (D). One of the main characteristics of ADMO is irregular cell shape (B, C, D).

Slika 1. Fotografije z vrstičnim elektronskim mikroskopom (SEM) Achnanthidium delmontii (ADMO). SEM: pogled na zunanji del valve z rafo in dobro vidnim pravokotnim praznim prostorom (puščica) (A), pogled na zunanji del valve brez rafe (B), pogled na zunanji del valve z rafo z bočne strani (C), pogled na notranji del valve brez rafe (D). Ena glavnih značilnosti ADMO je nepravilna oblika celic (B, C, D).

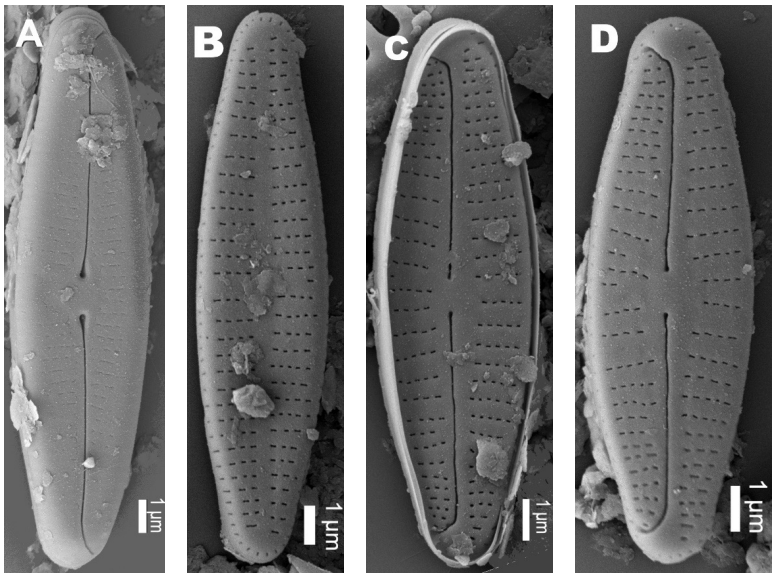


Figure 2. Scanning electron microscope (SEM) images of *Achnanthidium druartii* (ADRU). SEM: external views of the raphe valve (A, D), external view of the rapheless valve (B), internal view of the raphe valve (C).

Slika 2. Fotografije z vrstičnim elektronskim mikroskopom (SEM) *Achnanthidium druartii* (ADRU). SEM: pogled na zunanji del valve z rafo (A, D), pogled na zunanji del valve brez rafe (B), pogled na notranji del valve z rafo (C).

Occurrence of Achnanthidium delmontii and Achnanthidium druartii in Slovenia

Table 1 provides an overview of the sampling sites where ADMO and ADRU were detected, including national sampling site codes, date of sampling, and the relative abundance of both species expressed as a percentage of counted valves. The table also includes information on nutrient and organic matter loading at each site, as well as measures of species diversity and evenness, expressed as the Shannon-Wiener diversity index and the Evenness index, respectively.

Regarding rivers, a total of 247 phytobenthos samples collected at 175 sampling sites located on 87 rivers were included in the study. Among all investigated rivers, ADMO was present in 100 samples (41%) collected at 74 sampling sites (42%) from 40 rivers (46%) (Table 1). ADMO was a dominant species (more than 5% in relative abundance) in 56 phytobenthos samples (23%) collected at 45 sampling sites (26%) from 27 rivers (31%). The highest abundance of ADMO was recorded in the Bolska River (Figure 3), specifically at sampling sites Dolenja vas and Čeplje in August 2022, with relative abundances of 77% and 64%, respectively. Regarding ADMO representation, the Bolska River was followed by the Mirna River, where at the sampling site Dolenji Boštanj, the ADMO relative abundance reached 69%, also in August 2022.

ADRU was present in 32 river samples (13%) collected at 22 sampling sites (13%) from 12 rivers (14%) (Table 1). ADRU was a dominant species (more than 5% in relative abundance) in 11 phytobenthos samples (5%) collected at eight sampling sites (5%) from 4 rivers (5%). The highest abundance of ADRU was detected in the Drava River (Figure 4), specifically at sampling site Ruše in September 2021, with a relative abundance of 27%, followed by sampling site Ranca in August 2024 with 20%, and sampling site Tribej in September 2021 with a relative abundance of 19%.

In Slovenian lakes, 35 phytobenthos samples were collected at 32 sampling sites across 11 lakes. Among the two investigated species, ADMO was relatively rare, being detected in only five samples (14%) across three lakes. It was never dominant in any of the samples, with the highest recorded abundance reaching just 0.38% in the Gajševsko Lake (GaFB03, June 2023). In contrast, ADRU was more widespread and frequently encountered, being present in 15 samples (43%) in 8 of the 11 studied lakes. It showed dominance (relative abundance >5%) in 3 samples from two lakes. The highest relative abundance of ADRU was observed in Lake Slivnica (10% at SlFB04, May 2023), followed by Lake Pernica (6.6% at P2FB05, October 2024).

Table 1. List of sampling sites where invasive diatoms *Achnanthidium delmontii* (ADMO) and *Achnanthidium druartii* (ADRU) were detected, including national sampling site codes, date of sampling, relative abundance of both species expressed in percentage of counted valves, Trophic index (TI), Saprobic index (SI), ecological trophic status (EQR TI), ecological saprobic status (EQR SI), Shannon-Wiener diversity index (SW), and Evenness index (E). + indicates that the species was observed during the qualitative examination of the sample but not during valve counting. / indicates that the ecological status assessment methodology has not been developed. Blue colour indicates high ecological status, green indicates good ecological status, and yellow indicates moderate ecological status.

Tabela 1. Seznam vzorčnih mest z ugotovljeno prisotnostjo invazivnih diatomej *Achnanthidium delmontii* (ADMO) in *Achnanthidium druartii* (ADRU) z navedenimi nacionalnimi šiframi vzorčnih mest, datumom vzorčenja, relativno pogostostjo obeh vrst izraženo v odstotkih od preštetih polovic lupinic, trofičnim indeksom (TI), saprobnim indeksom (SI), ekološkim trofičnim (EQR TI) in ekološkim saprobnim (EQR SI) stanjem, Shannon-Wiennerjevim diverzitetnim indeksom (SW) in indeksom Evenness (E). Z znakom + je označeno, da je bila vrsta zaznana le med kvalitativnim pregledom vzorca in ne med štetjem polovic lupinic. Z znakom / je označeno, da metodologija vrednotenja ekološkega stanja ni razvita. Modra barva označuje zelo dobro ekološko stanje, zelena označuje dobro ekološko stanje in rumena zmerno ekološko stanje.

River/Lake	Sampling site	Date of sampling	ADMO (%)	ADRU (%)	TI	SI	EQR TI	EQR SI	SW	E
Mura	Bad Radkersburg	21.01.2021	33.84		2.63	2.03	0.71	0.60	3.45	0.77
Mura	Bad Radkersburg	30.08.2024	17.15		1.92	1.69	1.00	1.00	2.68	0.62
Mura	Ceršak	21.01.2021	11.04		2.90	2.09	0.61	0.58	3.96	0.82
Mura	Ceršak	30.08.2024	4.16	+	2.28	1.87	0.85	0.81	3.35	0.73
Mura	Mele	21.01.2021	6.13		2.93	2.07	0.61	0.58	3.84	0.78
Mura	Mele	30.08.2024	11.30		1.93	1.71	1.00	1.00	3.02	0.66
Mura	Mota	14.02.2023	0.78		2.71	1.87	0.69	0.80	3.85	0.78
Mura	Gibina	14.02.2023	5.86		3.15	2.16	0.47	0.55	3.75	0.77
Ledava	Domajinci	7.03.2019		2.39	2.72	1.89	0.75	0.77	4.07	0.75

River/Lake	Sampling site	Date of sampling	ADMO (%)	ADRU (%)	TI	SI	EQR TI	EQR SI	SW	E
Drava	Tribej	22.09.2021		18.70	2.52	1.94	0.75	0.71	4.4	0.82
Drava	Tribej	25.09.2024		17.81	2.34	1.89	0.83	0.77	3.85	0.74
Drava	Ruše	22.09.2021	12.08	26.53	2.28	1.79	0.85	0.93	3.86	0.75
Drava	Ruše	21.08.2024	3.28	13.68	2.19	1.76	0.89	0.96	3.96	0.77
Drava	Krčevina pri Ptuju	22.09.2021	21.97	1.98	2.04	1.66	0.96	1.00	3.12	0.67
Ptujsko jezero	Ranca	16.09.2021	0.39	13.80	1.83	1.51	1.00	1.00	3.87	0.76
Drava	Ranca	21.08.2024		19.68	2.55	1.69	0.74	1.00	4	0.75
Drava	Prepolje	6.08.2019	0.19	5.78	2.88	2.03	0.62	0.60	4.53	0.79
Drava	Prepolje	24.08.2022		1.93	2.80	1.90	0.65	0.76	4.53	0.85
Drava	Gorišnica	6.08.2019	0.77	4.44	2.67	1.90	0.70	0.76	4.37	0.82
Drava	Gorišnica	24.08.2022	5.08		2.83	1.98	0.64	0.66	4.54	0.83
Drava	Borl	16.09.2021	55.00	3.14	2.57	2.06	0.73	0.59	2.49	0.57
Drava	Borl	23.08.2024	12.28	1.56	2.52	1.71	0.75	1.00	3.84	0.75
Drava	Ormož	6.08.2019	4.79	1.80	2.63	1.98	0.71	0.66	5.01	0.83
Drava	Ormož	23.12.2020	4.23	0.38	2.54	1.99	0.75	0.64	4.41	0.79
Drava	Ormož	4.08.2022	2.47	0.38	2.95	1.94	0.59	0.70	4.61	0.83
Drava	Ormož	23.08.2024	5.81	6.97	2.54	1.77	0.75	0.95	4.39	0.79
Drava	Grabe	15.09.2021	26.21	+	1.50	1.48	1.00	1.00	2.03	0.55
Meža	Topla	17.07.2024	+		1.93	1.58	0.94	1.00	2.87	0.63
Mislinja	Mala vas	17.07.2024	0.39		1.85	1.53	1.00	1.00	2.33	0.57
Mislinja	Otiški vrh	17.07.2024	0.78		2.15	1.67	0.98	0.92	2.72	0.59
Dravinja	Videm pri Ptuju	6.08.2024	15.5		1.84	1.50	1.00	1.00	2.54	0.59
Pesnica	Zamušani	21.09.2023	1.89		2.28	1.98	0.96	0.74	3.98	0.85
Sava Dolinka	Zelenci	11.07.2023	1.75		1.43	1.41	1.00	1.00	3.07	0.61
Sava Bohinjka	nad izlivom Jezernice	29.08.2022	1.37		2.14	1.65	1.00	1.00	3.91	0.82
Sava Bohinjka	Bodešče	29.08.2022	4.89		2.54	2.02	1.00	0.75	2.66	0.58
Sava	Struževo	28.07.2021	6.9		1.39	1.51	1.00	1.00	2.72	0.72
Sava	Dragočajna	21.06.2022	1.36	0.39	1.78	1.65	1.00	1.00	3.71	0.71
Sava	Dragočajna	14.08.2024		9.23	2.45	1.79	0.70	0.77	4.68	0.87
Sava	Medno	14.08.2024	12.08		1.70	1.53	1.00	1.00	2.57	0.58
Sava	Šentjakob	26.07.2019	0.78		1.84	1.78	1.00	0.78	3.06	0.6
Sava	Kresnice	12.07.2023	4.9		1.82	1.56	1.00	1.00	2.53	0.58
Sava	Podkraj	16.08.2024	9.76		2.00	1.55	0.95	1.00	2.86	0.59
Sava	Vrhovo	20.08.2021	8.57		2.66	1.98	0.70	0.66	4.31	0.83
Sava	Vrhovo	23.06.2023	0.39		2.34	1.94	0.82	0.71	4.08	0.74
Sava	Brestanica	20.08.2021	1.86		2.81	2.03	0.65	0.59	4.04	0.74
Sava	Podgračeno	14.09.2021	0.77		2.53	1.99	0.75	0.64	3.95	0.76
Sava	Jesenice na Dolenjskem	21.08.2019	50.65		2.68	1.92	0.69	0.74	2.95	0.56
Sava	Jesenice na Dolenjskem	11.08.2020	1.36		2.23	1.59	0.88	1.00	3.1	0.6
Sava	Jesenice na Dolenjskem	14.09.2021	6.81		2.62	2.07	0.72	0.58	3.93	0.78
Sava	Jesenice na Dolenjskem	17.08.2022	4.64	0.77	2.85	2.01	0.63	0.61	3.98	0.79
Sava	Jesenice na Dolenjskem	9.10.2023	9.3		2.03	1.75	0.97	0.98	3.61	0.74
Sava	Jesenice na Dolenjskem	14.08.2024	16.01		1.61	1.48	1.00	1.00	1.93	0.49
Kokra	Kranj	26.07.2021	28.85		1.89	1.61	1.00	1.00	3.29	0.71
Sora	Medvode	22.06.2021	31.52		1.44	1.45	1.00	1.00	1.67	0.42

River/Lake	Sampling site	Date of sampling	ADMO (%)	ADRU (%)	TI	SI	EQR TI	EQR SI	SW	E
Poljanska Sora	Na Dobravi	1.07.2019	1.32		1.90	1.72	1.00	1.00	3.21	0.67
Poljanska Sora	Na Dobravi	22.06.2021	26.21		1.48	1.48	1.00	1.00	2.27	0.58
Selška Sora	Vešter	22.06.2021	6.83		1.76	1.59	1.00	1.00	3.31	0.78
Kamniška Bistrica	Ihan	26.08.2024	28.37		2.34	1.70	1.00	1.00	3.19	0.64
Kamniška Bistrica	Beričevo	26.08.2024	3.1		2.46	1.88	1.00	0.87	2.38	0.49
Pšata	Bišče	1.07.2021	13.23		2.74	1.99	0.52	0.73	4.09	0.79
Pšata	Bišče	11.07.2024	21.79		2.74	1.97	0.52	0.74	4.18	0.77
Mirna	Dolenji Boštanj	17.06.2019	0.39		2.60	2.06	0.58	0.71	4.05	0.76
Mirna	Dolenji Boštanj	1.08.2022	68.95	0.30	2.47	1.87	0.64	0.78	1.99	0.42
Sotla	Rigonce	14.09.2021		0.39	2.64	1.92	1.00	1.00	3.44	0.69
Bistrica	Zagaj	17.06.2019	25.58		2.06	1.58	1.00	1.00	3.48	0.73
Kolpa	Osilnica	4.07.2024	6.99		1.42	1.55	1.00	1.00	3.14	0.71
Kolpa	Radenci	14.06.2021	44.53		1.51	1.53	1.00	1.00	2.75	0.62
Kolpa	Radoviči (Metlika) - Bubnjarci	4.08.2021	24.43	0.38	2.11	1.60	0.68	1.00	4.03	0.76
Kolpa	Radoviči (Metlika) - Bubnjarci	4.07.2024	14.45	0.40	2.29	1.68	0.61	1.00	4.37	0.86
Lahinja	Geršiči	12.09.2023	+	5.56	2.32	1.67	0.76	1.00	4.23	0.81
Gruberjev prekop	Ljubljana	25.07.2022	0.39		2.73	2.02	0.56	0.75	4.26	0.81
Gruberjev prekop	Ljubljana	13.09.2023	5.09		2.76	1.99	0.55	0.76	3.83	0.79
Iščica	Ižanska cesta	17.08.2022	+		2.54	1.95	0.61	0.72	3.7	0.78
Iščica	Ižanska cesta	11.07.2024	0.78		2.56	1.86	0.60	0.75	3.21	0.69
Mali Graben	Dolgi most	21.06.2022	1.15		2.40	1.73	0.67	1.00	3.16	0.64
Gradaščica	Dvor	21.06.2022	15.59		2.15	1.73	1.00	1.00	3.29	0.71
Savinja	Grušovlje	16.07.2024	1.94		2.05	1.52	1.00	1.00	2.34	0.54
Savinja	Medlog	4.09.2024	5.03		1.98	1.57	1.00	1.00	2.46	0.57
Savinja	Veliko Širje	4.09.2024	17.58		2.05	1.63	1.00	1.00	3.03	0.65
Dreta	Spodnje Kraše	10.09.2021	15.67		2.16	1.70	1.00	1.00	3.48	0.7
Paka	Ločan	10.09.2021	8.43		2.60	1.87	0.77	1.00	3.82	0.75
Paka	Šoštanj	16.07.2024	2.73		2.47	1.93	1.00	1.00	3.23	0.67
Paka	Slatina	16.07.2024	10.4		3.05	2.54	0.69	0.60	3.06	0.73
Bolska	Čeplje	2.08.2022	63.99		2.51	1.95	1.00	0.92	2.12	0.45
Bolska	Dolenja vas	2.08.2022	76.67		2.03	1.81	1.00	1.00	1.59	0.33
Gračnica	Gračnica	25.08.2023	8.54		1.87	1.64	1.00	1.00	2.59	0.61
Krka	Soteska	20.08.2020	0.19		2.38	1.94	0.68	0.78	3.31	0.64
Krka	Krška vas	20.08.2020	1.74		2.50	1.86	0.92	1.00	4.32	0.8
Radulja	Grič pri Klevevžu	25.08.2023		3.50	1.85	1.65	1.00	1.00	2.97	0.64
Soča	Solkanski jez	30.08.2019	4.14		1.73	1.37	1.00	1.00	3.43	0.7
Soča	Solkanski jez	26.08.2020	4.82		1.77	1.47	0.99	1.00	3.12	0.69
Idrijca	nad Divjim jezerom	15.06.2021	3.88		1.41	1.49	0.91	1.00	3.09	0.7
Idrijca	Hotešk	18.09.2020	59.72		2.22	1.90	0.64	0.77	2.36	0.53
Trebuščica	Most pri Sovi	5.10.2023	8.15		1.71	1.59	0.74	1.00	3.02	0.72
Bača	Grapa	5.10.2023	8.81		1.55	1.44	1.00	1.00	1.39	0.37
Vipava	Velike Žablje	27.08.2020	1.51		2.18	1.88	0.83	0.87	2.91	0.63
Vipava	Velike Žablje	18.07.2023	0.77		2.39	1.82	0.73	1.00	2.2	0.45
Vipava	Miren	27.08.2020	5.71		2.66	1.90	0.43	0.73	3.92	0.76
Vipava	Miren	18.07.2023	5.87		2.63	1.94	0.44	0.71	4.05	0.8

River/Lake	Sampling site	Date of sampling	ADMO (%)	ADRU (%)	TI	SI	EQR TI	EQR SI	SW	E
Hubelj	Ajdovščina	5.03.2021	+		1.69	1.67	1.00	1.00	3.17	0.67
Nadiža	Robič	3.07.2024	3.14	0.39	1.65	1.65	0.98	1.00	3.18	0.76
Reka	Podgraje	10.03.2021	2.09		1.75	1.78	1.00	0.78	3.56	0.78
Reka	Topolc	21.06.2021	27.72		1.94	1.63	1.00	1.00	2.86	0.6
Reka	Topolc	7.09.2023	25.48		2.48	1.77	0.75	1.00	3.33	0.72
Reka	Cerkvenikov mlin	8.03.2021	6.73		2.39	1.98	1.00	0.82	4.21	0.83
Klivnik	Brid	25.02.2020		1.17	1.54	1.48	1.00	1.00	2.28	0.48
Molja	Zarečica	13.09.2023	6.65		2.36	1.81	0.81	0.77	4.33	0.8
Rižana	Dekani nad pregrado	17.06.2022	12.4		2.34	1.81	0.75	1.00	3.43	0.72
Rižana	Dekani nad pregrado	7.09.2023	14.85		1.60	1.53	1.00	1.00	3.25	0.72
Dragonja	Planjave	8.03.2021		4.31	1.37	1.46	1.00	0.80	3.29	0.71
Dragonja	Podkaštel	8.03.2021		5.34	1.65	1.71	0.98	0.72	3.74	0.74
Blejsko jezero	BlFB08	12.08.2019		0.20	2.41		0.43		3.52	0.68
Bohinjsko jezero	BOFB05	21.06.2022	+		1.32		0.75		3.87	0.74
Bohinjsko jezero	BOFB08	21.06.2022	+		1.25		0.77		3.77	0.74
Šmartinsko jezero	SmFB04	26.05.2023		1.23	2.02				4.64	0.87
Šmartinsko jezero	SmFB05	26.05.2023		0.97	1.59				3.04	0.61
Šmartinsko jezero	SmFB06	26.05.2023		1.95	1.57				3.77	0.77
Slivniško jezero	SlFB02	29.05.2023		5.34	2.12				3.8	0.76
Slivniško jezero	SlFB04	29.05.2023		10.02	2.30				3.77	0.75
Slivniško jezero	SlFB05	29.05.2023		4.30	2.43				3.91	0.76
Gajševsko jezero	GaFB02	14.06.2023	+		2.67				3.85	0.75
Gajševsko jezero	GaFB03	14.06.2023	0.38	+	2.64				4.05	0.77
Mola	MoFB02	1.08.2024		0.38	1.80				2.23	0.54
Vogršček	V2FB04	7.08.2024		0.60	1.62				2.65	0.65
Vogršček	V2FB06	7.08.2024		0.38	1.61				3.45	0.68
Klivnik	KLFB03	8.08.2024	+	+	1.68				2.62	0.63
Perniško jezero 2	P2FB03	23.10.2024		2.94	3.17				4.36	0.8
Perniško jezero 2	P2FB05	23.10.2024		6.61	2.98				2.3	0.54
Perniško jezero 2	P2FB06	23.10.2024		+	3.06				2.85	0.64

For the purpose of this study, SW and E of the samples with ADMO and/or ADRU were recorded (Table 1). Moreover, the correlations between ADMO and ADRU abundance and SW and E were calculated using the Pearson correlation coefficient. The results showed moderate negative correlations of -0.46 and -0.50 between ADMO abundance and SW, and ADMO abundance and E, respectively. These results indicate that the presence of ADMO in the samples is associated with lower diatom species diversity and lower diatom species evenness, which is most evident in the samples with the highest abundance of ADMO (Table 1). High abundance of ADMO in the samples, reaching up to 77%, is most probably due to its invasive character. According to Buczkó et al. (2022), AD, MO has a wide ecological range, which serves to confirm its potential invasive behaviour. High biodiversity and high species evenness are often associated with balanced environmental conditions and low anthropogenic pressure, which reflect good or high ecological status of water bodies. However, in the dataset used in this research, the river sections in which ADMO abundance was the highest (more than 60%) and diatom species diversity and evenness were the lowest are associated with good or high ecological saprobic and ecological trophic status (Table 1). Moreover, ADMO was present mainly in the upland river sections (Figure 5), which are, in most cases, hydromorphologically undisturbed, which

corresponds with the findings of Falasco et al. (2023). We can conclude that even if ADMO is very abundant, the consequent disturbance of the aquatic environment is not noticed, at least as far as the physical appearance of river sections (Figure 3) and ecological saprobic and trophic status assessment are concerned. These findings are in accordance with the findings from the literature (Buczkó et. al., 2022). In the lakes, ADMO was detected mainly during the qualitative inspection of the samples (Lake Bohinj, Gajševsko Lake and Lake Klivnik). With a slightly higher abundance of 0.4% ADMO was detected only in one sample in Gajševsko Lake in June 2023 (Table 1).

The results of the correlations between ADRU abundance and SW and E were the opposite compared with ADMO, showing a weak positive correlation of 0.25 and 0.23 between ADRU abundance and SW, and between ADRU abundance and E, respectively. Rimet et al. (2010) considered ADRU as an invasive species; however, in the dataset used in this research, ADRU was present in much lower proportions (up to 27%) compared to ADMO (up to 77%) and did not affect the species diversity and evenness of the diatom assemblages in the samples. These findings are in accordance with other studies in which the invasiveness of ADRU could not be confirmed (Ivanov, 2018). In the dataset used in this research, ADRU was present mainly in the lowland river sections, lakes and reservoirs (Figure 6), which corresponds with the findings of Ivanov (2018). ADRU reached the highest abundance in the Drava River, a river with a high number of reservoirs above the dams of Hydro-Power Plants, namely at the sampling sites Ranca, Ruše, and Tribej. In the dataset used in this research, the river sections in which ADRU abundance was the highest (>5%) are associated with good or high ecological saprobic and ecological trophic status (Table 1). Among the 11 lakes included in this study, ADRU was confirmed in eight, with the highest abundance of 10% recorded in Lake Slivnica in May 2023. In Slovenia, there are two natural lakes, namely the subalpine Lake Bled and the alpine Lake Bohinj. ADRU was found in Lake Bled in August 2019 with a low abundance of 0.2%; however, in Lake Bohinj, which is considered one of the most ecologically pristine lakes in Slovenia (good ecological status; Table 1), it was not found. Moreover, Lake Bled was included in the dataset used in this research in the years 2019 and 2022; however, ADRU was found only in one sample in 2019 and was not detected again in 2022.

The distribution of ADMO and ADRU in Slovenian rivers and lakes in the years 2019–2024, accompanied by the relative abundance of both species expressed in percentage, is presented in Figures 5 and 6. Buczkó et al. (2022) found that ADMO started to spread in the Danube River from the source downstream. In 2013, it was found in Germany and Austria, and three years later, it was found in the Hungarian section of the Danube River. However, this pattern was not confirmed in Slovenian rivers.

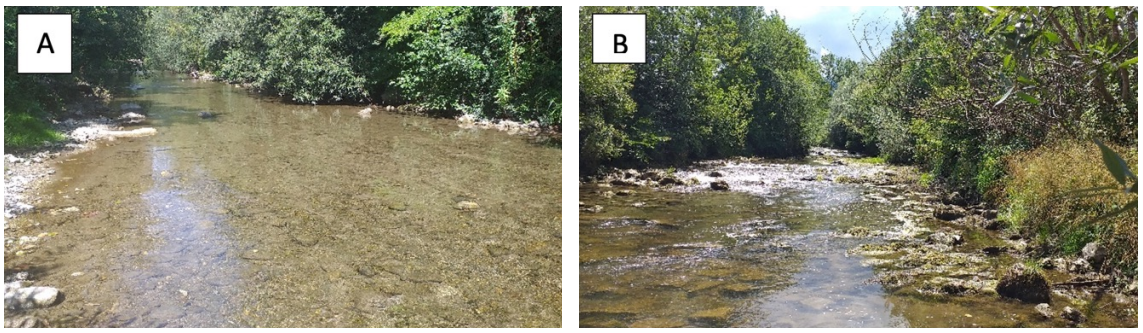


Figure 3. River Bolska has the highest occurrence of invasive benthic diatom *Achnanthidium delmontii* (ADMO) in Slovenia. A-Bolska-Čeplje and B-Bolska-Dolenja vas in August 2022. Photo: archive ARSO.

Slika 3. Reka Bolska z najvišjo številčnostjo invazivne bentoške diatomeje *Achnanthidium delmontii* (ADMO) v Sloveniji. A-Bolska-Čeplje in B-Bolska-Dolenja vas avgusta 2022. Foto: arhiv ARSO.

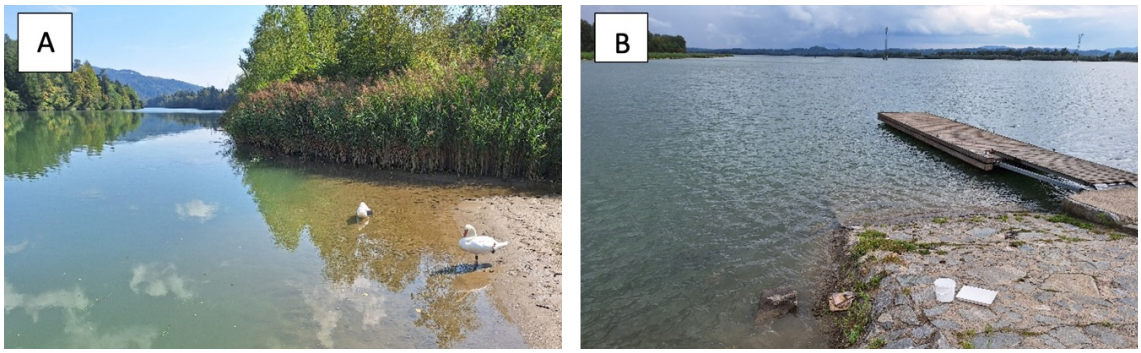


Figure 4. River Drava has the highest abundance of invasive alien diatom species, *Achnanthidium druartii* (ADRU), in Slovenia. A-Drava-Ruše and B-Drava-Ranca in August 2024. Photo: archive ARSO.

Slika 4. Reka Drava z najvišjo številčnostjo invazivne tujerodne vrste diatomeje *Achnanthidium druartii* (ADRU) v Sloveniji. A-Drava-Ruše in B-Drava-Ranca avgusta 2022. Foto: arhiv ARSO.

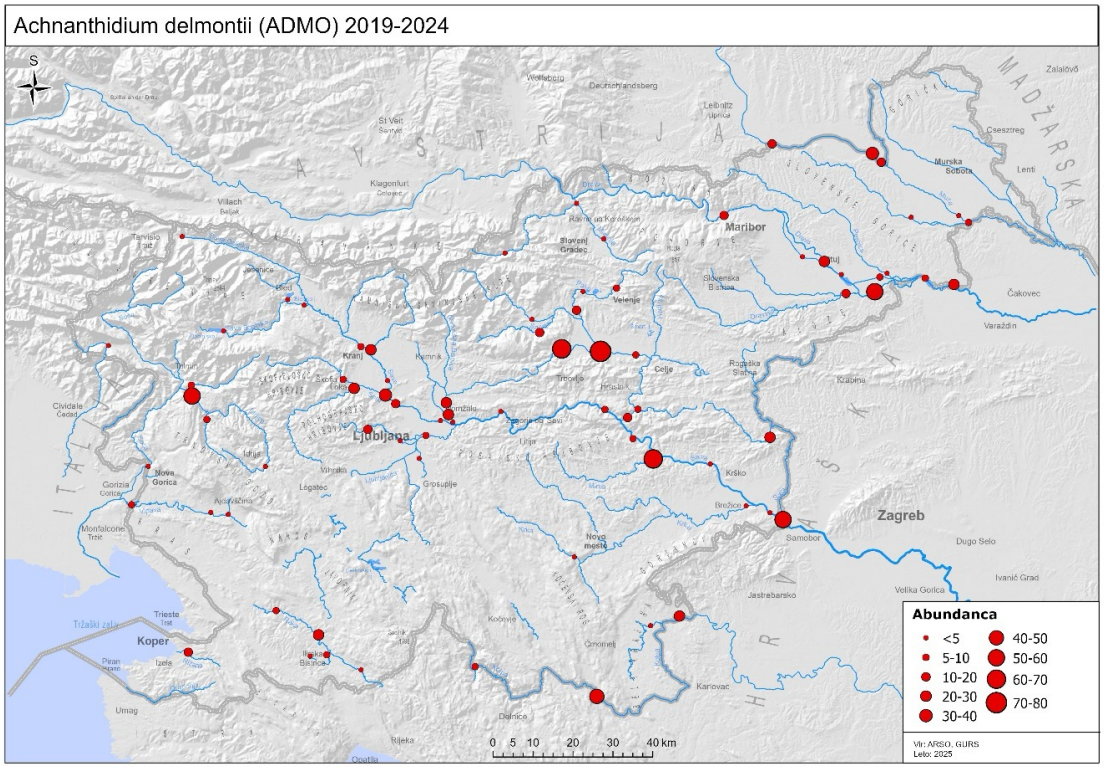


Figure 5. Distribution and relative abundance (in percentage) of *Achnanthidium delmontii* (ADMO) in Slovenia between 2019 and 2024.

Slika 5. Porazdelitev in relativna abundanca (v odstotkih) vrste *Achnanthidium delmontii* (ADMO) v Sloveniji med leti 2019 in 2024.

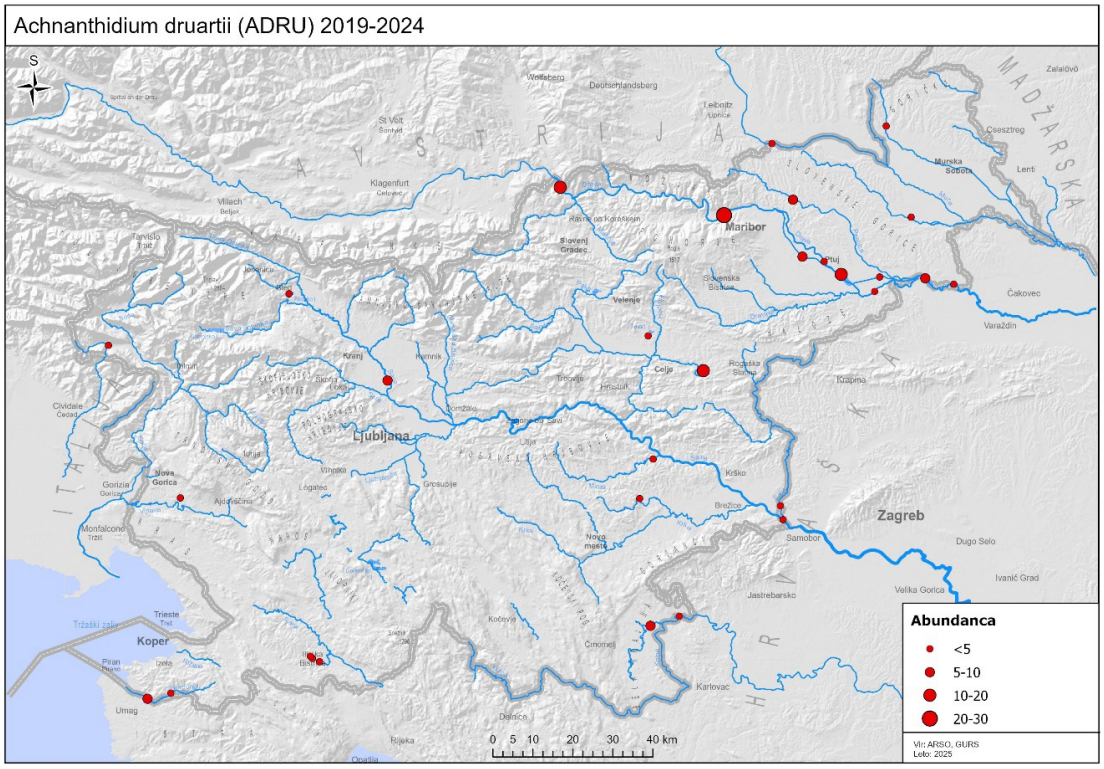


Figure 6 Distribution and relative abundance (in percentage) of *Achnanthidium druartii* (ADRU) in Slovenia between 2019 and 2024.

Slika 6. Porazdelitev in relativna abundanca (v odstotkih) vrste *Achnanthidium druartii* (ADRU) v Sloveniji med leti 2019 in 2024.

The first record of ADMO and ADRU in Slovenia is from the national monitoring of surface waters in 2019. The most probable reason is that in 2019, the Slovenian Environment Agency switched from the diatom taxonomy of Süßwasserflora von Mitteleuropa (Krammer and Lange-Bertalot, 1986, 1988, 1991a, 1991b) to the taxonomy of Freshwater Benthic Diatoms of Central Europe (Lange-Bertalot et al., 2017). In the monograph of Lange-Bertalot et al. (2017), both ADMO and ADRU were already included, while in older monographs, they are missing since both species were only formally described as new diatom species in 2012 (ADMO) and 2010 (ADRU). Although ADMO was formally first observed in Slovenia in 2019, it was most likely present in Slovenian freshwaters several years earlier and had been counted under *Achnanthes biasolettiana* Grunow, which is currently regarded as a synonym of *Achnanthidium pyrenaicum* (Hustedt) H. Kobayasi. One reason to assume the earlier occurrence of ADMO in Slovenia is that it had already been reported in the freshwaters of neighbouring countries: as early as 2013 in Austria (Buczkó et al., 2022) and Italy (Falasco et al., 2023), and in 2015 in Hungary (Buczkó et al., 2022). Buczkó et al. (2022) reported that in 2013, when ADMO was first recorded in German and Austrian sections of the Danube River, it was present in low abundances (<3%); however, by 2019, ADMO had become one of the most abundant and frequent diatom species found in the Danube River. Given that ADMO, when first recorded in Slovenian samples in 2019, was already present with very high abundances, reaching up to 51% in Sava - Jesenice na Dolenjskem (Table 1, Figure 5), we can conclude that it had already been present in Slovenia well before 2019. In contrast, according to our information, there are no records of ADRU occurring in neighbouring countries before 2019, and thus it may not have been present in Slovenian waters earlier. Furthermore, ADRU, when first recorded in Slovenian samples in 2019, showed a relative abundance

of up to 6% in Drava - Prepolje, which is 8.5 times lower than that of ADMO. There is a possibility that ADRU will be more widely distributed in Slovenian waters with a higher relative abundance after a certain amount of time, as was the case with ADMO. The data presented in this study show only the present state, which might be temporally biased. Although there are no records of ADRU in Croatia, Hungary, and Italy, we can conclude from its presence in Slovenian rivers near the border of the above-mentioned countries, such as the Kolpa, Sotla, Dragonja, Ledava, and Nadiža (Table 1, Figure 6), that ADRU is most likely present in the territory of those countries.

Although diatoms are useful indicators of various pressures such as nutrient and organic loading, acidification, or salinity in running and standing surface waters, ADMO and ADRU are not yet considered as indicator organisms. However, ongoing research is exploring their potential as indicator organisms, which would be reasonable given their increasing prevalence across Europe.

Conclusions

Altogether 247 phytobenthos samples collected at 87 rivers and 35 phytobenthos samples collected at 11 lakes in the frame of the national monitoring of surface water quality from 2019 to 2024 were included in this study. *Achnanthidium delmontii* (ADMO) was present in 100 river samples (41%) from 40 rivers (46%) and was a dominant species in 56 phytobenthos samples (23%) from 27 rivers (31%). The highest abundance of ADMO was recorded in the Bolska River at sampling sites Dolenja vas and Čeplje with relative abundances of 77% and 64%, respectively. *Achnanthidium druartii* (ADRU) was present in 32 river samples (13%) from 12 rivers (14%) and was a dominant species in 11 phytobenthos samples (5%) from 4 rivers (5%). The highest abundance of ADRU was detected in the Drava River at sampling site Ruše with a relative abundance of 27%. In lakes, ADMO was relatively rare, being detected in only five samples (14%) across three lakes and was never dominant in any of the lake samples. ADRU was present in 15 samples (43%) from 8 lakes and was a dominant species in 3 samples from two lakes. The highest relative abundance of ADRU was observed in Lake Slivnica with a relative abundance of 10%.

The results of this study show that the presence of ADMO in the samples is associated with lower diatom species diversity and evenness, indicating, together with very high relative abundances, its invasive character. However, the river sections in which ADMO abundance was the highest and diatom species diversity and evenness were the lowest are associated with good or high ecological saprobic and ecological trophic status. Moreover, ADMO was present mainly in the upland river sections, which are, in most cases, hydromorphologically undisturbed. Although ADMO was present in the investigated rivers in very high numbers, the consequent disturbance of the aquatic environment was not noticed, at least as far as the physical appearance of river sections and ecological saprobic and trophic status assessment are concerned. ADRU, which was present mainly in the lowland river sections and lakes, did not affect the species diversity and evenness of the diatom assemblages in the samples, and thus its invasive character could not be confirmed.

Author Contributions

Conceptualization, A.K.K.; methodology, A.K.K. and T.Š.; investigation, A.K.K. and T.Š.; resources, A.K.K.; data curation, A.K.K. and T.Š.; writing—original draft preparation, A.K.K.; writing—review and editing, A.K.K. and T.Š.; funding acquisition, A.K.K. All authors have read and agreed to the published version of the manuscript.

Acknowledgement

Special thanks are extended to Mr. Matej Cunder from the Slovenian Environment Agency for creating the distribution maps used in this study.

Funding

The authors acknowledge the financial support from the Slovenian Environment Agency and the Slovenian Research Agency (research core funding No. P2-0180).

Conflicts of Interest

The authors declare no conflict of interest.

References

ARSO, 2017. Program monitoringa kemijskega in ekološkega stanja voda, program za obdobje 2016 do 2021. Agencija Republike Slovenije za okolje, Ministrstvo za okolje in prostor, Ljubljana. https://www.gov.si/assets/organi-v-sestavi/ARSO/Vode/Stanje-voda/Program-monitoringa-kemijskega-in-ekoloskega-stanja-voda-za-obdobje-2016-do-2021.pdf (accessed 20.4.2025)

ARSO, 2022. Program monitoringa kemijskega in ekološkega stanja voda, program za obdobje 2022 do 2027. Agencija Republike Slovenije za okolje, Ministrstvo za okolje in prostor, Ljubljana. https://www.gov.si/assets/organi-v-sestavi/ARSO/Vode/Stanje-voda/Program-monitoringa-voda-2022-do-2027.pdf (accessed 20.4.2025)

Blanco, S., Ector, L., 2009. Distribution, ecology and nuisance effects of the freshwater invasive diatom *Didymosphenia geminata* (Lyngbye) M. Schmidt: a literature review. Nova Hedwigia, 88(3-4), 347–422. https://doi.org/10.1127/0029-5035/2009/0088-0347

Buczkó, K., Trábert, Zs., Stenger-Kovács, Cs., Tapolczai, K., Bíró, T., Duleba, M., ...Ács, É., 2022. Rapid expansion of an aquatic invasive species (AIS) in Central-European surface waters; a case study of *Achnanthidium delmontii*. Ecological indicators, 135, 108547. https://doi.org/10.1016/j.ecolind.2022.108547

Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. Off. J. Eur. Communities.

Falasco, E., Bona, F., Zoppi, M., La Morgia, V., 2023. Environmental factors controlling the presence and distribution of *Achnanthidium delmontii* in Mediterranean rivers (NW-Italy). Nova Hedwigia, 117(1-4), 119–141. https://doi.org/10.1127/nova_hedwigia/2023/0849

Guiry, M.D., Guiry, G.M. 14 March 2022. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. https://www.algaebase.org (accessed 5.5.2025)

Hasle, G.R., Fryxell, G.A., 1970. Diatoms: Cleaning and Mounting for Light and Electron Microscopy. Transactions of the American Microscopical Society, 89(4), 469-474. https://doi.org/10.2307/3224555

ICPDR, 2019. Biology, Phytobenthos, International commission for the protection of the Danube River, Discover Danube, Joint Danube survey 4, https://data.danubesurvey.org/jds4/biology/taxa?bqe_name=Phytobenthos (accessed 5.5.2025)

Ivanov, P.N., 2018. Two new diatom species from family Achnanthidiaceae in Bulgaria: *Achnanthidium druartii*, an invasive species in Europe and *Achnanthidium straubianum*, new to Bulgarian diatom flora. Phytologia Balcanica, 24(2), 195–199.

Krammer, K., Lange-Bertalot, H., 1986. Bacillariophyceae. 1. Teil. Naviculaceae. In: Ett, J., Gerloff, J., Heynig, H., Mollenhauer, D. (Eds.) Subwasserflora von Mitteleuropa, Gustav Fischer Verlag, Stuttgart, 2/1, pp. 1–876.

Krammer, K., Lange-Bertalot, H., 1988. Bacillariophyceae. 2. Teil: Bacillariaceae, Epithemiaceae, Surirellaceae. In: Ett, J., Gerloff, J., Heynig, H., Mollenhauer, D. (Eds.) Subwasserflora von Mitteleuropa, Gustav Fischer Verlag, Stuttgart, 2/2, pp. 1–610.

Krammer, K., Lange-Bertalot, H., 1991a. Bacillariophyceae. 3. Teil: Centrales, Fragilariaceae, Eunotiaceae. In: Ett, J., Gerloff, J., Heynig, H., Mollenhauer, D. (Eds.) Subwasserflora von Mitteleuropa, Gustav Fischer Verlag, Stuttgart, 2/3, pp. 1–576.

Krammer, K., Lange-Bertalot, H., 1991b. Bacillariophyceae. 4 Teil, Achnanthaceae, Kritische Ergänzungen Zu Navicula (Lineolatae) und Gomphonema. In: Ett, J., Gerloff, J., Heynig, H., Mollenhauer, D. (Eds.) Subwasserflora von Mitteleuropa, Gustav Fischer Verlag, Stuttgart, 2/4, pp. 1-468.

Lange-Bertalot, H., Hofmann, G., Werum, M., Cantonati, M., 2017. Freshwater Benthic Diatoms of Central Europe: Over 800 Common Species Used in Ecological Assessment. English edition with updated taxonomy and added species. In: Cantonati, M., Kelly., M.G., Lange-Bertalot, H. (Eds): Koeltz Botanical Books, Schmitten-Oberreifenberg, 942 pp.

MOP, 2016a. Metodologija vrednotenja ekološkega stanja vodotokov na podlagi fitobentosa in makrofitov. Ministrstvo za okolje in prostor, Ljubljana. https://www.gov.si/assets/ministrstva/MOP/Dokumenti/Voda/Ekolosko_stanje/metod_vredn_ekoloskega_stanja_vodotokov_fitobentosa_makrofitov.pdf (accessed 20.4.2025)

MOP, 2016b. Metodologija vrednotenja ekološkega stanja jezer na podlagi fitobentosa in makrofitov. Ministrstvo za okolje in prostor, Ljubljana. https://www.gov.si/assets/ministrstva/MOP/Dokumenti/Voda/Ekolosko_stanje/metod_vredn_ekoloskega_st_jezer_fitobentosa_makrofitov.pdf (accessed 20.4.2025)

Pérès, F., Barthès, A., Ponton, E., Costes, M., Ten-Hage, L., Le-Cohu, R., 2012. *Achnanthidium delmontii* sp. nov., a new species from French rivers. Fottea, Praha, 12, 189–198.

Rimet, F., Couté, A., Piuz, A., Berthon, V., Druart, J.-C., 2010. *Achnanthidium druartii* sp. nov. (Achnanthales , Bacillariophyta), a new species invading European rivers. Vie et Milieu - life and environment, 60(3), 185–195.

Rott, E., Pipp, E., Pfister, P., van Damm, H., Ortler, K., Binder, N., Pall, K., 1999. Indikationslisten Fur Aufwuchsalgen in Östereichen Fließgevessern, Teil 2: Trophienindikation So Vie Geochemische Präferenz, Taxonomische und Toxicologische Anmerkungen. Bundesministerium für Land und Forstwirtschaft, Wien, Austria.

Rott, E., Hofmann, G., Pall, K., Pfister, P., Pipp, E., 1997. Indikationslisten für Aufwuchsalgen in Fließgewässern in Österreich, Teil 1: Saprobielle indikation. Wasserwirtschaftskataster, Bundesministerium für Land- und Forstwirtschaft, Wien, Austria.

SIST EN 14407:2014. Water quality - Guidance for the identification and enumeration of benthic diatom samples from rivers and lakes.

Taylor, B.W., Bothwell, M.L., 2014. The Origin of Invasive Microorganisms Matters for Science, Policy, and Management: The Case of *Didymosphenia geminata*. BioScience, 64(6), 531–538. https://doi.org/10.1093/biosci/biu060

ABS

Original Research

Evaluation of selected functional traits in lactic acid bacteria isolated from Algerian Makatia goat milk for food applications

Ahmed Saci [1,*], Samia Gharbi [2], Fatima Djadouni [3], Noureddine Karkachi [4]

Abstract

While studies on the health aspects of lactic acid bacteria (LAB) may receive more attention from the scientific community, the focus on the technological aspects of these bacteria has also become equally important, as these bacteria possess physiological properties that qualify them for use in various food productions. This study examined some of these traits for 10 LAB isolates from Makatia goat milk in the Wilaya of Mascara in western Algeria. Tests were conducted to evaluate acidifying activity, enzymatic activity, growth capability at different pH levels, in high salt environments, different temperatures, exopolysaccharide (EPS) production, high-temperature tolerance, antagonistic activity, and antibiotic sensitivity. All isolates lacked amylolytic activity, while proteolytic activity varied between 14.33 and 32.67 mm of lysis zones, and lipolytic activity varied between 8.43 and 12.53 mm. Thermotolerance at 63 °C for 30 minutes was revealed for all 10 isolates, along with variable growth capacities under different conditions (high, low pH, salted environment, and different temperatures). Significant antagonistic activity ranged between 11.5 and 40.66 mm of inhibition zones against the tested indicator strains. All 10 LAB isolates from goat milk exhibited remarkable technological properties that may or may not be helpful in food technology. For example, they are desirable in the fermentation industry and undesirable in some pasteurisation-sensitive products or some meat food industries.

Keywords

Lactic acid bacteria, Technological properties, Goat milk, Food preservation, Food spoilage, Fermentation, Antimicrobial activity

1 Department of Biology, Faculty of Life and Nature Sciences, University of Oran 1 Ahmed BenBella, Oran 31100, Algeria.

2 Department of Biotechnology, Faculty of Life and Nature Sciences, University of Science and Technology of Oran, Mohamed-Boudiaf USTOMB, Oran 31000, Algeria.

3 Department of Biology, Faculty of Life and Nature Sciences, University of Mustapha Stambouli, Mascara 29000, Algeria.

4 Laboratory of Applied Microbiology, Department of Biology, Faculty of Life and Nature Sciences, University of Oran 1 Ahmed BenBella, Oran 31100, Algeria.

*** Corresponding author:**
E-mail address: saci.ahmed@edu.univ-oran1.dz

Citation: Saci, A., Gharbi, S., Djadouni, F., Karkachi, N., (2025). Evaluation of selected functional traits in lactic acid bacteria isolated from Algerian Makatia goat milk for food applications. Acta Biologica Slovenica 68 (4)

Received: 29.01.2025 / **Accepted:** 13.08.2025 / **Published:** 14.08.2025

https://doi.org/10.14720/abs.68.4.21735

Ocena izbranih funkcionalnih lastnosti mlečnokislinskih bakterij, izoliranih iz alžirskega kozjega mleka Makatia, za uporabo v živilih

Izvleček

Medtem ko študije o zdravstvenih vidikih mlečnokislinskih bakterij (LAB) morda pritegnejo več pozornosti znanstvene skupnosti, je enako pomembno tudi osredotočanje na tehnološke vidike teh bakterij, saj imajo fiziološke lastnosti, ki jih kvalificirajo za uporabo v različnih živilskih proizvodnjah. Ta študija je preučila nekatere od teh lastnosti za 10 izolatov LAB iz kozjega mleka Makatia v Wilaya v Mascari v zahodni Alžiriji. Opravili so se testi za oceno kisle aktivnosti, encimske aktivnosti, sposobnosti rasti pri različnih vrednostih pH, v okoljih z visoko vsebnostjo soli, pri različnih temperaturah, proizvodnje eksopolisaharidov (EPS), tolerance na visoke temperature, antagonistične aktivnosti in občutljivosti na antibiotike. Vsi izolati so bili brez amilolitične aktivnosti, proteolitična aktivnost pa je bila med 14,33 in 32,67 mm liznih con, lipolitična aktivnost pa med 8,43 in 12,53 mm. Vsi 10 izolati so pokazali termotoleranco pri 63 °C za 30 minut, skupaj z različnimi sposobnostmi rasti v različnih pogojih (visoka, nizka pH, slano okolje in različne temperature). Pomembna antagonistična aktivnost je bila med 11,5 in 40,66 mm inhibicijskih con proti testiranim indikatorjem. Vseh 10 izolatov LAB iz kozjega mleka je pokazalo izjemne tehnološke lastnosti, ki so lahko koristne v prehrambni industriji. Na primer, so zaželeni v fermentacijski industriji, vendar nezaželeni v nekaterih proizvodih, občutljivih na pasterizacijo, ali v nekaterih mesnih industrijah.

Ključne besede

Mlečnokislinske bakterije, tehnološke lastnosti, kozje mleko, konzerviranje živil, pokvarljivost živil, fermentacija, protimikrobna aktivnost

Introduction

LAB are considered the most interesting microbial group due to their extensive use as probiotics, bio-preservatives, or fermentation starter cultures (Tidona et al., 2020). Due to their enzymatic potential, LAB play a key role in various food productions. These enzymes directly contribute to developing organoleptic properties (flavour, consistency, texture, etc.) (García-Cano et al., 2019). In addition to influencing organoleptic properties, lactic acid bacteria possess important antioxidant and antimicrobial properties (Baureder and Hederstedt, 2013).

Regardless the number of technological characteristics of LAB, their ability to survive in different physicochemical conditions, such as storage and preservation conditions at different pH values, at different salt concentrations, at different heat treatments, and different storage temperatures, is considered as the key factor in their selection as potential sources for food preservation and processing (Sionek et al., 2024).

A lot of studies focus on the positive aspects of LAB, such as their probiotic and technological capabilities. The latter also plays an equally important role in food spoilage, particularly in those foods where manufacturing treatments do not target specific types of LAB. These foods are often subjected to spontaneous fermentation processes by present autochthonous microbiota or infected by undesirable contamination by LAB (uncontrolled manufacturing processes) (Ağagündüz et al., 2022; Peng et al., 2020; Vinderola, 2019).

Several raw materials for isolating LAB are available. Among these materials, goat milk occupies the smallest portion of the various scientific studies. Compared to other types of milk, goat milk has many health benefits for humans, such as high calcium content, low allergenic potential, and easy digestibility of its fat in the presence of bile (Nayik et al., 2021).

In the present study, we selected 10 isolates of LAB from 4 samples of milk from the Algerian Makatia goat breed. The Makatia goat is a purebred Algerian breed, native to the Saharan Atlas region, specifically the provinces of Laghouat and Djelfa. It is classified as a dual-purpose breed (meat and milk) and is renowned for its remarkable adaptability to harsh steppe environments. Intending to

contribute to detecting some technologically essential properties, such as their ability to grow under different conditions (pH, NaCl, temperature), enzymatic activity, and antimicrobial properties.

Materials and methods

Sample collection and preparation

Raw Makatia goat milk samples were collected from four healthy animals in the region of Mascara, western Algeria (Figure 1), during March 2024. The samples (50 mL) were collected aseptically into sterile containers and transported to the laboratory at 4 °C within 4 hours for immediate processing.

The pH of the goat milk samples was determined using a pH meter (Adwa AD1030, Szeged, Hungary) previously calibrated with a standard buffer (Webster, 2003).

Isolation and identification of lactic acid bacteria

Twenty-three isolates were obtained from four samples of raw goat milk from the Mascara province. Only 10 Gram-positive, catalase-negative, non-sporulating, and non-motile isolates were selected. 10 mL of each goat milk sample was diluted 10 times in sterile ¼ strength Ringer solution. 0.1 mL of each dilution was inoculated on the surface of an MRS agar (Liofilchem S.r.l., Italy) and then incubated at 30 °C for 48 hours under anaerobic conditions (2.5-litre anaerobic jar). Then, successive streaks were performed on MRS agar until pure cultures were obtained. These isolates were kept at -20 °C in MRS broth containing 25 % glycerol for further analysis (Haghshenas et al., 2017).

The morphology of the colonies, the Gram type, and the cellular morphology of the bacteria were determined for all 10 isolates Smith and Hussey, 2005. Motility was examined by inoculation in semi-solid agar (Patricia and Laura, 2011).

Figure 1. Geographic location of the sampling sites. The map displays the three communes (Sehaillia, Hachem, and Ain Farrah) within the Wilaya of Mascara, western Algeria, from which raw milk samples of the native Makatia goat breed were collected. The inset on the upper left provides a broader geographical context by showing the location of the Wilaya of Mascara within Algeria. The legend indicates the specific area of each commune. Map data is adapted from Google Maps.

Slika 1. Geografska lega mest vzorčenja. Zemljevid prikazuje tri občine (Sehaillia, Hachem in Ain Farrah) v provinci Mascara v zahodni Alžiriji, iz katerih so bili odvzeti vzorci surovega mleka avtohtone pasme koz Makatia. Vstavek v zgornjem levem kotu prikazuje širši geografski kontekst z lokacijo province Mascara v Alžiriji. Legenda označuje posebno območje vsake občine. Podatki zemljevida so prilagojeni iz Google Maps.

The catalase and oxidase tests were examined using 3 % hydrogen peroxide and oxidase strips (Merck Bactident oxidase 181, Darmstadt, Germany), respectively. *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as positive and negative controls for Gram type test, catalase activity and motility (Khadija et al. 2022).

Bacterial genomic DNA was extracted from isolates using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies Sdn Bhd, Selangor, DE, Malaysia) according to the manufacturer's protocol (Vivantis Technologies Sdn Bhd, 2010). The integrity and concentration of the extracted DNA were measured using a NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific, USA).

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers 27F (5'-AGA GTT TGA TCG TCC TGG CTC AG-3') and 1492R (5'-CCG TCA ATT CCT TTG AGT TT-3') (Edwards et al., 1989). PCR was conducted in a 50 µL reaction volume containing 1.25 U of Hot Start Taq DNA Polymerase (Solis Biodyne, Estonia), 1X reaction buffer with 1.5 mM $MgCl_2$, 0.3 µM of each primer, 25–50 ng of template DNA, and nuclease-free water. Amplification was performed in an iCycler thermocycler (Bio-Rad, USA) under the following conditions: an initial denaturation step at 94 °C for 12 minutes; followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute and 40 seconds; and a final extension step at 72 °C for 7 minutes. The presence and size of the PCR amplicons were verified by electrophoresis on a 1.5 % agarose gel stained with Midori Green Advance (Nippon Genetics, Japan) and visualised under UV light.

The purified PCR products were sent for bidirectional sequencing to Sarl GENE LIFE SCIENCES (Sidi Bel Abbès, Algeria). The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3130 Genetic Analyser (Applied Biosystems, USA), which corresponds to the Sanger sequencing method (Sanger et al., 1977). The resulting forward and reverse sequence chromatograms for each isolate were assembled and edited to generate a final consensus sequence using Bionumerics v3.5 software (Applied Maths, Belgium). The quality of sequences was visually assessed, and poor-quality regions were manually edited or trimmed. The consensus sequences were then identified by performing a homology search against the GenBank database using the National Centre for Biotechnology Information (NCBI) BLASTn tool.

Technological profiling

The acidifying activity of 10 isolates was examined over a time interval of 6 and 48 hours, using a rapid screening method to evaluate the acidifying activity of LAB (colourimetric assay), described by Ribeiro et al. (2021).

The α-amylase activity was examined by inoculating 10 µL of each fresh bacterial culture onto sterile filter paper discs placed on MRS agar devoid of glucose and supplemented with 0.3 % starch. After incubation at 30 °C for 48 hours, the Petri dishes were flooded with Lugol solution to detect α-amylase activity (The appearance of transparent yellow halos around bacterial cultures indicates starch decomposition) (Amapu et al., 2016).

The proteolytic activity was evaluated by inoculating 10 µL of a fresh culture of each isolate onto a sterile filter paper disc placed on the surface of an MRS agar plate supplemented with 1 % skimmed milk. After incubation at 37 °C for 48 hours, the diameters of the transparent zones (halos indicating positive proteolytic activity) were measured (Islam et al., 2021).

The lipolytic activity was assessed by inoculating 10 µL of a fresh culture of each isolate onto a sterile filter paper disc on an MRS agar plate surface enriched with 1 % Tween 80. After incubation at 37 °C for 48 hours, the diameters of the transparent zones (halos indicating positive lipolytic activity) were measured (Tanasupawat et al., 2015).

The ability of the isolates to produce EPS was examined by streaking pure and fresh cultures on Mayeux Sandine and Elliker (MSE) agar. The appearance of slimy and colloidal colonies after incubation at 37 °C for 48 hours indicates positive EPS production (Fguiri et al., 2016).

The ability of the isolates to grow in NaCl (weight/volume) solutions at 4, 6, and 10 % was evaluated by inoculating fresh culture colonies of each isolate into MRS broths supplemented with 4, 6, and 10 % NaCl (weight/volume) and bromocresol purple indicator. After incubation at 37 °C for 48 hours, the colour change from purple to yellow was evidence of cell growth (Ruiz et al., 2024).

The ability of the isolates to grow at different pH levels was tested by inoculating fresh colonies into several tubes containing MRS broths adjusted to different pH levels: 2.2, 4.5, 6.2, and 9.6. The appearance of turbidity after incubation at 37 °C for 48 hours indicates the cell viability of the isolates.

The growth of the isolates at different temperatures was examined by inoculating 50 µL of fresh cultures of each isolate into tubes containing 7 mL of MRS broth with bro-

mocresol purple indicator, four tubes for each isolate. Each series of these tubes was incubated at temperatures of 4, 10, 15, and 45 °C for 72 hours. Bacterial growth was observed in the colour change from purple to yellow (Kim et al., 2019).

The ability of the isolates to tolerate high temperatures was examined according to the method described by Hurtado-Bautista et al. (2021), with modifications (adaptation of the technique with LAB isolates and modification of the temperature exposure time). 100 µL of each bacterial isolate was inoculated into tubes containing 7 mL of MRS broth supplemented with bromocresol purple indicator. The tubes were immersed in a water bath previously set to 63 °C for 30 minutes. Then, they were incubated at 37 °C for 48 hours. The pH indicator's colour change indicated the bacterial cells' survival.

The antimicrobial activity of isolates against indicator strains (*Staphylococcus* (*S.*) aureus ATCC 25923, *Pseudomonas* (*P.*) *aeruginosa* ATCC 27853, *Escherichia* (*E.*) coli ATCC 25922, *Bacillus* (*B.*) *cereus* ATCC 10987, and *Candida* (*C.*) *albicans* ATCC 10231) was evaluated using the direct spot test. The goat milk isolates were inoculated as 0.5 mm diameter spots on an appropriate number of Petri dishes pre-filled with soft MRS agar (0.8 % MRS agar). Melted soft MRS agar (45 °C) in tubes was inoculated with 100 µL of each indicator strain. Then, the contents of each of these tubes were poured into the appropriate Petri dishes already inoculated with LAB isolates. The size of inhibition zones was measured after incubation at 37 °C for 24 hours (Akman et al., 2021).

The examination of the fermentation profile (sugar fermentation) was carried out using an API 50 CHL system according to the manufacturer's instructions (BioMérieux, Marcy-l'Étoile, France), aimed at identifying *Lactobacillus* and related genera (Ghanbari et al., 2009). Briefly, four fresh and pure colonies of each goat milk isolate were inoculated into API 50 CHL medium (7 mL). This inoculum was then inoculated into the gallery of wells (49 tests plus one control) up to the marker line, followed by the addition of 3 to 4 drops of sterile paraffin oil. The system was incubated at 37 °C for 24-48 hours. The colour change indicates a positive fermentation of carbohydrates.

Antibiotic resistance

The sensitivity of LAB isolates to antibiotics was evaluated against 10 antibiotics (Liofilchem S.r.l., Italy): penicillin (P) 10 µg, augmentin (AUG) 30 µg, ampicillin (AMP) 30 µg, cefepime (FEP) 30 µg, cefoxitin (FOX) 30 µg, ofloxacin (OFX) 5 µg, nalidixic acid (NA) 30 µg, chloramphenicol (C) 30 µg, erythromycin (E) 15 µg, and tetracycline (TE) 30 µg according to the Kirby-Bauer method (Hudzicki, 2009). According to Clinical and Laboratory Standards Institute guidelines (CLSI, 2020), the sensitivity data were categorized as resistant (R, zone diameter <14 mm), moderately susceptible (I, zone diameter between and including 14 to 20 mm), or susceptible (S, zone diameter >20 mm) (Meradji et al., 2023).

Statistical analysis

The results were expressed as mean ± standard deviation of three repetitions (n = 3). Statistical significance was demonstrated by Two-way ANOVA, accompanied by Duncan's post hoc test for the examination of acidifying capacity and antimicrobial activity, and One-way ANOVA, accompanied by Tukey's post hoc test for the assessment of proteolytic activity and lipolytic activity using GraphPad Prism 9.5.1 (733) software. The data for which the P value is less than 0.05 were statistically significant.

Results

Isolation and identification of lactic acid bacteria

The 10 isolates were examined based on their macroscopic, microscopic, and physiological characteristics. As expected for LAB, Gram staining revealed them as Gram-positive and rod-shaped. The motility, catalase, and oxidase tests were negative. The morphology of the colonies varied from one isolate to another (Table 1).

The 10 isolates named GL.01, GL.02, GL.03, GL.04, GL.05, GL.06, GL.07, GL.08, GL.09, and GL.10 were identified using 16S rRNA sequencing. Sequences obtained were subjected to NCBI blast tool in NCBI GenBank (https:// www. ncbi. nlm. nih.gov/genbank/) and were found to be *Lactiplantibacillus plantarum* (isolates GL.01 and GL.09), *Lactobacillus plantarum* (isolates GL.02 and GL.10), *Lactiplantibacillus pentosus* (isolate GL.07), *Lactobacillus fermentum* (isolate GL.03) and *Lactobacillus sakei* (isolate GL.06), *Lactiplantibacillus sp* (isolate GL.04, GL.05 and GL.08) These last three isolates showed a similarity percentage lower than the limit allowed for species identification (98.7 %). The % of similarity to the closest bacterial strains, together with their accession numbers, is shown in Table 2.

Table 1. Macroscopic, microscopic, and physiological characteristics of ten lactic acid bacteria isolates (GL.01-GL.10) collected from raw milk of Makatia goat breed samples, along with the pH values of the original samples.

Tabela 1. Makroskopske, mikroskopske in fiziološke lastnosti desetih izolatov mlečnokislinskih bakterij (GL.01-GL.10), zbranih iz surovega mleka vzorcev koz pasme Makatia, skupaj z vrednostmi pH izvirnih vzorcev.

			Macroscopic appearance				Microscopic appearance		Physiological tests		
Isolate	Animal milk sample sources	Sample pH	Form of colony	Size (mm) of colony	Colony margin	Colony color	Gram-Stain	Form	Catalase	Oxidase	Motility
GL.01			C	01	E	W	+	Rods	-	-	-
GL.02	01	6.42 ± 0.8	C	01	E	W	+	Rods	-	-	-
GL.03			C	01	E	W	+	Rods	-	-	-
GL.04	02	6.63 ± 1.4	C	01	E	Y. W	+	Rods	-	-	-
GL.05			C	01	E	W	+	Rods	-	-	-
GL.06	03	6.76 ± 0.6	C	01	E	W	+	Rods	-	-	-
GL.07			C	01	E	W	+	Rods	-	-	-
GL.08	04	6.15 ± 1.2	C	01	E	W	+	Rods	-	-	-
GL.09			C	01	E	Y. W	+	Rods	-	-	-
GL.10			C	01	E	W	+	Rods	-	-	-

The isolates were obtained from four Makatia goat milks collected from the western region of Algeria. Macroscopic appearance: C: Circular; E: Entire; W: White; Y.W: Yellowish white. Microscopic appearance and physiological tests: (+): Positive reaction (Gram-positive); (-): Negative reaction (for Catalase, Oxidase, and Motility tests). All isolates were identified as rod-shaped bacteria.

Table 2. Genotypic identification of lactic acid bacteria isolates from raw milk of the Algerian Makatia goat breed, based on 16S rRNA.

Tabela 2. Genotipska identifikacija izolatov mlečnokislinskih bakterij iz surovega mleka alžirske koze pasme Makatia na podlagi 16S rRNA.

	Molecular identification			
Isolates	Species	Strain	Similarity (%)	Accession number
GL.01	*Lactiplantibacillus plantarum*	2546	99.82	MT611578
GL.02	*Lactobacillus plantarum*	B_16LAB	99.83	MF405177
GL.03	*Lactobacillus fermentum*	KLAB15	99.36	KM485578
GL.04*	*Lactiplantibacillus sp*	1583	90.69	MT597488
GL.05*	*Lactiplantibacillus sp*	HBMSS8	96.48	MF662593
GL.06	*Lactobacillus sakei*	HBMSS7	99.32	MF662592
GL.07	*Lactiplantibacillus pentosus*	MYSDV3	98.92	MT152901
GL.08*	*Lactiplantibacillus sp*	HBMSS4	97.46	MF662589
GL.09	*Lactiplantibacillus plantarum*	MYSDV5	99.80	MT131268
GL.10	*Lactobacillus plantarum*	B_16LAB	100.00	MF405177

Identification was performed by comparing the 16S rRNA gene sequences of the isolates against the NCBI GenBank database using BLAST. The table shows the closest species match, the strain designation of that match, the percentage sequence similarity, and the corresponding GenBank accession number. (*) Isolates were identified to the genus level because their 16S rRNA gene sequence similarity was below the 98.7% threshold recommended for species delineation.

Technological profiling

The 10 isolates showed variable acidifying activities within the 6 to 8-hour incubation time range (Table 3). The acidifying activity values ranged from 5.00 to 6.11 after 6 hours of incubation and from 3.24 to 4.47 after 48 hours. After 6 hours of incubation, the isolate GL.03 showed the lowest pH value, while after 48 hours, the isolate GL.08 had the lowest pH value. A Two-way ANOVA comparison test, followed by a post hoc test (Sidak's multiple comparisons test), showed significant differences with $P < 0.5$.

A complete absence of starch hydrolysis activity appeared for the 10 isolates, represented by the absence of transparent or yellowish halos around the inoculated spots after adding Lugol's iodine (Table 3).

A varied proteolytic activity was revealed for the 10 isolates. Clear proteolysis zones ranged from 14.33 to 32.67 mm (Table 3). The isolate GL.02 exhibited the highest proteolytic activity with a proteolysis zone of 32.67 mm, while the isolate GL.07 showed the lowest activity with a proteolysis zone of 14.33 mm. A One-way ANOVA comparison test, followed by a post hoc test (Tukey's multiple comparisons test), showed significant differences with $P < 0.5$.

The 10 isolates showed lipolytic activities ranging from 8.43 to 12.53 mm of lipolytic zones (Table 3). In comparison with proteolytic activity, lipolytic activity was relatively modest. Isolate GL.02 showed the highest activity with a lipolysis zone of 12.53 mm, followed by isolates GL.10 and GL.06 with lipolysis zones of 12.33 mm and 11.17 mm, respectively, while the isolate GL.03 exhibited the lowest activity with a lipolysis zone of 8.43 mm. A One-way ANOVA comparison test, followed by a post hoc test (Tukey's multiple comparisons test), showed significant differences with $P < 0.5$.

Six isolates were revealed to be EPS production positive (GL.01, GL.04, GL.05, GL.06, GL.08, and GL.09), while four isolates were revealed to be EPS production negative (GL.02, GL.03, GL.07, and GL.10) (Table 3).

Table 3. Technological properties of lactic acid bacteria isolates from raw milk of the Algerian Makatia goat breed, including acidifying, enzymatic, and exopolysaccharide (EPS) production capabilities.

Tabela 3. Tehnološke lastnosti izolatov mlečnokislinskih bakterij iz surovega mleka alžirske koze pasme Makatia, vključno z zmožnostjo kislega delovanja, encimsko aktivnostjo in proizvodnjo eksopolisaharidov (EPS).

	Acidifying activity		Amylolytic activity	Proteolytic activity	Lipolytic activity	EPS production
	pH		Halos (mm)			
Isolates	06 h	48 h				
GL.01	5.21 ± 0.6^{a}	3.55 ± 0.3b	0.0	22.50 ± 0.5^{f}	9.12 ± 0.5^{lm}	+
GL.02	6.11 ± 0.1^{a}	4.21 ± 0.7b	0.0	32.67 ± 1.3^{c}	12.53 ± 0.1^{j}	-
GL.03	5.00 ± 1.3^{a}	3.28 ± 1.6b	0.0	26.50 ± 0.5^{e}	8.43 ± 1.5^{m}	-
GL.04	5.12 ± 0.7^{a}	3.49 ± 0.5b	0.0	25.33 ± 0.5^{e}	9.36 ± 0.5^{lm}	+
GL.05	5.36 ± 0.1^{a}	3.41 ± 0.4b	0.0	22.33 ± 1.0^{fg}	8.90 ± 1.2^{lm}	+
GL.06	6.10 ± 1.2^{a}	4.38 ± 0.3b	0.0	17.00 ± 0.5^{h}	11.17 ± 0.5^{jk}	+
GL.07	5.33 ± 0.4^{a}	3.64 ± 1.0b	0.0	14.33 ± 0.8^{i}	9.73 ± 0.5^{k}	-
GL.08	5.06 ± 1.5^{a}	3.24 ± 0.2b	0.0	20.33 ± 0.5^{g}	9.03 ± 1.4^{lm}	+
GL.09	5.56 ± 1.2^{a}	3.82 ± 0.6b	0.0	21.00 ± 0.5^{fg}	9.63 ± 1.0^{l}	+
GL.10	6.09 ± 0.3^{a}	4.47 ± 0.5b	0.0	30.17 ± 1.0^{d}	12.33 ± 0.5^{j}	-

Values are presented as mean ± standard deviation of three replicates. EPS: exopolysaccharide, (+): positive production, (-): negative production. For acidifying activity, within each row (isolate), values with different superscripts (a, b) are significantly different (Student's t-test, $p < 0.05$). For proteolytic and lipolytic activities, within each column, values followed by different superscripts (c-m) are significantly different (one-way ANOVA followed by Tukey's post-hoc test, $p < 0.05$). All isolates showed no amylolytic activity.

Eight out of 10 isolates demonstrated the ability to grow under different percentages of NaCl, 4, 6, and 10 %, for 48 hours (Table 4). As an exception, the isolates GL.02 and GL.10 (both identified as *Lactobacillus plantarum*) could not grow at 10% NaCl.

The 10 isolates revealed an ability to grow under different pH levels of 4.5, 6.2, and 9.6 and showed a complete absence of growth in highly acidic media (pH 2.2) (Table 4). All the isolates were grown in slightly acidic and alkaline media within a pH range of 4.5 to 9.6.

In a 72-hour incubation period, the 10 isolates could grow at 10, 15, and 45 °C (Table 4). In contrast, at a temperature of 4 °C, only the isolates GL.02 and GL.10 (*Lactobacillus plantarum*), and GL.03 (*Lactobacillus fermentum*) could grow.

After exposing all 10 bacterial isolates to 63 °C for 30 minutes, they all were able to grow (incubated at 37 °C for 48 hours) (Table 4).

A significant inhibition of indicator strains was revealed for all 10 isolates. Variably, *E. coli* and *P. aeruginosa* were the most sensitive to the antagonistic activity of 10 isolates, while *S. aureus* was the least sensitive. *B. cereus* was the most resistant strain against the antagonism of isolates GL.05 and GL.07. All 10 isolates exhibited satisfactory antagonistic effects against all the tested indicator strains, ranging from 11.5 to 40.66 mm (Figure 2). A Two-way ANOVA comparison test, followed by a post hoc test (Tukey's multiple comparisons test), showed significant differences with $P < 0.5$.

All 10 isolates showed distinctive fermentative profiles of various carbohydrates. In general, they have been able to ferment carbohydrates widespread in foods (Table 5).

The antibiotic sensitivity test showed different levels of resistance and sensitivity to 10 antibiotics. In short, all 10 isolates were entirely resistant to FEP (30 µg), moderately sensitive to AUG (30 µg), and AM (10 µg). For the rest of the antibiotics, the sensitivity varied (Table 6).

Table 4. Growth profiles and thermotolerance of lactic acid bacteria isolates from raw milk of the Algerian Makatia goat breed under various stress conditions.

Tabela 4. Profili rasti in toplotna toleranca izolatov mlečnokislinskih bakterij iz surovega mleka alžirske koze pasme Makatia v različnih stresnih pogojih.

	Growth under different conditions											
	NaCl (%) (48 h)			pH (48 h)				Temperature (°C) (72 h)				High-temperature tolerance (63 °C for 30 min)
Isolates	04	06	10	2.2	4.5	6.2	9.6	04	10	15	45	
GL.01	+	+	+	-	+	+	+	-	+	+	+	+
GL.02	+	+	-	-	+	+	+	+	+	+	+	+
GL.03	+	+	+	-	+	+	+	+	+	+	+	+
GL.04	+	+	+	-	+	+	+	-	+	+	+	+
GL.05	+	+	+	-	+	+	+	-	+	+	+	+
GL.06	+	+	+	-	+	+	+	-	+	+	+	+
GL.07	+	+	+	-	+	+	+	-	+	+	+	+
GL.08	+	+	+	-	+	+	+	-	+	+	+	+
GL.09	+	+	+	-	+	+	+	-	+	+	+	+
GL.10	+	+	-	-	+	+	+	+	+	+	+	+

Growth was assessed in MRS broth. (+): growth observed; (-): no growth observed. Standard incubation conditions were 37 °C for 48 h in MRS broth with a pH of 6.2. For NaCl tolerance, growth was tested in MRS broth with 4 %, 6 %, and 10 % (w/v) NaCl at 37 °C. For pH tolerance, growth was tested in MRS broth adjusted to pH 2.2, 4.5, 6.2, and 9.6, with incubation at 37 °C. For optimal growth temperature, growth was tested in standard MRS broth at 4 °C, 10 °C, 15 °C, and 45 °C. For the high-temperature (thermo) tolerance, isolates were exposed to 63 °C for 30 min in MRS broth and then incubated under standard conditions to check for survival.

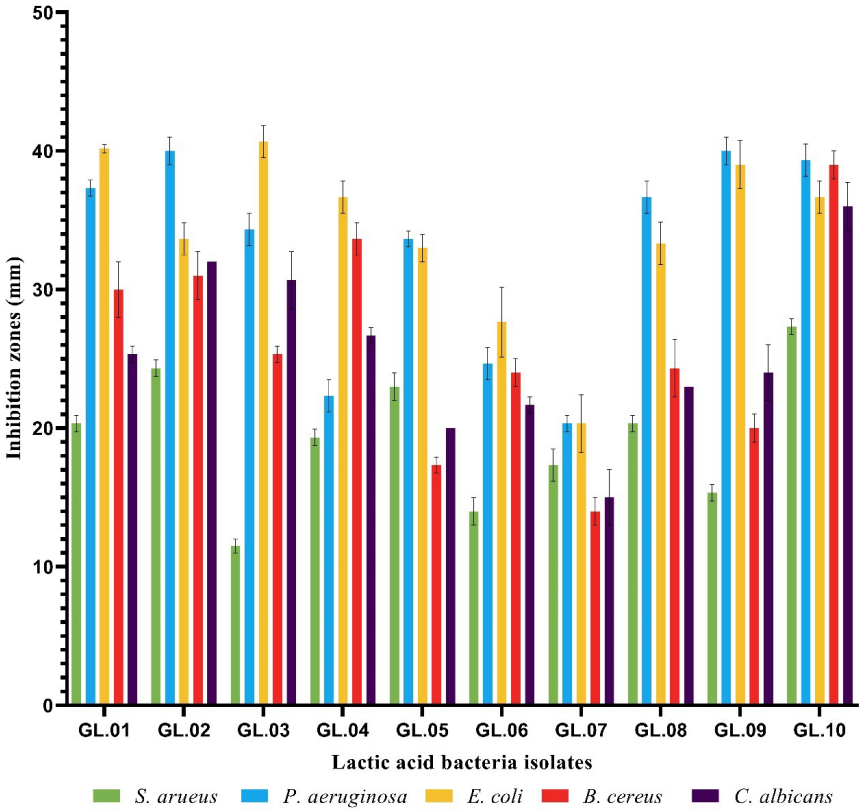


Figure 2. Antagonistic activity of ten lactic acid bacteria isolates against five pathogenic indicator strains. The LAB isolates were obtained from raw Algerian Makatia goat milk. Activity was measured as the diameter of the inhibition zone (mm) using a spot-on-lawn assay. Bars represent the mean of three independent replicates (n=3), and the error bars indicate the standard deviation. The indicator strains tested were *Staphylococcus (S.) aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 10987, and *Candida albicans* ATCC 10231.

Slika 2. Antagonistična aktivnost desetih izolatov mlečnokislinskih bakterij proti petim patogenim indikatorjem. Izolati LAB so bili pridobljeni iz surovega alžirskega kozjega mleka Makatia. Aktivnost je bila izmerjena kot premer inhibicijske cone (mm) z uporabo testa spot-on-lawn. Stolpci predstavljajo povprečje treh neodvisnih ponovitev (n = 3), napake pa so prikazane s standardnim odklonom. Testirani indikatorji so bili *Staphylococcus (S.) aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 10987 in *Candida albicans* ATCC 10231.

Table 5. Carbohydrate fermentation profiles of ten lactic acid bacteria isolates from raw Algerian Makatia goat milk were determined using the API 50 CHL system.

Tabela 5. Profili fermentacije ogljikovih hidratov desetih izolatov mlečnokislinskih bakterij iz surovega alžirskega kozjega mleka Makatia, določeni z uporabo sistema API 50 CHL.

Carbohydrates	Isolates									
	GL.01	GL.02	GL.03	GL.04	GL.05	GL.06	GL.07	GL.08	GL.09	GL.10
Control	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	+	-	-	-
Erythrol	-	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	-	+	+	+	+	+
Ribose	+	+	+	+	-	-	+	+	+	+

D-xylose	-	-	+	-	-	-	+	-	-	-
L-xilose	-	-	+	-	-	-	-	-	-	-
Adonitol	-	-	+	-	-	-	-	-	-	-
B-Methyl-D-Xyloside	-	-	+	-	+	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+
Sorbose	-	-	-	-	+	+	-	-	-	-
Rhamnose	-	+	-	-	-	-	W	-	W	+
Dulcitol	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	-	+	+	-	+	+	+	+
Sorbitol	+	+	-	+	-	-	+	+	+	+
1-methyl-d-mannoside	+	+	-	+	-	-	-	+	+	+
1-methyl-d-glucoside	-	+	-	W	-	-	+	-	-	+
N-acetyl-glucosamine	+	+	-	+	+	+	+	+	+	+
Amygdaline	+	+	-	+	+	-	+	+	+	+
Arbutin	+	+	-	+	+	-	+	+	+	+
Esculin	+	+	-	+	+	+	+	+	+	+
Salicin	+	+	-	+	W	-	+	+	+	+
Cellobiose	+	+	-	+	+	-	+	+	+	+
Maltose	+	+	+	+	+	-	+	+	+	+
Lactose	+	+	+	+	+	-	+	+	+	+
Melibiose	+	-	+	+	+	-	+	+	+	-
Saccharose	+	W	-	+	+	+	+	+	+	-
Trehalose	+	-	+	W	+	-	+	+	+	-
Inulin	-	-	-	-	-	-	-	-	-	-
Melezitose	+	-	-	+	+	-	+	W	W	-
Raffinose	+	-	+	+	+	-	+	-	W	-
Amidon	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-
Gentiobiose	+	+	-	+	W	-	+	+	+	w
D-turanose	+	-	-	+	-	-	W	+	+	-
D-lyxose	-	-	-	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-	-
D-arabitol	+	+	-	+	-	-	-	+	+	+
L-arabitol	-	-	-	-	-	-	-	-	-	-
Gluconate	-	+	+	-	+	W	W	-	-	+
2-keto-gluconate	-	-	-	-	-	-	-	W	-	-
5-keto-gluconate	-	-	-	-	-	W	-	W	-	-

Fermentation profiles were determined using the API 50 CHL system (bioMérieux, Marcy-l'Étoile, France). Strips were inoculated and incubated at 37 °C for 48 hours according to the manufacturer's instructions. Acid production, resulting in a colour change of the pH indicator, indicated fermentation. (+): positive fermentation; (-): negative fermentation; (W): weak positive fermentation. The control well (00) contains the basal medium without any carbohydrate and is a negative control.

Table 6. Antibiotic susceptibility profiles of lactic acid bacteria isolates from raw Algerian Makatia goat milk, determined by the Kirby-Bauer disk diffusion method.

Tabela 6. Profili občutljivosti na antibiotike izolatov mlečnokislinskih bakterij iz surovega alžirskega kozjega mleka Makatia, določeni z metodo difuzije diskov Kirby-Bauer.

Isolates	Antibiotics									
	P (10 µg)	AUG (30 µg)	AM (10 µg)	FEP (30 µg)	FOX (30 µg)	OFX (5 µg)	NA (30 µg)	C (30 µg)	E (15 µg)	TE (30 µg)
GL.01	S	S	S	R	I	R	I	S	R	I
GL.02	I	S	I	R	R	R	R	S	S	I
GL.03	S	S	S	R	I	I	R	S	S	S
GL.04	S	S	S	R	S	I	R	S	S	S
GL.05	S	S	S	R	S	R	R	S	S	S
GL.06	S	S	S	S	I	R	R	I	S	S
GL.07	S	I	S	R	R	S	R	S	S	R
GL.08	I	S	S	R	I	I	R	S	I	S
GL.09	I	S	S	R	I	S	R	S	I	S
GL.10	R	S	S	R	I	R	R	S	I	R

Susceptibility was tested on Mueller-Hinton agar using the Kirby-Bauer disk diffusion method. **R**, resistant (zone size <14 mm), **I**, intermediate (14 mm ≥ zone size ≤ 20 mm), **S**, sensitive (zone size > 20 mm). (CLSI). **P**: Penicillin (10 µg); **AUG**: Amoxicillin/Clavulanic acid (30 µg); **AM**: Ampicillin (10 µg); **FEP**: Cefepime (30 µg); **FOX**: Cefoxitin (30 µg); **OFX**: Ofloxacin (5 µg); **NA**: Nalidixic acid (30 µg); **C**: Chloramphenicol (30 µg); **E**: Erythromycin (15 µg); **TE**: Tetracycline (30 µg).

Discussion

This study analysed some technological traits of 10 different LAB isolates from 4 samples of Makatia goat milk in the Wilaya of Mascara (west of Algeria) that might be favourable for the food and agricultural industries' applications. Most important technological properties have been tested such as ability to convert/ferment raw material to novel food with new characteristics and keep it fresh, withstand harsh growth conditions (the ability to grow in a range of unfavorable temperature, pH, and NaCl presence), their ability to withstand heat treatment with high temperatures (63 °C/30 min), which is similar to the pasteurization process, and their hydrolytic ability.

Acidifying activity is one of the primary properties of starter microorganisms, particularly in dairy, meat, vegetable industry, and silage production (González et al., 2015). The ability to increase the acidity through the fermentation of LAB is one of the options to preserve foods and, at the same time, is one of the most widely exploited technological properties (Hitendra et al., 2016). On the other hand, this technological property is undesirable in some technologies due to the development of sour/untypical flavours, particularly in vinification and beer production (Wang et al., 2021), and is more considered as spoilage. The 10 isolates of LAB, analysed in our study, revealed a remarkable ability to reduce the pH value within 6 to 48 hours. After six hours of incubation, tested LAB were able to drop pH to 5.00 - 6.11, and after 48 h of incubation, the pH drop was even more evident, to 3.24 - 4.47. Abarquero et al. (2022) reported that the strains of 8 LAB species showed different acidifying capacities after 8 and 24 hours of incubation in milk, respectively, with the strains of *Lactococcus lactis* showing the most remarkable acidifying capacity with pH and total acidity values of 4.44. González et al. (2015) found that five strains/20 tested reduced pH values to less than six during 6 hours of incubation. Although different species of LAB possess different acidifying abilities, this trait is species-specific. This could be due to the diverse enzymatic potential that helps bacteria to specifically break down the carbon and nitrogen sources in the medium (Badis et al., 2004).

Among LAB, the most critical technological characteristics are their enzymatic activities. These characteristics are essential in fermentation and food processing (ripening and flavour development). Enzymatic activity is not necessarily linked to the fact that the bacterium is cultivable, as it can leave a group of metabolites and enzymes behind during autolysis. This trait is crucial for food preservation via fermentation (Luigi-Sierra et al., 2024; Yüce et al., 2017; Zambonelli et al., 2002). Although amylolytic activity can be a desirable trait in microorganisms, used as starters, LAB are known not to possess the necessary enzymatic systems (Meena et al., 2022; Owusu-Kwarteng et al., 2015). On the contrary, proteolytic activity is much more typical in LAB, as this mechanism contributes to the development of flavours and the nutritional quality of fermented products, particularly dairy products. Thanks to the production of peptides and biologically active amino acids, LAB also prevent the allergic effect of milk by producing non-epitopic peptides (Lopez-Kleine and Monnet, 2011; Pescuma et al., 2009). Our study revealed significant proteolytic activity with lysis zones as big as 14.33 to 32.67 mm. These results are even more promising compared to the results of Fguiri et al. (2017), who reported analysis of 10 isolates with lysis zones between 15 and 21 mm. Islam et al. (2021) reported that nine strains of LAB out of 11 possessed proteolytic activity, while *Pediococcus pentosaceus* and *Enterococcus faecalis* showed no proteolytic activity. In the study conducted by Abarquero et al. (2022), similar low proteolytic activity was revealed among the groups of LAB ($P > 0.05$), where the strains of *Lactococcus lactis* showed the highest values (close to 1 mmol Gly L− 1 milk). Further, due to their industrial potential, lipases are an important group of enzymes. They are widely used across diverse biotechnological domains, including food, pharmaceutical, detergent, agrochemical, oleochemical, textile, and cosmetic industries (Dinçer and Kıvanç, 2018). On the other hand, microorganisms that possess lipolytic potential are considered highly undesirable, particularly in the food industry. They cause food spoilage, especially those rich in fat, such as meat, fish, butter, and other high-fat dairy products. They degrade fat as a carbon source, directly or indirectly causing what is known as the rancidity phenomenon, which negatively alters the organoleptic properties of foods (Alford and Pierce, 1961; Anand, 2011). In our study, all 10 isolates exerted lipolytic activity, with lysis zones ranging from 8.43 to 12.53 mm. Isolates GL.02 and GL.10 (*Lactobacillus plantarum*) had the highest lipolytic activity, 12.33 and 12.53 mm, respectively. Other studies have also reported low lipolytic activities in several genera of LAB (Fguiri et al., 2017; Islam et al., 2021; Monfredini et al., 2012).

The ability of LAB to produce EPS continues to generate significant interest, particularly in the food industry. EPS are considered as ural thickeners capable of improving the rheological properties of foods. They also have health and nutritional benefits, such as regulating blood sugar, having antioxidant properties, and improving intestinal absorption and microbiome quality (Jurášková et al., 2022). In our study, six isolates (*Lactobacillus* and *Lactiplantibacillus* genera) out of 10 tested appeared as mucoid colloidal colonies, indicating their ability to produce EPS. Moreover, Islam et al. (2021) isolated and identified altogether 50 LAB strains from 18 goat milk samples. Among these 50 isolates, they selected 11 representative strains, which were further tested for technological and probiotic traits. Genus and species identification served as the basis for the selection of these 11 strains. One of the technological traits tested was also the ability to produce EPS. All 11 strains of tested LAB produced EPS, varied between 20 and 93 mg/L, where *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus* exerted the highest levels of EPS production, namely 93.0 and 90.7 mg/L, respectively. Chun-lei (2014) reported that EPS production in 11 strains of LAB (*Leuconostoc*, *Streptococcus*, and *Pediococcus* genera) was as high as 536.90 mg/L.

LABs are considered the primary spoilage agents in vacuum-packed meats and refrigerated foods because they produce undesirable metabolites that affect sensory and market quality (Silva et al., 2018). However, their ability to grow under different conditions, such as a wide range of temperatures and pH, high salinity, plays a crucial role in fermented food preservation, mainly due to the inability of the majority of spoilage microorganisms to grow under these hostile conditions and LAB inhibitory activity (Dillon, 2014). From a health and nutritional perspective, the ability of LAB to grow under unfavourable conditions, such as very low temperatures, like refrigeration temperature, gives them a long shelf life and, thus, significant probiotic potential (Fan et al., 2017). Generally, if we summarise the results of our study, 10 isolates could grow in the presence of high salt concentration, namely in a range of 4 to 10 % NaCl, except for the isolates GL.02 and GL.10 (*Lactobacillus plantarum*), which could not grow at 10 % NaCl. The 10 isolates were also able to grow at different temperatures, ranging from 10 to 45 °C, while only the isolates GL.02, GL.10, and GL.03

(*Lactobacillus* species) could grow at 4 °C as well. Our isolates were also able to grow at a wide range of pH levels (from 4.5 to 9.6) but not at pH 2.2. Several studies have shown that various LAB species can grow under different hostile conditions, such as unfavourable acidity, alkalinity, salinity, storage, and preservation temperatures (Fguiri et al., 2017; Islam et al., 2021; Johanningsmeier et al., 2012).

In some industries, LAB are essential food spoilage agents (cooked and vacuum-packed meat, varieties of thermally treated foodstuffs) (Franz, 1996). After being exposed to 63 °C for 30 minutes, all 10 isolates retained cultivability (at 37 °C/48h). Fossi and Ndjouenkeu (2017) found that two isolates of LAB (*Lactobacillus plantarum* and *Lactobacillus acidophilus*) were resistant to heat treatment (60 °C/60 minutes). Among LAB isolated from commercial sausages, four strains (*Lactobacillus plantarum*, *Pediococcus acidilacti, Lactobacillus curvatus*, and *Pediococcus pentosaceus*) survived heat treatment at 70 °C for 60 minutes (Pérez-Chabela et al., 2008). According to Kulkarni et al. (2018), the *Lactobacillus acidophilus* strain only survived 40 minutes at 65 °C.

It is well known that the antagonistic activity of LAB plays a crucial role as a selection factor for probiotic strains. This activity is essential not only for the putative probiotic potential but also for the technological and industrial potential. It is known that LAB produce a wide variety of metabolites, such as organic acids, bacteriocins, diacetyl, hydrogen peroxide, etc. (Dillon, 2014; Ibrahim et al., 2021) that have antimicrobial potential. So, our strains of LAB were no exception. *P. aeruginosa* and *E. coli* were the most sensitive indicator strains for the antagonism of the 10 isolates, with inhibition zones ranging from 20.33 to 40 mm and 20.33 to 40.66 mm, respectively. In comparison, *S. aureus* was the most resistant strain to the antagonism of the 10 isolates, with inhibition zones ranging from 11.5 to 27.33. In general, the antagonistic activity of 10 isolates varied between 11.5 and 40.66 mm. Owusu-Kwarteng et al. (2015) published that some *L. fermentum* strains' antagonistic activities against *S. aureus* ATCC 1448 were insufficient, with inhibition zones smaller than 4 mm. This may be because antimicrobial capacity is a highly strain-dependent property and not a constant characteristic of the entire bacterial species. The problem may not be only the weakness of *L. fermentum* but also the strength and resistance of *S. aureus*. Islam et al. (2021) found antagonistic activities of some LAB, particularly *L. plantarum*, with inhibition zones reaching up to 20 mm against five pathogens. The broad spectrum of activity against a wide range of pathogens is because *L. plantarum* produces a diverse and potent group of antimicrobial compounds, the most well-known of which are bacteriocins such as plantaricin. These compounds are often highly effective even at low concentrations. In a study of Divyashree et al. (2024), the antimicrobial activity of 6 *Lactobacillus* strains was tested against some enteric pathogens, such as *Salmonella typhi, Escherichia coli, Listeria monocytogenes, Salmonella paratyphi, Klebsiella pneumonia, Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Therefore, they prepared cell-free supernatants (CFS) of 6 lactobacilli strains that were used in the microdilution method in 96 96-well microtiter plate. All six lactobacilli CFS exhibited antagonistic properties against pathogens, achieving over 90 % inhibition, which was calculated according to the formula from the publication.

The different genera of LAB can metabolise a wide range of carbohydrates, including polysaccharides, which determines their industrial applications, whether in food or non-food sectors (Wang et al., 2021). The API 50 CHL system was used to examine the fermentation profile. The 10 isolates fermented various carbohydrates, including galactose, glucose, fructose, mannose, mannitol, sorbitol, and more. Also, none of the isolates metabolised the sugars numbered 16, 33, 36-38, 41-44, 46, and 48 (Table 5). Several studies have confirmed the ability of LAB to metabolise a wide range of carbohydrates and use them as energy sources (Gunkova et al., 2021; Islam et al., 2021; Ni et al., 2015; Ruiz et al., 2024).

Regarding the importance of health safety, evaluating antibiotic resistance is one of the selection criteria for interesting bacteria, either probiotic or technological. Before using a starter culture or a probiotic product, it is necessary to check that the bacterial strains involved do not possess high resistance or antibiotic resistance genes (Fguiri et al., 2017). The 10 isolates revealed varying levels of sensitivity and resistance depending on the types of antibiotics tested. Generally, they showed apparent resistance to FEP (30 µg) and NA (30 µg), while they were either sensitive or moderately sensitive to the other tested antibiotics. Many studies have examined the resistance of different genera of LAB to antibiotics. The results have always varied from one type to another, and according to the classes of antibiotics. However, it is important that, to date, no LAB have been reported to exhibit multiple antibiotic resistance (Amenu and Bacha, 2023; Choi et al., 2018; Meradji et al., 2023).

Conclusion

In this study, 10 LAB strains were isolated from 4 milk samples of Makatia breed goats in the Wilaya of Mascara (western Algeria). 4 isolates were identified as *Lactiplantibacillus plantarum*, two were identified as *Lactobacillus plantarum*, and 1 isolate each as *Lactobacillus fermentum, Lactiplantibacillus paraplantarum, Lactobacillus sakei*, and *Lactiplantibacillus pentosus*. The isolates exhibited hydrolytic activities, the ability to grow under different storage conditions, remarkable thermotolerance, and antimicrobial activity against pathogens. At the same time, all 10 isolates were not reported to possess multidrug resistance. These characteristics make 10 isolates useful in the food industry as starter cultures and natural alternatives to chemical preservatives. In certain specific sectors, the same characteristics may also serve as important factors to avoid the possibility of certain undesirable interactions. Therefore, more specialized and targeted studies are still necessary to determine the relevance of exploiting the technological properties of LAB with various food manufacturing or processing methods.

Author Contributions

Conceptualization, A.S. and S.G.; methodology, all authors; software, A.S.; validation, all authors; formal analysis, A.S. and S.G.; investigation, A.S. and S.G.; resources, Scientific Research Laboratories of the Universities of Oran 1 and Mascara, Algeria; data curation, all authors; writing—original draft preparation, all authors; writing—review and editing, all authors; visualization, A.S. and S.G.; supervision, S.G.; project administration, S.G. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to thank Prof. Madani Benyoucef, Director of the Laboratory of Geomatics, Ecology and Environment (LGEO2E), University of Mascara, Algeria, for his cooperation in providing the necessary research facilities to carry out this study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The authors declare that there is no conflict of interest.

References

Abarquero, D., Renes, E., Combarros-Fuertes, P., Fresno, J.M., Tornadijo, M.E., 2022. Evaluation of technological properties and selection of wild lactic acid bacteria for starter culture development. LWT, 171, 114121. https://doi.org/10.1016/j.lwt.2022.114121

Ağagündüz, D., Şahin, T.Ö., Ayten, Ş., Yılmaz, B., Güneşliol, B.E., Russo, P., Spano, G., Özogul, F., 2022. Lactic acid bacteria as pro-technological, bioprotective and health-promoting cultures in the dairy food industry. Food Bioscience, 47, 101617. https://doi.org/10.1016/j.fbio.2022.101617

Akman, P.K., Ozulku, G., Tornuk, F., Yetim, H., 2021. Potential probiotic lactic acid bacteria isolated from fermented gilaburu and shalgam beverages. LWT, 149, 111705. https://doi.org/10.1016/j.lwt.2021.111705

Alford, J.A., Pierce, D.A., 1961. Lipolytic Activity of Microorganisms at Low and Intermediate Temperatures. III. Activity of Microbial Lipases at Temperatures Below 0°C. Journal of Food Science, 26(5), 518–524. https://doi.org/10.1111/j.1365-2621.1961.tb00399.x

Amapu, T., Ameh, J., Ado, S., Abdullahi, I., Dapiya, H., 2016. Amylolytic Potential of Lactic Acid Bacteria Isolated from Wet Milled Cereals, Cassava Flour and Fruits. British Microbiology Research Journal, 13(2), 1–8. https://doi.org/10.9734/BMRJ/2016/24509

Amenu, D., Bacha, K., 2023. Probiotic potential and safety analysis of lactic acid bacteria isolated from Ethiopian traditional fermented foods and beverages. Annals of Microbiology, 73(1), 37. https://doi.org/10.1186/s13213-023-01740-9

Anand, S., 2011. Analytical Methods | Microbiological. In: Fuquay, J.W. (Ed.) Encyclopedia of Dairy Sciences, pp. 215–220. Elsevier. https://doi.org/10.1016/B978-0-12-374407-4.00021-2

Khadija, A., Omar, B., Abdessamad, E., Rachid, I., Imane, O., Hicham, H., Mohammed, O., 2022. Phenotypic and Genotypic Identification of the Most Acidifiers LAB Strains Isolated from Fermented Food. Biology Bulletin, 49(4), 260–270. https://doi.org/10.1134/S1062359022040045

Badis, A., Guetarni, D., Boudjema, B.M., Henni, D.E., Kihal, M., 2004. Identification and technological properties of lactic acid bacteria isolated from raw goat milk of four Algerian races. Food Microbiology, 21(5), 579–588.

Baureder, M., Hederstedt, L., 2013. Heme Proteins in Lactic Acid Bacteria. Advances in Microbial Physiology, 62, 1–43. https://doi.org/10.1016/B978-0-12-410515-7.00001-9

Choi, A.-R., Patra, J.K., Kim, W.J., Kang, S.-S., 2018. Antagonistic Activities and Probiotic Potential of Lactic Acid Bacteria Derived From a Plant-Based Fermented Food. Frontiers in Microbiology, 9, 1963. https://doi.org/10.3389/fmicb.2018.01963

Chun-lei, Z., 2014. Selection of exopolysaccharide-producing lactic acid bacteria isolates from Inner Mongolian traditional yoghurt. Mljekarstvo, 64(4), 254–260. https://doi.org/10.15567/mljekarstvo.2014.0404

CLSI, 2020. M100-Performance Standards for Antimicrobial Susceptibility Testing (Version 30th ed). www.clsi.org

Dillon, V.M., 2014. Natural anti-microbial systems | Preservative effects during storage. In: Batt, C.A., Tortorello, M.L. (Eds.) Encyclopedia of Food Microbiology pp. 941–947. Elsevier. https://doi.org/10.1016/B978-0-12-384730-0.00238-X

Dinçer, E., Kıvanç, M., 2018. Lipolytic Activity of Lactic Acid Bacteria Isolated from Turkish Pastırma. Anadolu University Journal Of Science And Technology –C Life Sciences and Biotechnology 7(1), 12–19. https://doi.org/10.18036/aubtdc.306292

Divyashree, S., Ramu, R., Sreenivasa, M.Y., 2024. Evaluation of new candidate probiotic *Lactobacillus* strains isolated from a traditional fermented food-multigrain-millet dosa batter. Food Bioscience, 57, 103450. https://doi.org/10.1016/j.fbio.2023.103450

Edwards, U., Rogall, T., Blöcker, H., Emde, M., Böttger, E.C., 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research, 17(19), 7843–7853. https://doi.org/10.1093/nar/17.19.7843

Fan, S., Breidt, F., Price, R., Pérez- Díaz, I., 2017. Survival and Growth of Probiotic Lactic Acid Bacteria in Refrigerated Pickle Products. Journal of Food Science, 82(1), 167–173. https://doi.org/10.1111/1750-3841.13579

Fguiri, I., Ziadi, M., Atigui, M., Ayeb, N., Arroum, S., Assadi, M., Khorchani, T., 2016. Isolation and characterisation of lactic acid bacteria strains from raw camel milk for potential use in the production of fermented Tunisian dairy products. International Journal of Dairy Technology, 69(1), 103–113. https://doi.org/10.1111/1471-0307.12226

Fguiri, I., Ziadi, M., Rekaya, K., Samira, A., Khorchani, T., 2017. Isolation and characterization of lactic acid bacteria strains from raw camel milk for potential use in the production of yogurt. J. Food Sci. Nutr, 3, 1–8.

Fossi, B.T., Ndjouenkeu, R., 2017. Probiotic potential of thermotolerant lactic acid bacteria isolated from "Gari"a cassava-based African fermented food. Journal of Applied Biology Biotechnology, 5(4), 1–5.

Franz, C., 1996. Thermotolerance of meat spoilage lactic acid bacteria and their inactivation in vacuum-packaged vienna sausages. International Journal of Food Microbiology, 29(1), 59–73. https://doi.org/10.1016/0168-1605(95)00022-4

García-Cano, I., Rocha-Mendoza, D., Ortega-Anaya, J., Wang, K., Kosmerl, E., Jiménez-Flores, R., 2019. Lactic acid bacteria isolated from dairy products as potential producers of lipolytic, proteolytic and antibacterial proteins. Applied Microbiology and Biotechnology, 103(13), 5243–5257. https://doi.org/10.1007/s00253-019-09844-6

Ghanbari, M., Rezaei, M., Jami, M., Nazari, R.M., 2009. Isolation and characterization of Lactobacillus species from intestinal contents of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*). Iranian Journal of Veterinary Research, 10(2), 152-157. https://doi.org/10.22099/ijvr.2009.1668

González, L., Cuadrillero, A.F., Castro, J.M., Bernardo, A., Tornadijo, M.E., 2015. Selection of lactic acid bacteria isolated from San Simón da Costa Cheese (PDO) in order to develop an autochthonous starter culture. Advances in Microbiology, 5(11), 748–759. https://doi.org/10.4236/aim.2015.511079

Gunkova, P.I., Buchilina, A.S., Maksimiuk, N.N., Bazarnova, Y.G., Girel, K.S., 2021. Carbohydrate Fermentation Test of Lactic Acid Starter Cultures. IOP Conference Series: Earth and Environmental Science, 852(1), 012035. https://doi.org/10.1088/1755-1315/852/1/012035

Haghshenas, B., Nami, Y., Almasi, A., Abdullah, N., Radiah, D., Rosli, R., Barzegari, A., Khosroushahi, A.Y., 2017. Isolation and characterization of probiotics from dairies. Iranian Journal of Microbiology, 9(4), 234.

Hitendra, J., Prasad, B.D.N., Murthy, G., Suvarna, H., 2016. Role of Lactic Acid Bacteria (LAB) in Food Preservation. International Journal of Current Microbiology and Applied Sciences, 5(8), 255–257. https://doi.org/10.20546/ijcmas.2016.508.026

Hudzicki, J., 2009. Kirby-Bauer disk diffusion susceptibility test protocol. American Society for Microbiology, 15(1), 1–23.

Hurtado-Bautista, E., Pérez Sánchez, L.F., Islas-Robles, A., Santoyo, G., Olmedo-Alvarez, G., 2021. Phenotypic plasticity and evolution of thermal tolerance in bacteria from temperate and hot spring environments. PeerJ, 9, e11734. https://doi.org/10.7717/peerj.11734

Ibrahim, S.A., Ayivi, R.D., Zimmerman, T., Siddiqui, S.A., Altemimi, A.B., Fidan, H., Esatbeyoglu, T., Bakhshayesh, R.V., 2021. Lactic Acid Bacteria as Antimicrobial Agents: Food Safety and Microbial Food Spoilage Prevention. Foods, 10(12), 3131. https://doi.org/10.3390/foods10123131

Islam, Md.Z., Uddin, Md.E., Rahman, Md.T., Islam, M.A., Harun-ur-Rashid, Md., 2021. Isolation and characterization of dominant lactic acid bacteria from raw goat milk: Assessment of probiotic potential and technological properties. Small Ruminant Research, 205, 106532. https://doi.org/10.1016/j.smallrumres.2021.106532

Johanningsmeier, S.D., Franco, W., Perez- Diaz, I., McFeeters, R.F., 2012. Influence of Sodium Chloride, pH, and Lactic Acid Bacteria on Anaerobic Lactic Acid Utilization during Fermented Cucumber Spoilage. Journal of Food Science, 77(7), 397-404 https://doi.org/10.1111/j.1750-3841.2012.02780.x

Jurášková, D., Ribeiro, S.C., Silva, C.C.G., 2022. Exopolysaccharides Produced by Lactic Acid Bacteria: From Biosynthesis to Health-Promoting Properties. Foods, 11(2), 156. https://doi.org/10.3390/foods11020156

Kim, C., Alrefaei, R., Bushlaibi, M., Ndegwa, E., Kaseloo, P., Wynn, C., 2019. Influence of growth temperature on thermal tolerance of leading foodborne pathogens. Food Science Nutrition, 7(12), 4027–4036. https://doi.org/10.1002/fsn3.1268

Kulkarni, S., Haq, S.F., Samant, S., Sukumaran, S., 2018. Adaptation of Lactobacillus acidophilus to Thermal Stress Yields a Thermotolerant Variant Which Also Exhibits Improved Survival at pH 2. Probiotics and Antimicrobial Proteins, 10(4), 717–727. https://doi.org/10.1007/s12602-017-9321-7

Lopez-Kleine, L., Monnet, V., 2011. Lactic acid bacterial proteolytic systems. Applied microbiology and biotechnology, 71(4), 394-406.

Luigi-Sierra, M.G., Ramayo-Caldas, Y., Guan, D., Amills, M., 2024. Short communication: Comparing the microbiota diversity from the core, middle part and rind of six Spanish commercial goat cheeses. Livestock Science, 285, 105496. https://doi.org/10.1016/j.livsci.2024.105496

Meena, K.K., Taneja, N.K., Jain, D., Ojha, A., Kumawat, D., Mishra, V., 2022. In Vitro Assessment of Probiotic and Technological Properties of Lactic Acid Bacteria Isolated from Indigenously Fermented Cereal-Based Food Products. Fermentation, 8(10), 529. https://doi.org/10.3390/fermentation8100529

Meradji, M., Bachtarzi, N., Mora, D., Kharroub, K., 2023. Characterization of Lactic Acid Bacteria Strains Isolated from Algerian Honeybee and Honey and Exploration of Their Potential Probiotic and Functional Features for Human Use. Foods, 12(12), 2312. https://doi.org/10.3390/foods12122312

Monfredini, L., Settanni, L., Poznanski, E., Cavazza, A., Franciosi, E., 2012. The spatial distribution of bacteria in Grana-cheese during ripening. Systematic and Applied Microbiology, 35(1), 54–63. https://doi.org/10.1016/j.syapm.2011.07.002

Nayik, G.A., Jagdale, Y.D., Gaikwad, S.A., Devkatte, A.N., Dar, A.H., Dezmirean, D.S., Bobis, O., Ranjha, M.M.A.N., Ansari, M.J., Hemeg, H.A., Alotaibi, S.S., 2021. Recent Insights Into Processing Approaches and Potential Health Benefits of Goat Milk and Its Products: A Review. Frontiers in Nutrition, 8, 789117. https://doi.org/10.3389/fnut.2021.789117

Ni, K., Wang, Y., Li, D., Cai, Y., Pang, H., 2015. Characterization, Identification and Application of Lactic Acid Bacteria Isolated from Forage Paddy Rice Silage. PLOS ONE, 10(3), e0121967. https://doi.org/10.1371/journal.pone.0121967

Owusu-Kwarteng, J., Tano-Debrah, K., Akabanda, F., Jespersen, L., 2015. Technological properties and probiotic potential of *Lactobacillus fermentum* strains isolated from West African fermented millet dough. BMC Microbiology, 15(1), 261. https://doi.org/10.1186/s12866-015-0602-6

Patricia, S., Laura, C., 2011. Motility Test Medium Protocol. American Society for Microbiology. (Accessed: 2011, November 1).

Peng, K., Koubaa, M., Bals, O., Vorobiev, E., 2020. Recent insights in the impact of emerging technologies on lactic acid bacteria: A review. Food Research International, 137, 109544. https://doi.org/10.1016/j.foodres.2020.109544

Pérez-Chabela, M. de L., Totosaus, A., Guerrero, I., 2008. Evaluation of thermotolerant capacity of lactic acid bacteria isolated from commercial sausages and the effects of their addition on the quality of cooked sausages. Food Science and Technology, 28, 132–138.

Pescuma, M., Hébert, E. M., Dalgalarrondo, M., Haertlé, T., Mozzi, F., Chobert, J.-M., Font De Valdez, G., 2009. Effect of Exopolysaccharides on the Hydrolysis of β-Lactoglobulin by Lactobacillus acidophilus CRL 636 in an in Vitro Gastric/Pancreatic System. Journal of Agricultural and Food Chemistry, 57(12), 5571–5577. https://doi.org/10.1021/jf9006505

Ribeiro, S.C., Coelho, M.C., Silva, C.C.G., 2021. A rapid screening method to evaluate acidifying activity by lactic acid bacteria. Journal of Microbiological Methods, 185, 106227. https://doi.org/10.1016/j.mimet.2021.106227

Ruiz, M.J., Medina, L.M., Palacio, M.I., Vega, M.F., Etcheverría, S., Frizzo, L.S., Etcheverría, A.I., 2024. Investigation of some probiotic and technological properties of lactic acid bacteria strains isolated from artisanal sheep milk cheese and their growth in goat milk. Small Ruminant Research, 238, 107329. https://doi.org/10.1016/j.smallrumres.2024.107329

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences, 74(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Silva, A.P.R.D., Longhi, D.A., Dalcanton, F., Aragão, G.M.F.D., 2018. Modelling the growth of lactic acid bacteria at different temperatures. Brazilian Archives of Biology and Technology, 61, e18160159. https://doi.org/10.1590/1678-4324-2018160159

Sionek, B., Szydłowska, A., Trząskowska, M., Kołożyn-Krajewska, D., 2024. The Impact of Physicochemical Conditions on Lactic Acid Bacteria Survival in Food Products. Fermentation, 10(6), 298. https://doi.org/10.3390/fermentation10060298

Smith, A.C., Hussey, M.A., 2005. Gram stain protocols. American society for microbiology, 1(14), 113-144.

Tanasupawat, S., Phoottosavako, M., Keeratipibul, S., 2015. Characterization and lipolytic activity of lactic acid bacteria isolated from Thai fermented meat. Journal of Applied Pharmaceutical Science, 5(3), 6–12. https://doi.org/10.7324/JAPS.2015.50302

Tidona, F., Francolino, S., Ghiglietti, R., Locci, F., Carminati, D., Laforce, P., Giraffa, G., 2020. Characterization and pre-industrial validation of *Streptococcus thermophilus* strains to be used as starter cultures for Crescenza, an Italian soft cheese. Food Microbiology, 92, 103599. https://doi.org/10.1016/j.fm.2020.103599

Vinderola, G., 2019. Lactic acid bacteria: Microbiological and functional aspects (5th edition). CRC Press. https://doi.org/10.1201/9780429057465.

Vivantis Technologies Sdn Bhd. (2010). GF-1 Bacterial DNA Extraction Kit.

Wang, Y., Wu, J., Lv, M., Shao, Z., Hungwe, M., Wang, J., Bai, X., Xie, J., Wang, Y., Geng, W., 2021. Metabolism Characteristics of Lactic Acid Bacteria and the Expanding Applications in Food Industry. Frontiers in Bioengineering and Biotechnology, 9, 612285. https://doi.org/10.3389/fbioe.2021.612285

Webster, D., 2003. pH – Principles and measurement. In: Caballero, B. (Ed.) Encyclopedia of Food Sciences and Nutrition, pp. 4501–4507. Elsevier. https://doi.org/10.1016/B0-12-227055-X/00913-5

Yüce, S., Tahtaci, S.A., Kılıç, G.B., 2017. The hydrolytic enzymes produced by halophilic lactic acid bacteria. GIDA – Journal of Food, 42(3), 242-251.

Zambonelli, C., Chiavari, C., Benevelli, M., Coloretti, F., 2002. Effects of lactic acid bacteria autolysis on sensorial characteristics of fermented foods. Food Technology and Biotechnology, 40(4), 347–351.

ABS

Original Research

Taxonomical implications of fruit and seed morphology in wild species of *Diospyros* L. (Ebenaceae) in Odisha, Eastern India

Sabita Tudu [1], Jeevan Kumar Jena [1], Sifan Priyadarshini [1], Sudhanshu Sekhar Dash [2] and Sudam Charan Sahu [1,*]

Abstract

The family Ebenaceae exhibits a diverse array of characteristics and evolutionary histories. The members of the genus *Diospyros* L. are mostly dioecious and exhibit a great variation in their leaf, flower, fruit and seed morphology. These morphological variations often lead to ambiguity in species delimitation and misidentification in the genus *Diospyros*. As a pilot study, a detailed morphological and morphometric assessment was carried out in ten species of *Diospyros* growing wild in Odisha, Eastern India. Twenty (20) taxonomically important morphological characters of fruits and seeds were selected to study the similarity and variations among the taxa. The phenetic clustering divided the studied taxa of *Diospyros* into three groups at a cophenetic correlation coefficient (r) of 0.89, which provided insights into species differentiation and their closest taxonomic relationship. Moreover, the principal component analysis (PCA) indicated that the length of the calyx lobe and the size of the seed were significant in explaining the total variation among the studied taxa. The study came up with a dichotomous key and revealed the distinct morphological characters of fruits and seeds, contributing to a refined understanding of the *Diospyros* species complex. To clarify the taxonomic ambiguity, fruit and seed morphological characteristics provide an important tool for accurate species identification and enriching our understanding of the systematics of these specific Ebenaceae members.

Keywords

Diospyros L., Ebenaceae, morphology, phenetic clustering, Principal Component Analysis (PCA), Odisha

1 Biosystematics Laboratory, Department of Botany, Maharaja Sriram Chandra Bhanja Deo University, Baripada-757003, Odisha, India

2 Botanical Survey of India, CGO Complex, Sector -1, Salt Lake, Kolkata, West Bengal, India

*** Corresponding author:**
E-mail address: sudamsahu.bdk@gmail.com

Citation: Tudu, S., Jena, J. K., Priyadarshini, S., Dash, S. S., Sahu, S. C., (2025). Taxonomical implications of fruit and seed morphology in wild species of *Diospyros* L. (Ebenaceae) in Odisha, Eastern India. Acta Biologica Slovenica 68 (4)

Received: 19.04.2025 / **Accepted:** 18.08.2025 / **Published:** 20.08.2025

https://doi.org/10.14720/abs.68.4.22558

Taksonomske posledice morfologije plodov in semen divjih vrst *Diospyros* L. (Ebenaceae) v Odishi, vzhodna Indija

Izvleček

Družina Ebenaceae ima raznolike značilnosti in evolucijsko zgodovino. Člani rodu *Diospyros* L. so večinoma dvodomni in se med seboj zelo razlikujejo po morfologiji listov, cvetov, plodov in semen. Te morfološke razlike pogosto vodijo do nejasnosti pri razmejevanju vrst in napačni identifikaciji v rodu *Diospyros*. Kot pilotna študija je bila izvedena podrobna morfološka in morfometrična ocena desetih vrst *Diospyros*, ki rastejo v divji naravi v Odishi v vzhodni Indiji. Za preučevanje podobnosti in razlik med taksoni je bilo izbranih dvajset (20) taksonomsko pomembnih morfoloških značilnosti plodov in semen. Fenetično združevanje je preučevane taksone *Diospyros* razdelilo v tri skupine s kophenetičnim korelacijskim koeficientom (r) 0,89, kar je omogočilo vpogled v razlikovanje vrst in njihovo najbližjo taksonomsko sorodnost. Poleg tega je analiza glavnih komponent (PCA) pokazala, da sta dolžina listne čaše in velikost semena pomembna za pojasnitev skupnih razlik med preučevanimi taksoni. Študija je prinesla dihotomni ključ in razkrila različne morfološke značilnosti plodov in semen, kar je prispevalo k boljšemu razumevanju kompleksa vrst *Diospyros*. Morfološke značilnosti plodov in semen so pomembno orodje za natančno določanje vrst in bogatijo naše razumevanje sistematike teh posebnih članov družine Ebenaceae.

Ključne besede

Diospyros L., Ebenaceae, morfologija, fenetično združevanje, analiza glavnih komponent (PCA), Odisha

Introduction

The taxonomically diverse family Ebenaceae exhibits a wide range of variation in vegetative and reproductive characteristics, leading to challenges in classification and identification. Due to the rich species diversity and dioecious habit, the family was subjected to several variable classifications with respect to the status of genera from time to time by various taxonomists (Bentham and Hooker, 1876; Clarke, 1882; Bakhuizen van den Brink, 1933; White, 1988; Singh, 2005). Recent studies reveal that the family Ebenaceae represents three genera, such as *Diospyros* L., *Euclea* L. and *Lissocarpa* Benth, distributed mostly in pantropical regions of the world (Utteridge and Jennings, 2021; WFO, 2025). Among these genera, *Diospyros* L. is the largest genus, consisting of about 787 species globally (Govaerts, 2021). Members of this genus are distributed pantropically in the warmer regions of Southeast Asia, including China, Japan, Korea, India, and Pakistan, as well as parts of the Middle East and Africa. *Diospyros* species generally prefer warmer climates, whereas a few can also be found in temperate regions (Shashwathi and Krishnamurthy, 2024). These species exhibit a wide range of diversity, thriving in ecosystems such as dry forests, rainforests, mixed forests, lowland forests, and dry deciduous forests (Turner et al., 2013; Rauf et al., 2017).

In India, the genus *Diospyros* comprises about 66 taxa (Singh, 2005; POWO, 2025; WFO, 2025). It is estimated that 17 taxa are endemic to India, and their distribution is mostly concentrated in the peninsular region, North-Eastern region and Andaman and Nicobar Islands (Gamble, 1922; Haines, 1922; Singh, 2005; POWO, 2025; WFO, 2025). Members of this genus are taxonomically characterised by tree or shrub, dioecious, evergreen or semi-evergreen; simple, alternate, coriaceous leaves; axillary cyme or pistillate flower solitary; fleshy with persistent calyx; 1-16 seeded fruit (Singh, 2005; Abd El Halim et al., 2014; Schatz and Lowry, 2018; Samuel et al., 2019). In addition to the taxonomic diversity, the species of *Diospyros* are also recognised for their economic value, providing high-quality timber, medicinal compounds and edible fruits (Nematollahi et al., 2012; Butt et al., 2015; Ullah et al., 2015; Ribeiro et al., 2023).

The state of Odisha, located on the eastern coast of India, is home to a rich diversity of flora, including several species of *Diospyros* L. The varied climatic conditions and ecosystems ranging from coastal plains to dense tropical evergreen forests support this diversity. The work carried out by Saxena and Brahmam (1995) was the last effort in

the field of conventional taxonomy on the genus *Diospyros* from Odisha. Subsequently, significant contributions to the taxonomy of the genus have been made, such as the monograph of Indian Diospyros (Singh, 2005); the work of Schatz and Lowry (2020) for the Malagasy region; Rindyastuti et al. (2021) for Indonesian *Diospyros* L. These studies have advanced our understanding of the genus through detailed evaluation of morphological traits, including leaves, flowers, and fruits. However, morphological inconsistencies, particularly in vegetative and floral characters, continue to complicate species identification. Consequently, fruit morphology has emerged as a more consistent and reliable diagnostic feature for distinguishing among *Diospyros* species (Schatz and Lowry, 2018; Sandratriniaina et al., 2023). Moreover, seed morphology has been investigated in terms of shape, size, germination, and the results showed that seed characteristics clearly have taxonomic relevance (Ahmed and Qaiser, 1989; Corral et al., 1989; Karaismailoglu and Erol, 2018; Abdel et al., 2021; Ozbek and Uzunhisarcıklı, 2023).

The numerical taxonomy employs statistical and computational techniques to analyse morphological data, providing an objective framework for species delimitation (Sneath, 2001). By utilising multivariate analysis, this approach facilitates the reconstruction of more precise phylogenetic relationships, offering clear insights into evolutionary connections. In the case of *Diospyros*, numerical taxonomy can significantly contribute to identifying key morphological characteristics that are vital for distinguishing species and resolving confusion in species groups. Accordingly, this study was carried out to improve the accuracy in species identification based on comparative key characters and statistical measures of fruit and seed morphology. The present study aimed to a) analyse the fruit and seed morphological characteristics of *Diospyros* L. taxa found in Odisha; b) determine the closest relationship among the studied taxa, and c) find out the key characters used for delimitation of *Diospyros* species complex.

Materials and Methods

Study area

The present study was conducted in Odisha, located on the eastern coast of India (17°49' to 22°34' N, 81°27' to 87°29' E) (Figure 1). Odisha accounts for 4.87% (1,55,707 km^2) of India's total geographical area. The state features four distinct physiographic regions: the Northern Plateau, Eastern Ghats, Central Table Land, and Coastal Plains (Government of Odisha, 2025). Nearly one-third of Odisha is forested, covering 37.34% of the state's total land area. The forest cover extends over 58,136.869 km^2 (ISFR, 2023). Odisha experiences a tropical monsoon climate, with hot summers and substantial monsoonal rainfall. The average annual temperature ranges between 25°C and 28°C, with rainfall varying from 1,200 mm to 1,600 mm. Soil types include mixed red and black soils in the Central Table Land, and red, yellow, and fertile alluvial soils in the Coastal Plains. These climatic and soil variations support diverse vegetation, including tropical dry deciduous, mixed, moist deciduous, and semi-evergreen forests (Champion and Seth, 1968; Saxena and Brahmam, 1994).

Field survey and Specimen collection

Several field tours were carried out across different regions of the state during the period from January 2022 to September 2024. Fresh plant samples were collected for all ten *Diospyros* species from 13 distinct localities across the state of Odisha. For each species, a minimum of 5 to 10 individuals were sampled per locality. The location coordinates of each specimen were recorded using a handheld Global Positioning System (GPS) with World Geodetic System 1984 (a geographic coordinate system) (Table 1). The preliminary identification was made through detailed observation of the collected specimen, along with a survey of the regional and national herbaria (CSIR-IMMT, Bhubaneswar; Central National Herbarium, Howrah (CAL), regional floras (Saxena and Brahmam, 1995; Haines, 1922), monograph and available literature. Voucher specimens for all collected taxa were prepared following the standard protocol (Kottapalli et al., 2016) and deposited in the Herbarium at the Department of Botany, MSCB University, Baripada (MSCBUB). These vouchers are available for future reference and taxonomic validation.

During field surveys, the habitat characteristics of each *Diospyros* species were recorded in situ at the point of specimen collection. Data included associated vegetation and forest types (i.e., moist deciduous, dry deciduous or coastal forest) following Champion and Seth's (1968) forest type classification. Further, the information regarding local names, growth form, fruiting phenology, and local uses of *Diospyros* species was obtained through informal interviews with local communities residing near the sampling

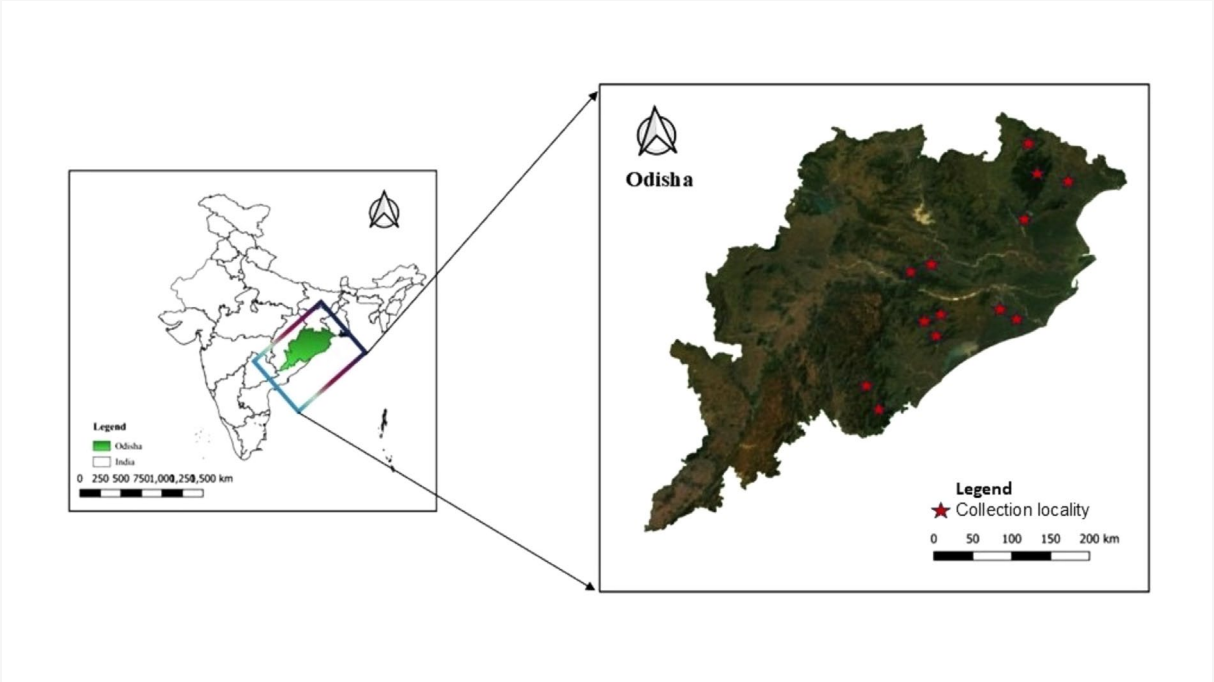


Figure 1. Map of the study area with collection localities. (Source: Map generated using QGIS v3.34).

Slika 1. Zemljevid območja raziskave z lokacijami zbiranja. (Vir: zemljevid, izdelan z uporabo QGIS v3.34).

sites. A total of 25 informants (aged between 30 and 70 years), including forest-dependent villagers and traditional knowledge holders, were interviewed using a purposive sampling approach. The key questions included: (i) What is the local name of this tree? (ii) Is the tree known for any particular use (e.g., fruit, timber, medicine)? (iii) During which months does it bear fruit? and (iv) What is its local significance or perceived abundance? Responses were documented through note-taking during direct conversations in the *Odia* and *Santali* languages. Additionally, the threat status and distribution data were presented by referring to the IUCN Red List data and Plants of the World Online (POWO, 2025), respectively.

Morphological analysis

Morphological characteristics of fruits and seeds were analysed and described according to the Kew Plant Glossary (Beentje, 2016). The key characters that were examined include infructescence pedicel; shape, colour, surface of fruit; calyx format, number of calyx-lobe, calyx-lobe format, margin, aestivation of fruiting calyx; quantity, format, colour of seeds and endosperm (Table 2). Endosperm type: "Ruminate" indicates irregular intrusions of the seed coat into the endosperm, while "uniform" indicates a uniform and uninterrupted endosperm. Colour of fruits and seeds was determined visually in daylight and compared against the Royal Horticultural Society (RHS) Colour Chart to ensure consistency and reduce subjectivity.

Morphometric analysis

For morphometric analysis, fruit length, fruit diameter, length and width of fruiting calyx, seed length and width were measured using a digital calliper. Fruit length was measured from the base of the fruit to the apex, excluding the calyx; fruit diameter was taken at the widest cross-section. Calyx length and width were measured from the base to the tip of the lobes and across the widest span of the calyx, respectively. Seed length and width were measured along the longest and widest axes, respectively. All characters were measured on mature fruits and were expressed as the mean of 10 measurements. "Thin" pericarp refers to a thickness of <2 mm; "Thick" is >2 mm and rigid. Additionally, the number of seeds per fruit and the number of calyx lobes were documented through direct observation.

Table 1. Details of coordinates (World Geodetic System 1984), specimen collection locality, and accession number were deposited for *Diospyros* L.

Tabela 1. Podrobnosti o koordinatah (Svetovni geodetski sistem 1984), kraju zbiranja vzorcev in številki dostopa so bile deponirane za *Diospyros* L.

Species Name	Coordinates	Locality	Accession No.
D. candolleana Wight	18°57′51.92"N 84°22′22.41"E	Mahendragiri, Gajapati	MSCBUB/01510
D. chloroxylon Roxb.	20°18'00.08"N 85°43'59.08" E	Chandaka Wildlife Sanctuary	MSCBUB/01511
	20°03'34.66"N 85°12'43.77"E	Beguniapatana, Nayagarh	MSCBUB/01512
D. ebenum Koenig.	20°36'13.96"N 85°01'43.95"E	Satkosia, Angul	MSCBUB/01513
	19°57'12.98"N 85°07'29.72"E	Similijhar, Nayagarh	MSCBUB/01514
D. exculpta Buch. -Hum.	19°00′58.94"N 84°14′47.52"E	Raikaguma, Gajapati	–
D. ferrea (Willd.) Bakh.	20°18'00.92"N 85°43'00.46" E	Chandaka Wildlife Sanctuary	MSCBUB/01515
	19°57'45.00"N 86°15'39.71"E	Astaranga, Puri	MSCBUB/01516
D. malabarica (Desr.) Kostel.	21°17'24.03"N 86°14'39.91"E	Pitanau, Keonjhar	MSCBUB/01517
	21°55'33.78"N 86°27'15.97"E	Similipal BR, Mayurbhanj	–
	20°36'02.26"N 85°01'40.92"E	Satkosia WS, Angul	MSCBUB/01518
D. melanoxylon Roxb.	20°35'20.04"N 84°46'48.27"E	Padmadala, Angul	–
	22°15'41.04"N 86°26'43.33"E	Argarghati, Mayurbhanj	MSCBUB/01519
	21°50'15.05"N 86°35'08.35"E	Dukura, Mayurbhanj	MSCBUB/01520
	20°16'17.90"N 85°09'44.42"E	Khandapada, Nayagarh	–
D. montana Roxb.	21°52'13.35"N 86°30'33.86"E	Similipal BR, Mayurbhanj	MSCBUB/01521
	22°15'55.10"N 86°26'30.36"E	Argarghati, Mayurbhanj	–
	21°50'52.39" N 86°33'30.34"E	Dukura, Mayurbhanj	MSCBUB/01522
	19°57'26.43"N 85°07'27.19"E	Similijhar, Nayagarh	–
D. ovalifolia Wight.	19°57'11.25"N 85°07'29.05"E	Simili jhar, Nayagarh	MSCBUB/01523
D. sylvatica Roxb.	21°55'55.95"N 86°32'25.63"E	Similipal BR, Mayurbhanj	MSCBUB/01524
	22°15'55.23"N 86°26'21.86"E	Argarghati, Mayurbhanj	–
	21°51'09.25"N 86°32'56.80"E	Dukura, Mayurbhanj	–
	19°57'38.10"N 85°07'32.88"E	Similijhar, Nayagarh	MSCBUB/01525

Statistical analysis

The phenetic relationship and the key characters for morphological variation in *Diospyros* were investigated using two statistical analyses. Firstly, the Cluster Analysis (CA) was conducted using the Unweighted Pair Group Method (UPGMA) to establish the phenetic relationship that explains the closest relationship among the studied *Diospyros* species. Secondly, principal components analysis (PCA) was performed to identify the effects of selected characters on the delimitation of the *Diospyros* species complex. In total, 20 taxonomically important characters with 34 morphological attributes and eight morphometric attributes were selected and codified numerically (Table 2). The cluster analysis was performed using statistical software PAST (Paleontological Statistics) ver. 4.03. whereas PCA was plotted by ggbiplot in the R environment.

Results

Species Diversity

The present study documented a total of ten wild-growing taxa belonging to the genus *Diospyros* L. in Odisha, Eastern India (Table 3). This diversity encompasses a range of trees and shrubs, and was found to be adopted in different habitats. The species *D. melanoxylon* Roxb. was a notable species within the studied taxa, which primarily thrives in mixed deciduous forests. Likewise, the species *D. ebenum* Koenig., *D. montana* Roxb. and *D. sylvatica* Roxb. were found to share the same habitat across the moist deciduous forest type of Odisha. The species *D. malabarica* (Desr.) Kostel is typically distributed near streams, where it coexists with other tree species. In contrast, the two representative shrub species were *D. ferrea* (Willd.) Bakh. and *D. chloroxylon*

Table 2. List of morphological and morphometric attributes selected for the present study.

Tabela 2. Seznam morfoloških in morfometričnih lastnosti, izbranih za to študijo.

Characters	Structure analysed	States/measurements
Morphological attributes		
Fruit	Pedicel	Sessile (1), subsessile (2)
	shape	Globose (1), ovoid (2)
	colour	Pale green (1), olive red (2), yellow (3), red (4), black (5), orange (6), salmon (7)
	surface	glabrous (1), pubescent (2)
Fruiting calyx	calyx format	Flat (1), campanulate (2), bowl-shaped (3)
	calyx lobe format	Triangular (1), rounded (2), ovate (3)
	margin	Entire (1), rolled (2), wavy (3)
	aestivation	Valvate (1), contorted (2)
	texture of calyx	Thin leafy (1), thick leafy (2), woody (3)
Seed	seed format	Oblong (1), rounded (2), elliptic (3)
	colour	Brown (1), black (2)
	endosperm	Ruminate (1), uniform (2)
Morphometric attributes		
Fruit	length	cm
	diameter	cm
Fruiting calyx	no. of calyx lobe	3 to 5
	length	cm
	diameter	cm
Seed	seed quantity	1 to 8
	length	cm
	diameter	cm

Roxb., mostly found in coastal forest areas. The diversity of the remaining three species, such as *D. candolleana* Wight., *D. exculpta* Buch. -Hum. and *D. ovalifolia* Wight, was found to be confined within a small geographical area (Figure 2).

Phenology and IUCN status

The baseline information on local names, growth form, phenology, threat status, local importance, and distribution of the studied taxa is presented in Table 3. Regarding phenological events, the present study documented the fruiting period for all taxa. The fruits of the *Diospyros* species developed after flowering and ripened at varying times based on species and local climate. However, the study observed that peak fruiting maturity for most species occurred between April and May. In case of the two shrub species, *D. chloroxylon* Roxb. and *D. ferrea* (Willd.) Bakh., the fruiting period peaked from September to October. Additionally, seed germination begins between June and July, following fruit maturation. As per the distribution, the collected *Diospyros* taxa were mostly found to be distributed in India and Sri Lanka. Notably, *D. candolleana* and *D. exculpta* are endemic to India. Concerning the threat status, *D. candolleana* has been categorised as Vulnerable (VU) based on criteria A2cd of the IUCN Red List. In contrast, the IUCN status of the remaining species indicated that *D. chloroxylon, D. exculpta, D. ferrea, D. malabarica, D. melanoxylon, D. montana, D. ovalifolia*, and *D. sylvatica* were listed as Not Evaluated (NE), while *D. ebenum* was categorised as Data Deficient (DD).

Economic Importance

The documentation on local importance indicated that *Diospyros* species were traditionally valued for their edible fruit, timber, medicinal properties, and fodder. Timber from species such as *D. candolleana, D. malabarica*, and the well-known ebony *D. ebenum* was employed in building materials and artisanal craftsmanship. Certain species, including *D. malabarica, D. melanoxylon* and *D. montana*, were recognised for their medicinal properties used for treating stomach ailments. The foliage of *D. melanoxylon*, known as bidi leaf (used for smoking purposes), contrib-

uted to local livelihoods through its use in bidi production. Additionally, species like *D. ferrea* and *D. ovalifolia* were utilised as fodder for livestock, underscoring their significance in local agriculture.

Morphological characterisation

The present morphological analysis showed remarkable differences in fruit, fruiting calyx and seeds (Table 4). All the fruits were fleshy and indehiscent. There was also a variation observed in fruit shape that varies from globose to ovoid. The pre-mature fruits were green, but a gradual variation was found with yellow, orange, red, olive red, black and salmon colour. The fruit surface was mostly glabrous in the young and mature states of all taxa. However, the fruit surface of *D. melanoxylon* and *D. ovalifolia* was pubescent in young fruit. The analyses of fruiting calyx showed significant taxonomical differences in format, aestivation and texture of the calyx lobe. As per the observation, four major types of calyx format were observed: flat, campanulate, flattened triangularly, and bowl-shaped, among the ten species. Of these, about seven species showed flat calyx format, while *D. malabarica* was flattened triangularly. Besides, *D. chloroxylon* and *D. ferrea* showed campanulate-type calyx format, and *D. ebenum* showed bowl-shaped calyx format. Ovate, rounded, triangular calyx lobes were observed in these species, with the exception of *D. malabarica*, which showed an ovate-triangular calyx lobe.

Among the seed characters, endosperm showed potential distinguishing tools for the delimitation of taxa. The majority of the species had uniform endosperm, while the four species, i.e., *D. candolleana, D. exculpta, D. melanoxylon* and *D. sylvatica*, were characterised by ruminate endosperm. The observed seed format was rounded, elliptic and oblong. Among the ten species, nine had brown-coloured seeds, except *D. montana*, which had black-coloured seeds.

Morphometric analysis

The fruit, calyx and seeds of ten wild-growing *Diospyros* taxa were critically examined for the analysis of the morphometric variations. The analysis included length and diameter of fruit, number of calyx lobes, length and width of calyx lobes, seed quantity, seed length, and seed width, which were represented in Table 5. The study came up with a variation in fruit size of 2.46±1.57 × 1.95±1.41 cm (length × diameter), where *D. ferrea* (0.98±0.04 cm) was the smallest fruit and *D. malabarica* (6.06±0.08 cm) was found to be the largest one. A significant difference in seed quantity per fruit was observed within the individual species of the studied taxa. The seed number varies from 1 (*D. ferrea; D. ovalifolia*) to 8 (*D. malabarica*), and the remaining species have an average of 4 seeds within the taxa. A negligible variation was found in the number of calyx lobes per fruit within the same taxa. However, the species like *D. melanoxylon* and *D. sylvatica* exhibit minimum variation, where the number of calyx lobes ranges from 5 to 6 and 3 to 4, respectively.

Phenetic relationship

The dendrogram, generated by cluster analysis of morphological characters, clustered the studied taxa into three groups (Figure 3). The first group comprises three taxa, namely *D. ovalifolia, D. chloroxylon* and *D. ferrea*, at 0.3 taxonomic distance. In the second group, *D. exsculpta, D. candolleana* and *D. melanoxylon* were clustered at 0.2 taxonomic distance. In the last group, clustering of *D. ebenum, D. malabarica, D. montana* and *D. sylvatica* was found at about 0.29 taxonomic distance. This dendrogram exhibited a cophenetic correlation coefficient (r) of 0.89, which was considered a good fit for the observed data.

Principal component analysis (PCA)

The plotted result of Principal component analysis shows the key diagnostic traits and patterns of variation that assisted in distinguishing among the species of *Diospyros* (Figure 4). The analysis resulted in the separation of variables along PC1 and PC2, explained with a variance of 38% and 29%, respectively. The PC1 was found to have a weighted contrast between the length of the calyx lobe and the seed width. Both the characters exhibited higher influence in variation with a positive coefficient (loading coefficient=0.37). On the other hand, the number of calyx lobes, texture of calyx lobes, endosperm, calyx lobe margin and fruit surface are found to show inverse relationships with principal components. The second Principal component was dominated by calyx lobe format, fruit shape, aestivation of calyx and seed colour, with the highest positive loading. Moreover, there was an overlapping variable, i.e., fruit diameter and length of seed, which shows more similarity among the original variables. The ellipses in the PCA biplot represented two distinct clusters of species, providing independent support for morphological groupings based on trait loadings.

Table 3. Details on the baseline information of *Diospyros* species in Odisha, Eastern India.

Tabela 3. Podrobnosti o osnovnih podatkih vrst *Diospyros* v Odishi, vzhodna Indija.

Species Name	Local name	Growth form	Fruiting Phenology	Threat Status	Local importance	Distribution
D. candolleana Wight.	Kendu	Tree	March-June	VU	Timber, fodder	India
D. chloroxylon Roxb.	Kosai kendu	Shrub	Oct.-Dec.	NE	Edible fruit	India, Bangladesh
D. ebenum Koenig.	Kendu	Tree	June	DD	Timber	India, Sri Lanka
D. exculpta Buch. -Hum.	Kendu	Tree	Oct.-Dec.	NE	Timber	India
D. ferrea (Willd.) Bakh.	Guakoli	Shrub	Sep.	NE	Edible fruit, fodder	India, Sri Lanka, Myanmar, Malaysia, Indonesia, Australia, Africa
D. malabarica (Desr.) Kostel.	Mankada kendu	Tree	June	NE	Edible fruit, timber, medicine	Indo-Malesia
D. melanoxylon Roxb.	Kendu	Tree	May	NE	Edible fruit, foliage, timber, medicine	India, Sri Lanka
D. montana Roxb.	Halada	Tree	Apr.	NE	Fish poisoning, medicine	Indo-Malesia
D. ovalifolia Wight.	Kendu	Small tree	Apr.	NE	Fodder	India, Sri Lanka, Bangladesh
D. sylvatica Roxb.	Kalucha	Tree	March	NE	Edible fruit, timber, medicine	India, Sri Lanka

[Abbr.: VU- Vulnerable; DD- Data Deficient; NE- Not evaluated. Distribution data were compiled from Plants of the World Online (POWO, 2025), and threat status was referenced from the IUCN Red List (2024).]

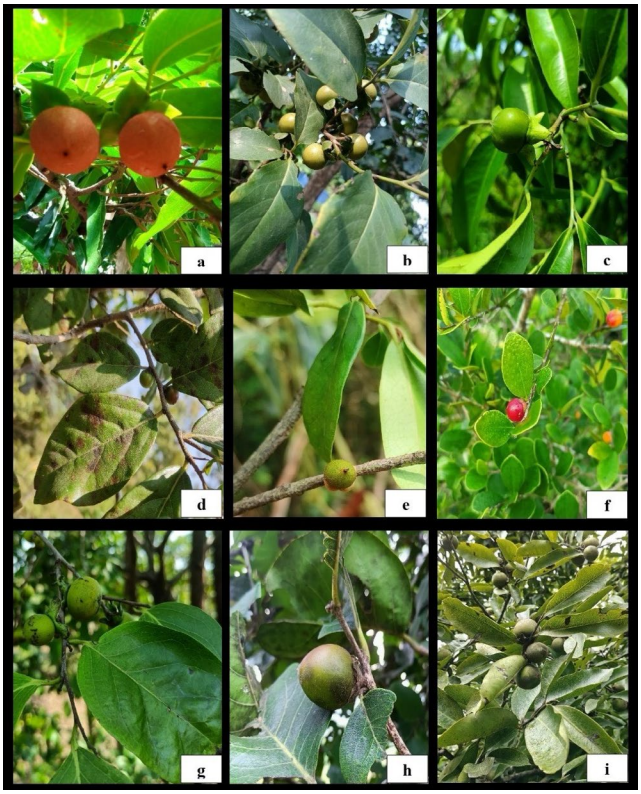


Figure 2. Field photographs of *Diospyros* species in the fruiting stage: a) *D. malabarica*, b) *D. montana*, c) *D. ebenum*, d) *D. chloroxylon*, e) *D. ovalifolia*, f) *D. ferrea*, g) *D. sylvatica*, h) *D. melanoxylon*, and i) *D. candolleana*.

Slika 2. Terenske fotografije vrst *Diospyros* v plodnem obdobju: a) *D. malabarica*, b) *D. montana*, c) *D. ebenum*, d) *D. chloroxylon*, e) *D. ovalifolia*, f) *D. ferrea*, g) *D. sylvatica*, h) *D. Melanoxylon* in i) *D. candolleana*.

Table 4. Fruit morphological characters of *Diospyros* L. in Odisha, Eastern India.

Tabela 4. Morfološke značilnosti plodov *Diospyros* L. v Odishi, vzhodna Indija.

Fruit					
Species Name	**Pedicel**	**Fruit colour (mature)**	**Fruit shape**	**Surface (young fruit)**	**Surface (mature fruit)**
D. candolleana Wight.	sessile	pale yellow	ovoid	glabrous	glabrous
D. chloroxylon Roxb.	sessile	olive red	globose	glabrous	glabrous
D. ebenum Koenig.	subsessile	yellow	ovoid	glabrous	glabrous
D. exculpta Buch. -Hum.	sessile	yellow	ovoid	glabrous	glabrous
D. ferrea (Willd.) Bakh.	sessile	red	globose	glabrous	glabrous
D. malabarica (Desr.) Kostel.	subsessile	salmon	ovoid	Red scurf	glabrous
D. melanoxylon Roxb.	sessile	yellow	ovoid	pubescent	glabrous
D. montana Roxb.	subsessile	black	globose	glabrous	glabrous
D. ovalifolia Wight.	subsessile	orange	globose	pubescent	glabrous
D. sylvatica Roxb.	subsessile	black	globose	glabrous	glabrous
Fruiting calyx	**Calyx format**	**Calyx lobe format**	**Calyx lobe margin**	**Aestivation**	**Texture of calyx**
D. candolleana Wight.	sessile	pale yellow	ovoid	glabrous	glabrous
D. chloroxylon Roxb.	sessile	olive red	globose	glabrous	glabrous
D. ebenum Koenig.	subsessile	yellow	ovoid	glabrous	glabrous
D. exculpta Buch. -Hum.	sessile	yellow	ovoid	glabrous	glabrous
D. ferrea (Willd.) Bakh.	sessile	red	globose	glabrous	glabrous
D. malabarica (Desr.) Kostel.	subsessile	salmon	ovoid	Red scurf	glabrous
D. melanoxylon Roxb.	sessile	yellow	ovoid	pubescent	glabrous
D. montana Roxb.	subsessile	black	globose	glabrous	glabrous
D. ovalifolia Wight.	subsessile	orange	globose	pubescent	glabrous
D. sylvatica Roxb.	subsessile	black	globose	glabrous	glabrous
Seed (mature)	**Seed format**	**Seed colour**	**Endosperm**		
D. candolleana Wight.	oblong	brown	ruminate		
D. chloroxylon Roxb.	rounded	brown	uniform		
D. ebenum Koenig.	elliptic	brown	uniform		
D. exculpta Buch. -Hum.	elliptic	brown	ruminate		
D. ferrea (Willd.) Bakh.	rounded	brown	uniform		
D. malabarica (Desr.) Kostel.	elliptic	brown	uniform		
D. melanoxylon Roxb.	elliptic	brown	ruminate		
D. montana Roxb.	elliptic	black	uniform		
D. ovalifolia Wight.	rounded	brown	uniform		
D. sylvatica Roxb.	oblong	brown	ruminate		

Table 5. Fruit morphometric characters of *Diospyros* L. in Odisha, Eastern India.

Tabela 5. Morfometrične značilnosti plodov *Diospyros* L. v Odishi, vzhodna Indija.

Species Name	Fruit length (cm)	Fruit diam. (cm)	No. of calyx-lobe	Length of calyx lobe (cm)	Width of calyx-lobe (cm)	Seed quantity	Length of seed (cm)	Width of seed (cm)
D. candolleana	2.7±0.22	1.8±0.1	5	1.05±0.07	0.67±0.05	4	1.2±0.05	0.8±1.3
D. chloroxylon	1.03±0.05	0.94±0.12	4	0.36±0.05	0.47±0.05	3	0.7±0.05	0.5±0.1
D. ebenum	2.03±0.05	1.9±0.09	4	0.95±0.12	0.62±0.05	4	1.22±0.05	0.6±0.07
D. exculpta	3.5±0.23	2.5±0.57	5	2.45±0.07	0.8±0.09	5	1.07±0.09	0.72±0.05
D. ferrea	0.98±0.04	0.7±0.11	3	0.42±0.05	0.38±0.12	1–4	0.7±0.09	0.62±0.04
D. malabarica	6.06±0.08	5.5±0.4	4	3±0.07	2.5±0.57	8	2.5±0.08	1.2±0.05
D. melanoxylon	3.4±0.21	2.42±0.22	5	2.5±0.05	0.9±0.14	4–6	1.5±0.05	0.91±0.14
D. montana	2.4±0.06	1.99±0.09	4	1.5±0.08	0.87±0.13	6	1.06±0.08	0.6±0.09
D. ovalifolia	1.04±0.11	0.92±0.15	4	0.5±0.05	0.45±0.05	1–2	0.91±0.09	0.45±0.07
D. sylvatica	1.5±0.07	0.9±0.12	4	0.8±0.03	0.85±0.15	6	1±0.05	0.75±0.05

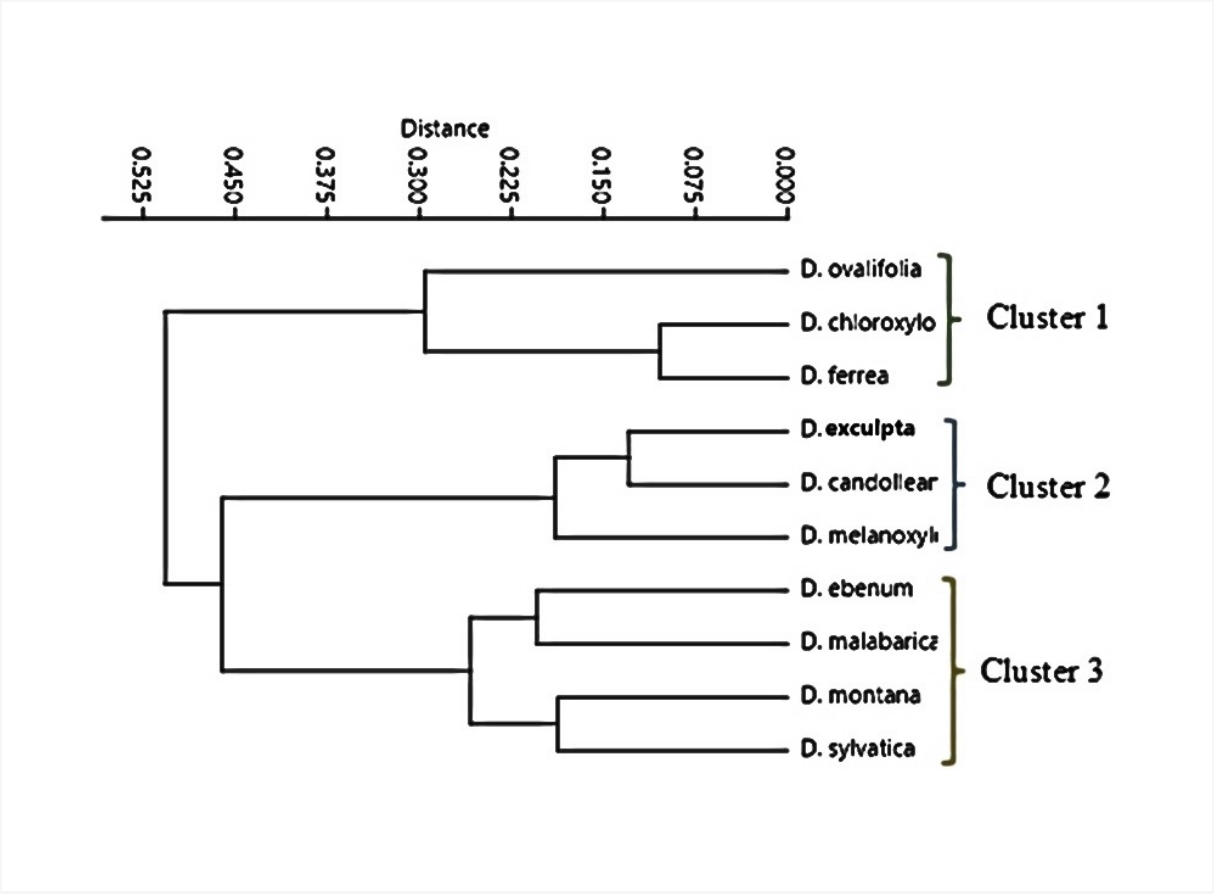


Figure 3. A dendrogram was produced through cluster analysis of the examined characters.

Slika 3. Dendrogram je bil izdelan s pomočjo skupinske analize preučevanih značilnosti.

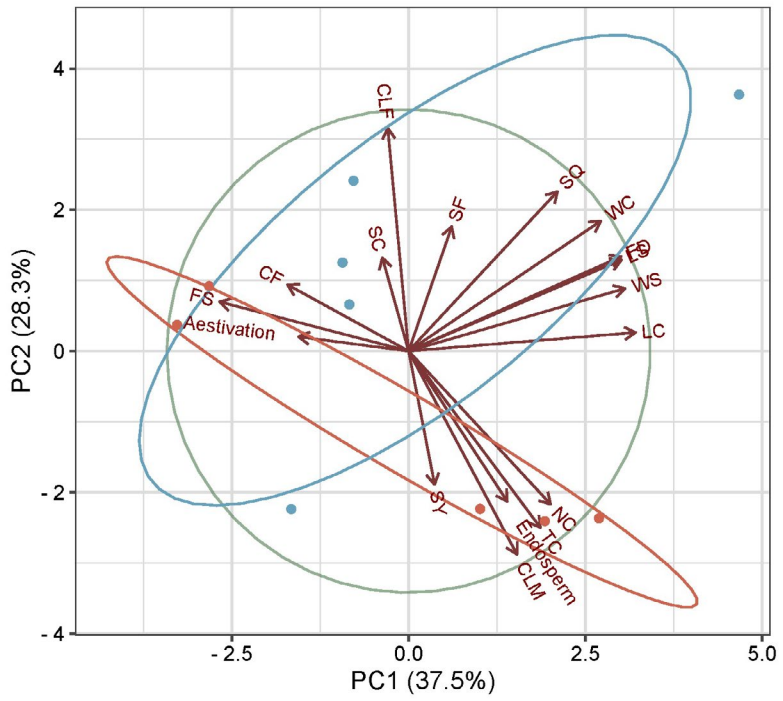


Figure 4. Loading plot showing the contribution of fruit and seed morphological characters in the first two Principal components. The ellipses represent 95% confidence intervals for the individual groups of samples. [Calyx format (CF); Calyx lobe format (CLF); Calyx lobe margin (CLM); Texture of calyx (TC); Fruit shape (FS); Fruit Surface (SY); Seed colour (SC); Seed format (SF); Fruit diameter (FD); Number of calyx lobe (NC); length of calyx lobe (LC); width of calyx lobe (WC); seed quantity (SQ); seed length (LS) and seed width (WS)].

Slika 4. Diagram prikazuje prispevek morfoloških značilnosti plodov in semen v prvih dveh glavnih komponentah. Elipse predstavljajo 95-odstotne interval zaupanja za posamezne skupine vzorcev. [Oblika keliha (CF); Oblika kelihovega lista (CLF); Rob kelihovega lista (CLM); Tekstura keliha (TC); Oblika ploda (FS); Površina ploda (SY); Barva semena (SC); Oblika semena (SF); Premer ploda (FD); Število cvetnih listov (NC); Dolžina cvetnega lista (LC); Širina cvetnega lista (WC); Količina semen (SQ); Dolžina semena (LS) in širina semena (WS)]

Key to the species of the genus *Diospyros* L.

Here, an identification key was prepared based on constant diagnostic characters of fruit and seed morphology for more natural and practical segregation of taxa. Further, a photo plate was also provided to support the morpho-taxonomical information on collected *Diospyros* species (Figure 5; Figure 6).

1. Fruiting calyx lobe 3 to 4 2
1. Fruiting calyx lobe 5..... 8

2. Calyx-lobe contorted ..... *D. chloroxylon*
2. Calyx-lobe valvate 3

3. Fruiting calyx bowl-shaped ..... *D. ebenum*
3. Fruiting calyx flat or campanulate 4

4. Fruiting calyx woody ..... *D. ovalifolia*
4. Fruiting calyx thin to thick leafy 5

5. Endosperm ruminate ..... *D. sylvatica*
5. Endosperm uniform 6

6. Size of mature fruit up to 1 × 1 cm; calyx lobe format rounded ..... *D. ferrea*
6. Size of mature fruit more than 1 × 1 cm; calyx lobe format ovate-triangular 7

7. Matured fruit black in colour ..... *D. montana*
7. Matured fruit salmon in colour ..... *D. malabarica*

8. Young fruit surface glabrous; calyx lobe margin reflexed 9
8. Young fruit surface pubescent; calyx lobe margin wavy ..... *D. melanoxylon*

9. Seeds oblong, shining, margin obscure..... *D. candolleana*
9. Seeds elliptic, compressed, margin prominent ..... *D. exculpta*

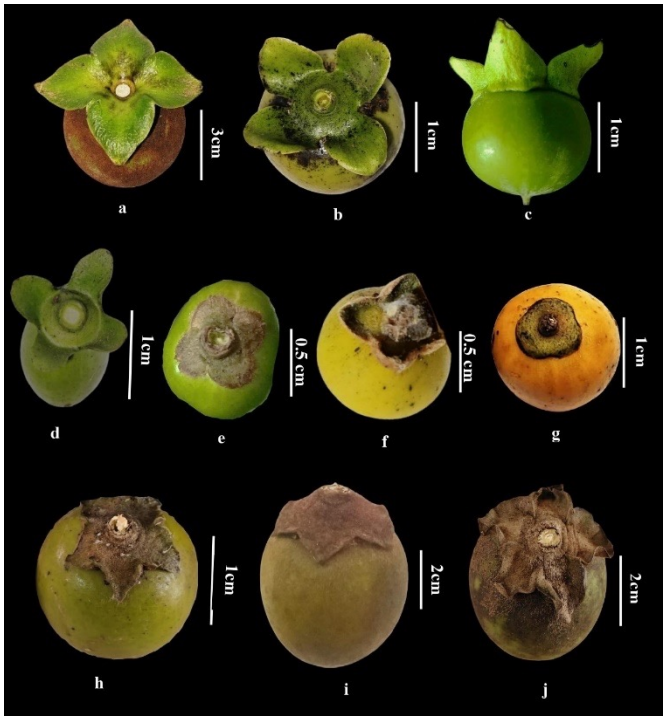


Figure 5. Comparative fruit morphology of *Diospyros* L. species: a) *D. malabarica*, b) *D. montana*, c) *D. ebenum*, d) *D. sylvatica*, e) *D. chloroxylon*, f) *D. ovalifolia*, g) *D. ferrea*, h) *D. exculpta*, i) *D. melanoxylon*, and j) *D. candolleana*. All fruits were photographed at the premature stage, preserving the diagnostic characters used for species identification.

Slika 5. Primerjalna morfologija plodov vrst *Diospyros*: a) *D. malabarica*, b) *D. montana*, c) *D. ebenum*, d) *D. sylvatica*, e) *D. chloroxylon*, f) *D. ovalifolia*, g) *D. ferrea*, h) *D. exculpta*, i) *D. melanoxylon*, in j) *D. candolleana*. Vsi plodovi so bili fotografirani v nezrelem stanju, da so ohranili diagnostične značilnosti, ki se uporabljajo za identifikacijo vrst.

Figure 6. Comparative seed morphology of Diospyros L. species: a) *D. malabarica*, b) *D. montana*, c) *D. ebenum*, d) *D. sylvatica*, e) *D. chloroxylon*, f) *D. ovalifolia*, g) *D. ferrea*, h) *D. exculpta*, i) *D. melanoxylon*, and j) *D. candolleana*. Seed images were captured from mature fruits, except *D. candolleana*, which was photographed at the immature stage.

Slika 6. Primerjalna morfologija semen vrst Diospyros L.: a) *D. malabarica*, b) *D. montana*, c) *D. ebenum*, d) *D. sylvatica*, e) *D. chloroxylon*, f) *D. ovalifolia*, g) *D. ferrea*, h) *D. exculpta*, i) *D. melanoxylon*, in j) *D. candolleana*. Slike semen so bile posnete z zrelih plodov, razen *D. candolleana*, ki je bila fotografirana v nezrelem stanju.

Discussion

The genus *Diospyros* L. exhibits considerable species richness in India, with most taxa having highly localised distributions and minimal geographic overlap (Bakhuizen van den Brink, 1933; Singh, 2005). The present study documented the occurrence of ten wild-growing *Diospyros* taxa in Odisha, predominantly of Indian origin. Their occurrence patterns generally correspond with previous floristic accounts for the state (Gamble, 1922; Haines, 1922; Saxena and Brahmam, 1995; Singh, 2005), indicating broad stability in distribution over time. The majority of taxa are concentrated in the forested uplands of the Eastern Ghats, while a few, such as *D. ferrea* and *D. chloroxylon*, also occur in lowland coastal forests. Notably, this study documented new distributional localities for *D. chloroxylon, D. ebenum, D. exculpta*, and *D. ovalifolia* (Table 1), providing updated field confirmations that strengthen and extend earlier reports for the region.

The surveyed *Diospyros* species in Odisha show diverse local uses, largely centred on timber, edible fruits, fodder, and medicinal applications. Timber-yielding taxa such as *D. candolleana, D. ebenum*, and *D. malabarica* are valued for their durability and fine grain, echoing earlier reports from southern and central India (Gamble, 1881; Troup, 1986). Edible fruits from species like *D. chloroxylon, D. ferrea, D. melanoxylon* and *D. sylvatica* are consumed seasonally and occasionally sold in local markets, a pattern also noted by Mahapatra and Panda (2009). Certain species, including *D. montana* and *D. malabarica*, are employed in traditional medicine (Ahmed, 1993; Joshi, 1993; Singh, 1993; Jayaraman, 1996; Achiwa et al., 1997; Prajapati et al., 2003), while *D. montana* is additionally used as a fish poison, a practice recorded in parts of the Western Ghats (Narayanan et al., 2011). In addition to these known uses, this survey recorded two novel observations. Ripe fruits of *D. candolleana* were observed being consumed by the giant squirrel (*Ratufa indica*) and bonnet macaque (*Macaca radiata*), suggesting a potential role for these mammals in seed dispersal. Furthermore, *D. montana* was cited in local ethnomedicinal practice as a treatment for gastric ulcers, a use not previously reported in the literature. Although the uses recorded are generally consistent with regional ethnobotanical patterns, the present survey expands both the geographic and functional scope of existing records and confirms the persistence of traditional knowledge in rural communities. Such information, when integrated with conservation assessments, may help prioritise species that are both ecologically significant and culturally valued.

Previous taxonomic studies of *Diospyros* have largely emphasised vegetative and floral traits for species identification (Putri and Chikmawati, 2015; Schatz and Lowry, 2020; Gnonlonfin et al., 2022; Meeprom et al., 2024). In contrast, the present work demonstrates that fruit and seed morphology can serve as a reliable and distinct diagnostic tool for species delimitation, offering advantages where vegetative or floral material is unavailable. Similar approaches have been successfully applied in other plant families, including *Araceae* (Camelo et al., 2023), *Apocynaceae* (Sarikaya and Guven, 2024), *Rosa* L. (Zarkovic et al., 2024), and *Cistus* L. (Gokmen et al., 2024). The addition of morphometric analysis further strengthened the discriminatory power of fruit and seed traits, as also reported in other taxa (Sonibare et al., 2004; Abdulrahaman and Oladele, 2005; Nandini et al., 2015). A notable contribution of this study is the resolution of morphological overlap between *D. montana* and *D. sylvatica*. While both species share similarities in fruit and calyx form, morphometric evaluation revealed significant differences in fruit length, fruit diameter, and calyx lobe length, providing key traits for accurate identification. However, seed size was found to be less diagnostic. This clarification addresses an area of uncertainty in the regional taxonomy of the genus.

The clustering analysis revealed phenetic groupings that in many cases align with known morphological or phylogenetic relationships within *Diospyros*. The close association between *D. melanoxylon, D. exculpta* and *D. candolleana* was found to be consistent with shared fruit and calyx traits previously noted in regional floras (Saxena and Brahmam, 1995), and may reflect a relatively recent divergence within Indian lineages. Similarly, the grouping of *D. ebenum, D. malabarica, D. montana*, and *D. sylvatica* parallels patterns observed in molecular phylogenetic studies from Southeast Asia, where these taxa cluster within the same clade despite ecological differences (Duangjai et al., 2006). The separation of *D. ferrea* and *D. chloroxylon* from *D. ovalifolia* supports earlier morphological classifications based on fruit size and calyx structure (Singh, 2005), suggesting that these traits are evolutionarily conserved within certain subgroups.

Moreover, the PCA shows the most significant character that explains the total variation among the studied taxa. This analysis indicated that the length of the calyx, seed length, seed width, fruit diameter, and seed quantity are the most significant features with the highest relative

variation rate, which can be useful in the delimitation of the *Diospyros* species complex. These traits likely hold strong taxonomic value because they are linked to reproductive structures, which tend to be more genetically constrained and less influenced by local environmental variation than vegetative traits (Sonibare et al., 2004). The present study confirms that fruit and seed morphology, when analysed qualitatively and quantitatively, offers a reliable and effective tool for taxonomic delimitation (Camelo et al., 2023; Belo et al., 2024), and they provide a regional baseline for integrating morphometric data into broader systematic and phylogenetic frameworks.

While these findings advance our understanding of *Diospyros* taxonomy in the region, the study acknowledges certain limitations. Phenetic groupings based solely on morphological data should be validated using molecular techniques such as DNA barcoding or phylogenetic analysis to strengthen species delimitation. Cytological studies, including chromosome counts and palynological analyses, could provide additional insights into evolutionary relationships and genetic diversity. Integrating these complementary approaches would offer a more robust, multidimensional framework for resolving species boundaries in *Diospyros*.

Conclusion

The present study provided more comprehensive diagnostic characters of fruits and seed morphology of *Diospyros* at the species level. The analysis of the shape, size, colour, and texture of the calyx lobe, fruit, and seed shows a clear understanding of the diversity of the *Diospyros* species of Odisha. Moreover, the result obtained from statistical measures indicated that the length of the calyx lobe and size of the seed were significant in explaining the total variation among the studied taxa. The resulting phenetic clustering provided insights into species differentiation and their closest taxonomic relationship, which can aid in more accurate species delimitation. Although this study is important in understanding the diversity and relationships of species within *Diospyros*, there is growing recognition of the value of integrating diverse methods, including molecular and ecological studies. As such, further studies are necessary to resolve taxonomic issues within this genus. Besides, the study documented some taxa of *Diospyros* that had a high demand for edible fruit and timber. Conservation assessment of such valuable species should be of prime importance. Therefore, the baseline information on distribution, phenology, economic importance and taxonomic key will boost the conservation manager to take further steps towards their conservation and sustainable utilisation for the benefit of the local people.

Author Contributions

Conceptualization, S.C.S., S.S.D., and S.T.; methodology, S.C.S., S.S.D., S.T., and S.P.; software S.T., J.K.J.and S.P.; validation, S.T., S.P., J.K.J., and S.C.S.; formal analysis, ST; investigation, S.T., J.K.J., and S.P.; resources, S.C.S.; data curation, S.T., J.K.J.and S.P.; writing—original draft preparation, S.T.; writing—review and editing, S.C.S., S.S.D., and J.K.J.; visualization, S.T. and S.C.S.; supervision, S.C.S. and S.S.D.; project administration, S.C.S.; funding acquisition, S.C.S.. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The authors acknowledge Ministry of Environment, Forest and Climate Change (MoEFandCC), Government of India for financial assistance (Sanction Order No. BSI-298/01/29/2024/ABG-Tech/278/5778). Thanks are due to the PCCF (Wildlife) and Chief Wildlife Warden, Odisha for granting permission to carry out the study. We are also thankful the forest department staff for their cooperation during field work.

Funding

The study was supported by Ministry of Environment, Forest and Climate Change (MoEFandCC), Government of India under Sanction Order No. BSI-298/01/29/2024/ABG-Tech/278/5778.

Conflicts of Interests

The authors have no competing interests to declare that are relevant to the content of this study.

References

Abd El Halim, A.M., Hafeez, R.H., Safwat, A.A., 2014. Taxonomic Revision of Ebenaceae in Egypt. Current Science International, 3(4), 414–425.

Abdel, K.K., Al-Ruzayza, S., Farid, A., 2021. Taxonomic significances of seed morphology in some tribes of subfamily Malvoideae (Malvaceae) in Saudi Arabia. Aust. J. Crop Sci., 15(8), 1204–1216. https://doi.org/10.21475/ ajcs.21.15.08p3360.

Abdulrahaman, A.A., Oladele, F.A., 2005. Stamata, trichomes and epidermal cells as diagnostic features in six species of genus *Ocimum* L. (Lamiaceae). Nigerian Journal of Botany, 18, 214–223.

Achiwa, Y., Hibasami, H., Katzuzaki, H., Imai, K., Komia, Y., 1997. Inhibitory effect of persimmon (*Diospyros kaki*) extract and related polyphenol compounds on growth of human lymphoid leukemia cells. Biosci. Biotech. Biochem., 61(7), 1099–1101.

Ahmed, R., Qaiser, M., 1989. Seed morphological studies of some common plants of Karachi. Pak J Bot., 21(2), 218–246.

Ahmed, R.U., 1993. Medicinal plants used in ISM-their procurement, cultivation, regeneration and import/export aspects-a report. Today and Tomorrow Printers, Publishers, New Delhi.

Bakhuizen van den Brink, R.C., 1933. Enumeration of Malayan Ebenaceae. The Garden's Bulletin Straits Settlements, 7(2), 161–189.

Beentje, H., 2016. The Kew Plant Glossary, an Illustrated Dictionary of Plant Terms, 2nd Ed. Kew Publishing. Royal Botanic Garden, Kew.

Belo, D.P., Louzada, R.B., Buril, M.T., 2024. Alpha-taxonomy underestimates the diversity in *Jacquemontia* (Convolvulaceae): a new hypothesis for *Jacquemontia evolvuloides* complex based on morphological, anatomical, and morphometric analyses, Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 158(4), 613–631. https://doi.org/10.1080/11263504.2024.2347853

Bentham, G., Hooker, J.D., 1876. Genera plantarum ad exemplaria imprimis in Herberiis Kewensibus servata definita. Vol. 2, Lovell Reeve, Co., Williams, Norgate, pp. 662–666.

Butt, M.S., Sultan, M.T., Aziz, M., Naz, A., Ahmed, W., Kumar, N., Imran, M., 2015. Persimmon (*Diospyros kaki*) fruit: Hidden phytochemicals and health claims (Review). EXCLI Journal, 14, 542–561.

Camelo, M.D.C., Temponi, L.G., Coelho, M.A.N., Baumgratz, J.F.A., 2023. Fruit morphology in Anthurium sect. Pachyneurium from Brazil (Araceae) and its taxonomic implications. Flora, 309, 152406. https://doi.org/10.1016/j.flora.2023.152406.

Champion, H.G., Seth, S.K., 1968. A Revised Survey of the Forest Types of India. The Manager of Publications, Delhi.

Clarke, C.B., 1882. Ebenaceae. In: J. D. Hooker (Ed.) Flora of British India Vol. 3, L. Reeve, Co., London, pp. 549–572.

Corral, R., Perez-Garcia, F., Pita, J.M., 1989. Seed morphology and histology in four species of *Cistus* L. (Cistaceae). Phytomorphology, 39, 75–80.

Duangjai, S., Wallnofer, B., Samuel, R., Munzinger, J., Chase, M.A., 2006. Generic delimitation and relationships in Ebenaceae Sensu Lato: Evidence from six plastid DNA regions. American Journal of Botany, 93(12), 1808–1827.

Frades, I., Matthiesen, R., 2010. Overview on Techniques in Cluster Analysis. Methods in molecular biology (Clifton, N.J.), 593, 81–107.

Gamble, J.S., 1881. A Manual of Indian Timbers, Calcutta, pp. 453–463.

Gamble, J.S., 1922. Flora of the Presidency of Madrasi, Vol. 2 (Part IV). West Newman, Co., Adlard, Son, London.

Gnonlonfin, L., Biaou, H., Ouinsavi, C.A.I.N., 2022. Morphological variation in *Diospyros mespiliformis* (Ebenaceae) among different habitats in Benin, West Africa. Journal of Ecology and the Natural Environment, 14(2), 44–55.

Gokmen, F.S., Ozbek, F., Duman, H., 2024. Morphological features of pollen, fruits, and seeds of Turkish *Cistus* species (Cistaceae), Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 158(4), 650–667. http://doi.org/ 10.1080/11263504.2024.2347864

Govaerts, R., 2021. World Checklist of Ebenaceae. Facilitated by the Royal Botanic Gardens, Kew. (Accessed on 01.12.2024)

Government of Odisha. 2025. Odisha Profile: Topography. https://odisha.gov.in/odisha-profile/topography (Accessed on 01.01.2025)

Haines, H.H., 1922. The Botany of Bihar, Orissa, Part IV. Adlard, Son, West Newman Ltd., London.

ISFR, 2023. India state of forest report 2023. Ministry of Environment, Forest and Climate Change. Forest Survey of India. https://www.fsi.nic.in/ (Accessed on 01.12.2024)

IUCN, 2024. (International Union for Conservation of Nature, 2024). The IUCN Red List of Threatened Species. https://www.iucnredlist.org (Accessed on 01.12.2024)

Jayaraman, U., 1996. Economic importance of the genus Diospyros L. (Ebenaceae) in India. Ind. For., 122, 1040–1044.

Joshi, P., 1993. Ethnomedicine of the Kathodias-A monkey eating tribe in Rajasthan. Glimpses of Plant Research, 10, 75-95.

Karaismailoğlu, M.C., Erol, O., 2018. Seed structure and its taxonomic implications for genus *Thlaspi* sensu lato sections *Nomisma, Thlaspi*, and *Pterotropis* (Brassicaceae). Turkish Journal of Botany, 42(5), 591 – 609.

Kottapalli, S., Krishna, H., Venumadhav, K., Nanibabu, B., Jamir, K., Ratnamma, B.K., Jena, R., Babarao, D.K., 2016. Preparation of herbarium specimen for plant identification and voucher number. Roxburghia, 6(1-4), 111-119.

Mahapatra, A.K., Panda, P.C., 2012. Wild edible fruit diversity and its significance in the livelihood of indigenous tribals: Evidence from eastern India. Food Security, 4(2), 219-234.

Maroyi, A., 2018. *Diospyros lycioides* Desf.: Review of its botany, medicinal uses, pharmacological activities and phytochemistry. Asian Pacific Journal of Tropical Biomedicine, 8(2), 130-136.

Meeprom, N., Duangjai, S., Utteridge, T.M.A., Culham, A., Puglisi, C., 2024. Notes on South-East Asian *Diospyros* L. (Ebenaceae, Ericales): commonly misidentified species in mainland South-East Asia. European Journal of Taxonomy, 932, 225–251.

Nandini, D., Ravikumar, B.S., Prasanna, K.K., 2015. Morphometric Analysis of Datura Plant to Understand Variation and Similarities among Four Major Species. Medicinal, Aromatic Plants, 4(209), 2167. https://doi.org/10.4172/2167-0412.1000209.

Narayanan, M.K.R., Mithunlal, S., Sujanapal, P., Kumar, N.A., Sivadasan, M., Alfarhan, A.H., Alatar, A.A., 2011. Ethnobotanically important trees and their uses by Kattunaikka tribe in Wayanad Wildlife Sanctuary, Kerala, India. Journal of Medicinal Plants Research, 5(4), 604–612.

Nematollahi, A., Aminimoghadamfarouj, N., Wiart, C., 2012. Reviews on 1, 4-naphthoquinones from *Diospyros* L. Journal of Asian natural products research, 14(1), 80–88.

Ozbek, F., Uzunhisarcıklı, M.E., 2023. Taxonomic significance of seed macro-micromorphology of Turkish *Alcea* L. (Malvaceae) through light microscopy and scanning electron microscopy. Microsc Res Tech., 86(12), 1551–1567. https://doi.org/10.1002/jemt.24385.

Pearson, R.S., Brown, H.P., 1981. Commerical timbers of India: their distribution, supplies, anatomical structure, physical and mechanical properties and uses. Vol. 2, A. J. Reprint Agency, New Delhi.

POWO. 2025. Plants of the World Online. Royal Botanic Gardens, Kew. http://www.plantsoftheworldonline.org/. (accessed on 01.03.2025)

Prajapati, N.D., Purohit, S.S., Sharma, A.K., Kumar, T., 2003. In: A handbook of Medicinal plants-A complete source book, Agrobios (India), Jodhpur, pp. 200–202

Putri, E.K., Chikmawati, T., 2015. Leaf flushing as taxonomic evidence of some *Diospyros* species. Floribunda, 5(2), 31–47.

Rauf, A., Uddin, G., Patel, S., Khan, A., Halim, S.A., Bawazeer, S., Ahmad, K., Muhammad, N., Mubarak, M.S., 2017. *Diospyros*, an under-utilized, multi-purpose plant genus: A review. Biomedicine and Pharmacotherapy, 91, 714–730.

Ribeiro, A., Serrano, R., da Silva, I.B.M., Gomes, E.T., Pinto, J.F., Silva, O., 2023. The Genus *Diospyros*: A Review of Novel Insights into the Biological Activity and Species of Mozambican Flora. Plants, 12(15), 2833. https://doi.org/10.3390/plants12152833

Rindyastuti, R., Hapsari, L., Wibowo, A.T., 2021. Analysis of morphological characteristics and phenetic relationship of ebony (*Diospyros* spp.) in Indonesia. Biodiversitas, 22(7), 2739–2754.

Samuel, R., Turner, B., Duangjai, S., Munzinger, J., Paun, O., Barfuss, M.H.J., Chase, M.W., 2019. Systematics and evolution of the Old World Ebenaceae, a review with emphasis on the large genus *Diospyros* and its radiation in New Caledonia. Botanical Journal of the Linnean Society, 189(2), 99–114.

Sandratriniaina, N.A., Ramanantsialonina, R.N., Rakouth, B., Wiemann, M.C., Hermanson, J.C., Ravaomanalina, B.H., 2023. Young stem and leaf anatomy of 15 Malagasy-endemic *Diospyros* species (Ebenaceae): taxonomic implications. Botanical Journal of the Linnean Society, 203(1), 94–109. https://doi.org/10.1093/botlinnean/boad013

Sarikaya, E., Güven, S., 2024. Contributions to the taxonomy of Periplocoideae and Asclepiadoideae (Apocynaceae) in Türkiye based on fruit and seed morphology. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 158(5), 963–977. http://doi.org/1-15. 10.1080/11263504.2024.2383449.

Saxena, H.O., Brahmam, M., 1994. Flora of Orissa, Vol. I. Orissa Forest Development Corporation Ltd., Bhubaneswar.

Saxena, H.O., Brahmam, M., 1995. Flora of Orissa, Vol. II. Orissa Forest Development Corporation Ltd., Bhubaneswar.

Schatz, G., Lowry, P., 2020. Taxonomic studies of *Diospyros* L. (Ebenaceae) from the Malagasy region. IV. Synoptic revision of the Squamosa group in Madagascar and the Comoro Islands. Adansonia, 42(10), 201–218.

Schatz, G.E., Lowry II, P.P., 2018. Novitates neocaledonicae. IX. Taxonomic notes on the New Caledonian *Diospyros* (Ebenaceae) with new synonymy and the description of two new species. Candollea, 73(1), 91–110.

Shashwathi, H.S., Krishnamurthy, Y.L., 2024. Taxonomic notes and illustration of *Diospyros crumenata*, a Critically Endangered and endemic tree species of the Western Ghats. Plantasia, 38(12), 42–46.

Singh, V., 2005. Monograph on Indian *Diospyros* L. (persimmon, ebony) Ebenaceae. Botanical Survey of India, Kolkata, India, pp. 63–235

Singh, V.K., 1993. Selected Indian folk medicinal claims and their relevance in primary health care programme. Glimpses Plant Res, 10, 147-152.

Sneath, P.H.A., 2001. Numerical Taxonomy. In: Boone, D.R., Castenholz, R.W., Garrity, G.M. (Eds.) Bergey's Manual of Systematic Bacteriology. Springer, New York, NY. https://doi.org/10.1007/978-0-387-21609-6_6

Sonibare, M.A., Jayeola, A.A., Egunyomi, A., 2004. A morphometric analysis of the genus *Ficus* Linn. (Moraceae). African J Biotechnol., 3(4), 229–235.

Troup, R.S., 1986. Indian woods and their uses. Soni Reprints Agency, Delhi, pp. 127–130.

Turner, B., Munzinger, J., Duangjai, S., Temsch, E.M., Stockenhuber, R., Barfuss, M.H., Samuel, R., 2013. Molecular phylogenetics of New Caledonian Diospyros (Ebenaceae) using plastid and nuclear markers. Mol. Phylogenet. Evol., 69(3), 740–763.

Ullah, Z., Rauf, A., Tariq, M., Tahir, A.A., Ayub, K., Ullah, H., 2015. Phytochemical, spectroscopic and density functional theory study of Diospyrin, and non-bonding interactions of Diospyrin with atmospheric gases. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 141, 71–79.

Utteridge, T.M.A., Jennings, L.V.S., 2021. Trees of New Guinea, Kew Publishing. Royal Botanic Gardens, Kew.

WFO. The World Flora Online plant list. Available online: https://wfoplantlist.org/plant-list (accessed on 01.03.2025).

White, F., 1988. The taxonomy, ecology and chorology of African Ebenaceae II. Bull. Jard. Bot. Belg., 58, 325–448.

Zarkovic, L.D., Hinic, S.S., Matejic, J.S., Veljic, M.M., Marin, P.D., Dzamic, A.M., 2024. Fruit micromorphology and morphometry of eight wild- growing roses in Serbia. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology, 158(4), 823–835. http://doi.org/10.1080/11263504.2024.2370269

ABS

Original Research

Carvacrol Enhances Cell Viability by Reducing Apoptosis in A549 Lung Epithelial Cells Exposed to Elastase

Khojasteh Hoseininejad [1], Sabah Mahmoudi Nejad Mounfared [2], Fereshteh Nejad Dehbashi [3], Maryam Radan [1,*]

Abstract

Chronic obstructive pulmonary disease (COPD) represents a significant global health issue characterised by mechanisms such as inflammation, apoptosis, and oxidative stress. Carvacrol, a phenolic compound known for its antioxidant and anti-inflammatory effects, may mitigate damage caused by oxidative stress and apoptosis. This research aimed to explore the protective effects of carvacrol against elastase-induced apoptosis and oxidative stress in A549 lung epithelial cells. A549 cells were cultured and treated with elastase (60 mU/ml) along with varying concentrations of carvacrol (0.1, 0.2, and 0.4 mmol/L) for 24 hours. Cytotoxicity was evaluated using the MTT assay. Malondialdehyde (MDA) levels were measured as a marker of oxidative stress, while total antioxidant capacity (TAC) and the expression of apoptotic genes BAX and BCL2 were assessed via RT-PCR. Both H_2O_2 and elastase significantly reduced cell viability, increased MDA levels and BAX expression, and decreased TAC and BCL2 expression. Treatment with carvacrol in combination with elastase led to a dose-dependent increase in cell viability, a reduction in MDA levels and BAX expression, and an enhancement in TAC and BCL2 expression, with the strongest protective effect observed at 0.4 mmol/L. Pearson's correlation analysis revealed a positive correlation between TNF-α and BAX expression (r = 0.43, P = 0.002), a negative correlation between TNF-α and BCL2 expression (r = -0.62, P = 0.0031), and a negative correlation between BAX and BCL2 expression (r = -0.56, P = 0.007). Carvacrol demonstrated significant protective effects on pulmonary epithelial cells exposed to elastase-induced oxidative stress, reducing apoptosis and enhancing antioxidant capacity. While our in vitro results suggest potential mechanisms that could be targeted in COPD, we acknowledge that these findings are preliminary. Further studies involving in vivo models and clinical trials are essential to validate the therapeutic relevance of these observations.

Keywords

A549 Cell, Apoptosis, Carvacrol, Elastase, Oxidative Stress

1 Cellular and Molecular Research Centre, Persian Gulf Physiology Research Centre, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

2 School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

3 Cellular and Molecular Research Centre, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

*** Corresponding author:**
E-mail address: radan.maryam@yahoo.com

Citation: Hoseininejad, K., Mahmoudi Nejad Mounfared, S., Nejad Dehbashi, F. , Radan, M., (2025). Carvacrol Enhances Cell Viability by Reducing Apoptosis in A549 Lung Epithelial Cells Exposed to Elastase. Acta Biologica Slovenica 68 (4)

Received: 13.05.2025 / **Accepted:** 16.07.2025 / **Published:** 01.10.2025

https://doi.org/10.14720/abs.68.4.22698

Karvakrol izboljša celično preživetje z zmanjšanjem apoptoze v pljučnih epitelnih celicah A549, izpostavljenih elastazi

Izvleček

Kronična obstruktivna pljučna bolezen (KOPB) je pomemben globalni zdravstveni problem, za katerega so značilni mehanizmi, kot so vnetje, apoptoza in oksidativni stres. Karvakrol, fenolna spojina, znana po svojih antioksidativnih in protivnetnih učinkih, lahko ublaži poškodbe, povzročene z oksidativnim stresom in apoptozo. Namen te raziskave je bil proučiti zaščitne učinke karvakrola proti apoptozi, povzročeni z elastazo, in oksidativnemu stresu v pljučnih epitelnih celicah A549. Celice A549 so bile gojene in obdelane z elastazo (60 mU/ml) skupaj z različnimi koncentracijami karvakrola (0,1, 0,2 in 0,4 mmol/L) v 24 urah. Citotoksičnost je bila ocenjena z testom MTT. Ravni malondialdehida (MDA) so bile izmerjene kot marker oksidativnega stresa, medtem ko so bila skupna antioksidativna sposobnost (TAC) in izražanje apoptotičnih genov BAX in BCL2 ocenjena z RT-PCR. Tako H_2O_2, kot elastaza sta znatno zmanjšala celično vitalnost, povečala ravni MDA in izražanje BAX ter zmanjšala TAC in izražanje BCL2. Zdravljenje s karvakrolom v kombinaciji z elastazo je povzročilo odvisno od odmerka povečanje celične vitalnosti, zmanjšanje ravni MDA in izražanja BAX ter povečanje TAC in izražanja BCL2, pri čemer je bil najmočnejši zaščitni učinek opazen pri 0,4 mmol/L. Pearsonova korelacijska analiza je pokazala pozitivno korelacijo med TNF-α in izražanjem BAX (r = 0,43, P = 0,002), negativno korelacijo med TNF-α in izražanjem BCL2 (r = -0,62, P = 0,0031) in negativno korelacijo med BAX in ekspresijo BCL2 (r = -0,56, P = 0,007). Karvakrol je pokazal pomembne zaščitne učinke na pljučne epitelne celice, izpostavljene oksidativnemu stresu, povzročenemu z elastazo, saj je zmanjšal apoptozo in povečal antioksidativno sposobnost. Čeprav naši rezultati in vitro kažejo na možne mehanizme, na katere bi se lahko osredotočili pri KOPB, se zavedamo, da so ti rezultati le predhodni. Za potrditev terapevtske pomembnosti teh ugotovitev so nujne nadaljnje študije z in vivo modeli in kliničnimi študijami.

Ključne besede

A549 celica, apoptoza, karvakrol, elastaza, oksidativni stres

Introduction

Chronic obstructive pulmonary diseases (COPDs) encompass conditions such as emphysema, characterised by the destruction and enlargement of alveoli; chronic bronchitis, marked by persistent cough and increased mucus production; and small airway disease, involving heightened resistance in the smaller bronchioles. The underlying mechanisms include inflammation, apoptosis, cellular dysfunction, extracellular matrix degradation, and oxidative stress. The lungs are continuously exposed to oxidising agents, and elevated oxidative stress is considered a major contributor to the pathogenesis of pulmonary disorders (Barnes, 2022).

Elastase, a protease, disrupts the protease–antiprotease balance and is widely used in animal models to induce COPD and emphysema. Studies have shown that elastase promotes apoptosis in pulmonary epithelial cells, increases mucin secretion, and elevates inflammatory mediators such as interleukin-8 (IL-8) in bronchial epithelial cells. These mediators, in turn, stimulate neutrophil recruitment, exacerbating inflammation (Voynow and Shinbashi, 2021; Chakraborty and Bhattacharyya, 2013). Elastase also enhances the expression of cathepsin B and matrix metalloproteinase-2 (MMP-2) in macrophages, contributing to tissue remodelling and impaired mucociliary clearance (Greenlee et al. 2007; McKelvey et al. 2021).

Furthermore, elastase induces oxidative stress and free radical formation, which upregulate MUC5AC expression — a gene promoting excessive mucin secretion and airway obstruction (Shao and Nadel, 2005; Li and Ye, 2020). A free radical is defined as a molecule with an unpaired electron, making it highly reactive. These molecules initiate chain reactions by interacting with lipids, proteins, and DNA, amplifying oxidative damage. Antioxidants can interrupt this process by scavenging free radicals (Andrade et al. 2005).

Among phytochemicals, polyphenols represent a major group found in plant-based foods and are known for their antioxidant properties. Diets rich in polyphenols have been associated with reduced oxidative stress and improved cellular defence mechanisms through modulation of intracellular signalling pathways (Rana et al. 2022).

Carvacrol, a monoterpenoid phenol ($C_{10}H_{14}O$), has a boiling point of ~238 °C and a density of 0.976 g/cm^3 at 20 °C and 0.975 g/cm^3 at 25 °C. It is a small, lipophilic molecule capable of crossing the blood–brain barrier. Both in vitro and in vivo studies have shown that carvacrol exhibits a broad range of pharmacological effects, including antioxidant, antibacterial, antifungal, anticancer, anti-inflammatory, hepatoprotective, spasmolytic, and vasodilatory activities (Naghdi Badi et al. 2017; Suntres et al. 2015).

Carvacrol neutralises various reactive species such as peroxyl, superoxide, hydrogen peroxide, and nitric oxide radicals—mainly due to its hydroxylated aromatic ring. Its mild acidity enhances reactivity with free radicals. Additionally, Carvacrol has been shown to modulate immune responses and reduce inflammation, thereby attenuating cell death. Phenolic compounds like carvacrol may decrease pro-inflammatory cytokines (IL-4, TGF-β, IL-17) and increase anti-inflammatory cytokines such as IFN-γ and FOXP3 (Imran et al. 2022).

Given elastase's established role in inducing oxidative damage in pulmonary epithelial cells and the limited research on carvacrol's protective effects in this context, the present study aims to evaluate the potential of carvacrol in mitigating elastase-induced oxidative stress and apoptosis in human A549 lung epithelial cells.

Material and Methods

In this study, various parameters of pulmonary epithelial cells in cell culture medium were investigated using the A549 cell line. All experiments were performed in three independent biological replicates (n = 3) unless otherwise stated. Each biological replicate was conducted on separate days using independently cultured cells. Technical triplicates were also included for each condition in assays such as MTT, ELISA, and RT-PCR to ensure measurement accuracy.

Grouping:

1. Negative control group cultured under standard conditions (37 °C, 5% CO_2, and humidified atmosphere) in RPMI-1640 medium without any treatment for 24 hours.
2. Positive control group receiving H_2O_2 (a well-known oxidant) 100 µM for 24 hours (Vilema-Enriquez et al., 2016).
3. Group receiving elastase 60 mU/mL for 24 hours (Hou et al., 2014).
4. Group receiving elastase 60 mU/mL plus Carvacrol 0.1 mmol/L for 24 hours
5. Group receiving elastase 60 mU/mL plus Carvacrol 0.2 mmol/L for 24 hours
6. Group receiving elastase 60 mU/mL plus Carvacrol 0.4 mmol/L for 24 hours (Yin et al., 2012).

Lung epithelial cell culture

The A549 cell line, derived from lung epithelial cells, was obtained from the Pasteur Institute of Iran. These cells were cultured at a density of 1.06 × 10^6 cells in 100 mm dishes using RPMI-1640 medium supplemented with 10% FBS, with a total volume of 10 ml. The cultures were incubated at 37°C in a 5% CO2 environment, and the medium was refreshed every two days. Once the flasks reached 80-90% confluency, the cells were passaged. Following the establishment of the required number of flasks and cells, the experimental groups were organised, ensuring an equal distribution of cells across each group before initiating the treatment phase. After the designated study period, the cells were taken out of the incubator, and the necessary measurements were conducted in accordance with established protocols.

Cell treatment

Human neutrophil elastase (HNE) was obtained from Sigma-Aldrich (USA; Catalogue No. E8140). The enzyme had a specific activity of ≥4 units/mg protein, as provided by the manufacturer. The CAS number used in this study was 9004-06-2. A549 cells were treated with elastase at a concentration of 60 mU/mL for a duration of 24 hours, based on preliminary dose–response testing to determine sub-cytotoxic oxidative stress induction. Also, A549 cells were treated with Carvacrol (co-treatment) for 24 hours. Carvacrol was added at final concentrations of 0.1, 0.2, and 0.4 mmol/L concurrently with the oxidative agents (elastase or H_2O_2) directly into the culture medium. This co-treatment approach was used to evaluate the protective effects of carvacrol against oxidative stress and apoptotic responses.

Evaluation of cytotoxicity based on MTT analysis

The MTT assay was employed to assess the metabolic activity of A549 cells following treatment with elastase and Carvacrol, serving as an indirect indicator of cell viability. The experiment consisted of three main phases: cell culture, treatment, and MTT analysis. After 24 hours of incubation, the medium was replaced with 200 µl of fresh medium containing MTT solution (0.5 mg/ml; Sigma-Aldrich, USA), and the plates were incubated at 37 °C in a 5% CO_2 atmosphere for 2–4 hours. During this period, mitochondrial succinate dehydrogenase in metabolically active cells reduced MTT to purple formazan crystals, which were visible under a microscope. Because these crystals are insoluble in aqueous solution, they were dissolved using dimethyl sulfoxide (DMSO) prior to colourimetric measurement. Absorbance was read using a microplate reader, and metabolic activity was expressed as a percentage relative to the control group (untreated cells). A reduction in metabolic activity was interpreted as an indicator of compound-induced cytotoxicity. Cells were treated with Carvacrol at concentrations of 0.1, 0.2, and 0.4 mmol/L for 24 hours at 37 °C.

Measurement of Total Antioxidant Capacity (TAC) and Malondialdehyde (MDA) in Lung Epithelial Cells

At the end of the treatment period, the culture supernatants were collected and stored at −70 °C until analysis. TAC and MDA levels were measured using commercial assay kits (Kiazist, Iran) according to the manufacturer's instructions. Total antioxidant capacity was assessed based on the ability of antioxidants in the sample to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The reaction was measured colourimetrically at a wavelength of 593 nm. Trolox was used as the calibration standard, and results were expressed in Trolox equivalents (pg/mL). MDA, a marker of lipid peroxidation, was quantified using the thiobarbituric acid reactive substances (TBARS) method. Absorbance was recorded at 532 nm. The standard curve was prepared using 1,1,3,3-tetramethoxypropane (TMP), and MDA concentrations were reported in pg/mL.

RT-PCR analysis for apoptosis gene expression (BAX- BCL2) in lung epithelial cells

Total RNA was extracted from A549 cells using the RNA extraction kit and reverse-transcribed into cDNA using the cDNA synthesis kit, following the manufacturer's protocol.

Quantitative real-time PCR was performed using SYBR Green Master Mix on a PCR system. Gene expression was normalised to GAPDH using the 2-ΔΔCt method. GAPDH was selected based on its stable expression across all treatment conditions. Reactions were performed in triplicate. Melt curve analysis was conducted to ensure primer specificity.

Data Analysis

The data obtained were analysed using SPSS version 22 software. Prior to analysis, the assumptions of normality and homogeneity of variances were assessed using the Shapiro-Wilk test and Levene's test, respectively. Data meeting these assumptions were subjected to one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. The correlation between different variables was calculated using Pearson's correlation coefficient. Statistical significance was set at $p < 0.05$.

Table 1. Primer sequences, annealing temperatures, and amplification efficiencies used for RT-PCR analysis.

Tabela 1. Sekvence primerjev, temperature annealinga in učinkovitost amplifikacije, uporabljene za analizo RT-PCR.

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Annealing Temp (°C)	Efficiency (%)
BAX	TTTGCTTCAGGGTTTCATCC	CAGTTGAAGTTGCCGTCAGA	60	97.8
BCL2	GGTGAACTGGGGGAGGATTGT	CAGGCTGGAAGGAGAAGATGC	60	98.2
TNF-α	CCAGGGACCTCTCTCTAATC	ATGGGCTACAGGCTTGTCACT	58	96.7
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC	58	98.5

Results

Carvacrol reduces oxidative stress marker levels in A549 cells exposed to elastase

Table 1 presents the metabolic activity across various experimental groups, with the control group serving as the reference point at 100%. In the H_2O_2 group, a notable reduction in metabolic activity of 28.24% was recorded, highlighting the detrimental impact of oxidative stress induced by H_2O_2. Conversely, the elastase (ELS) group demonstrated partial mitigation of the harmful effects of H_2O_2, raising metabolic activity to 48.20%. Furthermore, in the groups receiving both ELS and Carvacrol (CR), a dose-dependent enhancement in metabolic activity was observed. Specifically, the combination of ELS and CR at a dose of 0.1 mmol/L resulted in metabolic activity increasing to 64.73%. As the CR dosage was escalated to 0.2 and 0.4 mmol/L, metabolic activity further improved to 72.99% and 78.29%, respectively. This pattern suggests that higher doses of CR confer a more pronounced protective effect (Figure 1). The optical density (OD) values from the MTT assay, measured spectrophotometrically, were used to calculate the percentage of metabolic activity relative to the control group.

Carvacrol reduces oxidative stress marker levels in A549 cells exposed to elastase

The concentration of malondialdehyde (MDA), a well-established marker of oxidative stress, was measured across experimental groups. The control group exhibited an MDA level of 3.10 pg/mL, reflecting normal cellular conditions. Exposure to H_2O_2 significantly increased MDA concentration to 8.16 pg/mL, indicating elevated oxidative stress. Treatment with elastase (ELS) reduced MDA levels to 6.23 pg/mL, suggesting partial mitigation of H_2O_2-induced oxidative damage ($p < 0.01$ compared to the H_2O_2 group). Moreover, co-treatment with ELS and Carvacrol (CR) further decreased MDA levels in a dose-dependent manner. Specifically, at CR doses of 0.1, 0.2, and 0.4 mmol/L, MDA levels were reduced to 6.11, 5.36, and 4.17 pg/mL, respectively,

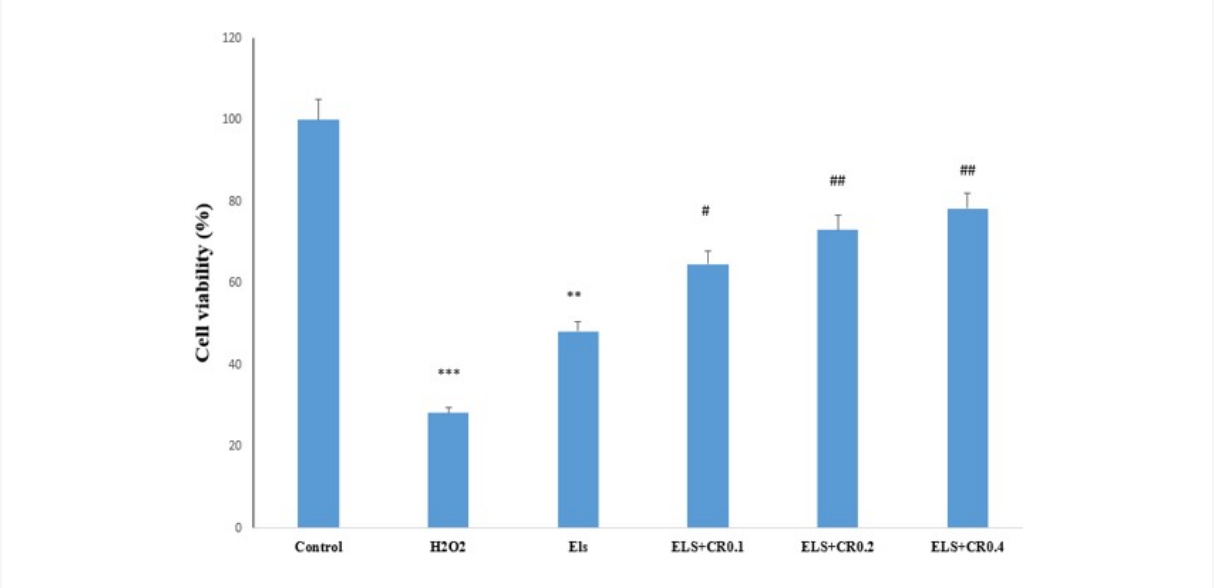


Figure 1. Measurement and comparison of cell viability in all study groups, including control, H_2O_2, Elastase (ELS), Elastase plus 0.1 mmol/L Carvacrol, Elastase plus 0.2 mmol/L Carvacrol and Elastase plus 0.4 mmol/L Carvacrol. The y-axis represents cell viability (%). Data are mean ± SEM (n = 3). One-way ANOVA: F (5,12) = 41.65, P < 0.001. Tukey's post hoc test. **p < 0.01, ***p < 0.001 Vs control group and #p < 0.05, ##p < 0. 01 Vs ELS group.

Slika 1. Merjenje in primerjava celične vitalnosti v vseh študijskih skupinah, vključno s kontrolno skupino, H_2O_2, elastazo (ELS), elastazo plus 0,1 mmol/L karvakrol, elastazo plus 0,2 mmol/L karvakrol in elastazo plus 0,4 mmol/L karvakrol. Os y predstavlja celično vitalnost (%). Podatki so povprečje ± SEM (n = 3). Enosmerna ANOVA: F (5,12) = 41,65, P < 0,001. Tukeyjev post hoc test. **p < 0,01, ***p < 0,001 v primerjavi s kontrolno skupino in #p < 0,05, ##p < 0,01 v primerjavi s skupino ELS.

demonstrating a progressive reduction in oxidative stress. These findings highlight the protective effect of Carvacrol against oxidative stress-induced lipid peroxidation in A549 cells (Figure 2).

Carvacrol improves total antioxidant capacity in A549 cells exposed to elastase

As demonstrated in Figure 3, the control group exhibited the highest TAC value at 10.28 pg/mL, reflecting a healthy and normal antioxidant status. In contrast, both the H_2O_2 and ELS groups demonstrated a marked reduction in TAC levels, with the H_2O_2 group measuring 4.96 pg/mL and the ELS group at 5.19 pg/mL, both significantly lower than the control group, highlighting the detrimental impact of oxidative stress on total antioxidant capacity. Conversely, in groups receiving a combination of ELS and CR at varying doses, there was a progressive increase in TAC levels. The ELS+CR 0.1 group recorded a TAC value of 5.71 pg/mL, indicating a relative enhancement compared to the H_2O_2 and ELS groups. The TAC level further rose to 6.24 pg/mL in the ELS+CR 0.2 group, marked by a significant difference from other groups. Ultimately, the ELS+CR 0.4 group achieved the highest TAC value of 7.81 pg/mL, representing the most substantial improvement among the treatment groups.

Carvacrol reduces BAX gene expression in A549 cells exposed to elastase. Figure 4 illustrates the expression level of the BAX (pro-apoptosis) gene across different groups. The control group shows the lowest BAX gene expression, indicating a normal physiological state. The H_2O_2 group demonstrates a significant increase, attributed to the induction of oxidative stress and apoptosis. Similarly, the ELS group shows a significant increase in BAX expression. In the ELS+CR groups with different doses, a gradual reduction in BAX gene expression is observed. The ELS+CR 0.1 group shows a relative decrease. In the ELS+CR 0.2 group, BAX expression is significantly reduced, and in the ELS+CR 0.4 group, it reaches the lowest level among the treatment groups. These results indicate that oxidative stress increases BAX gene expression; however, treatment with CR in combination with ELS leads to a significant, dose-dependent reduction in gene expression and apoptotic effects.

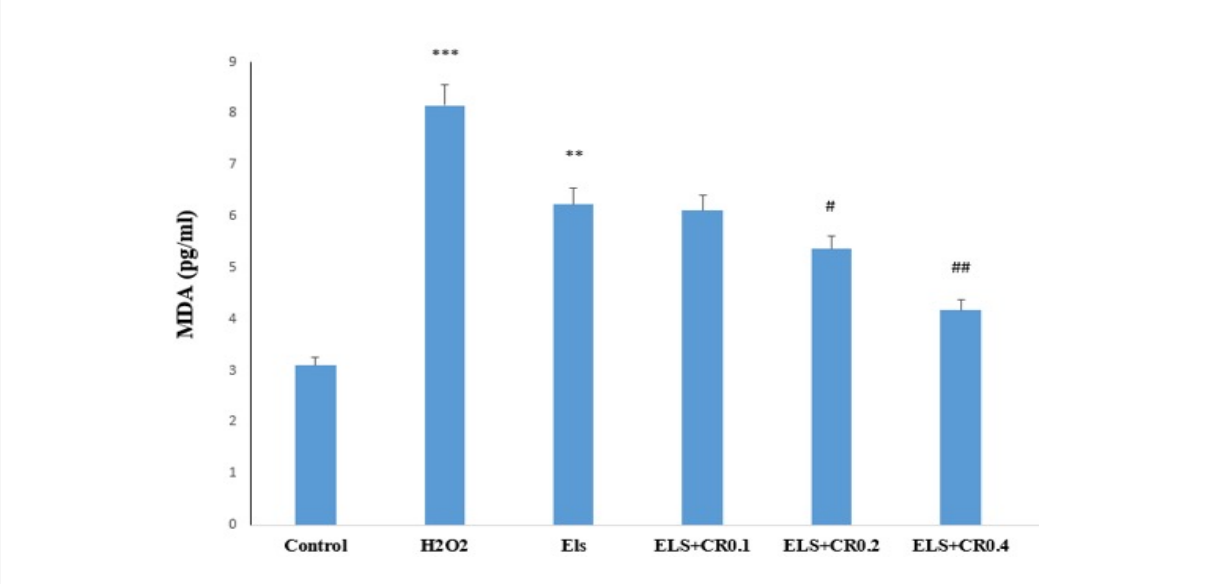


Figure 2. Oxidative stress index (MDA) in different groups, including control, H_2O_2, Elastase (ELS), Elastase plus 0.1 mmol L Carvacrol, Elastase plus 0.2 mmol L Carvacrol and Elastase plus 0.4 mmol L Carvacrol. The y-axis represents MDA (pg/mL). Data are mean ± SEM (n = 3). One-way ANOVA: F (5,12) = 31.707, P < 0.001. Tukey's post hoc test. **p < 0.01, ***p < 0.001 Vs control group and #p < 0.05, ##p < 0. 01 Vs ELS group.

Slika 2. Indeks oksidativnega stresa (MDA) v različnih skupinah, vključno s kontrolno skupino, H_2O_2, elastazo (ELS), elastazo plus 0,1 mmol L karvakrola, elastazo plus 0,2 mmol L karvakrola in elastazo plus 0,4 mmol L karvakrola. Os y predstavlja MDA (pg/mL). Podatki so povprečje ± SEM (n = 3). Enosmerna ANOVA: F (5,12) = 31,707, P < 0,001. Tukeyjev post hoc test. **p < 0,01, ***p < 0,001 v primerjavi s kontrolno skupino in #p < 0,05, ##p < 0,01 v primerjavi s skupino ELS.

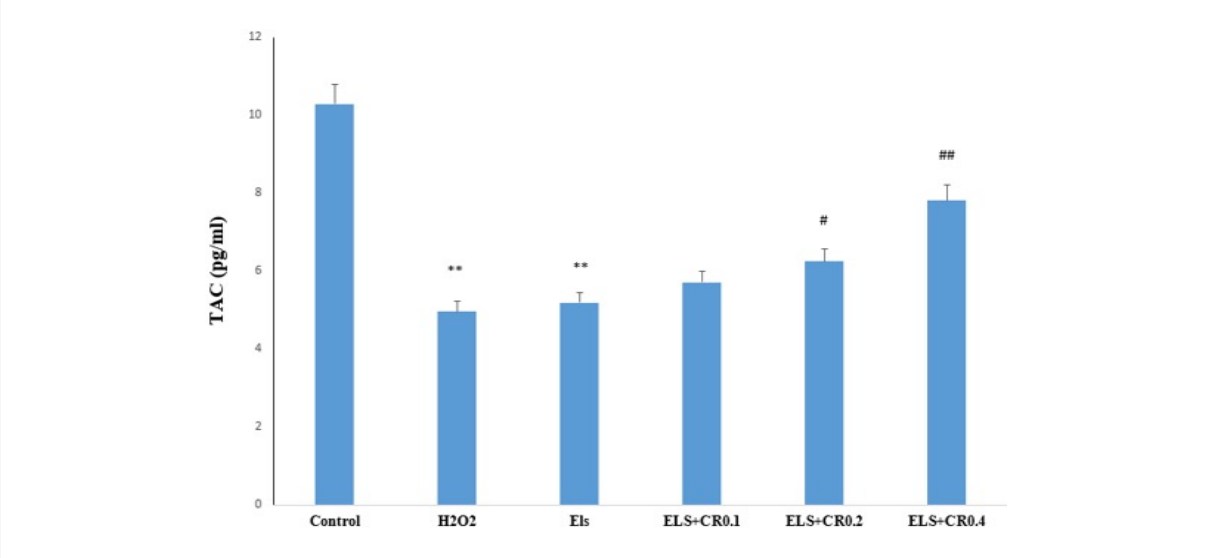


Figure 3. Total antioxidant capacity (TAC) in different groups, including control, H_2O_2, Elastase (ELS), Elastase plus 0.1 mmol L Carvacrol, Elastase plus 0.2 mmol L Carvacrol and Elastase plus 0.4 mmol L Carvacrol. The y-axis represents TAC (pg/ml). Data are mean ± SEM (n = 3). One-way ANOVA: F (5,12) = 51.37, P < 0.001. Tukey's post hoc test. **p < 0.01 Vs control group and #p < 0.05, ##p < 0. 01 Vs ELS group.

Slika 3. Skupna antioksidativna sposobnost (TAC) v različnih skupinah, vključno s kontrolno skupino, H_2O_2, elastazo (ELS), elastazo plus 0,1 mmol L karvakrola, elastazo plus 0,2 mmol L karvakrola in elastazo plus 0,4 mmol L karvakrola. Os y predstavlja TAC (pg/ml). Podatki so povprečje ± SEM (n = 3). Enosmerna ANOVA: F (5,12) = 51,37, P < 0,001. Tukeyjev post hoc test. **p < 0,01 v primerjavi s kontrolno skupino in #p < 0,05, ##p < 0,01 v primerjavi s skupino ELS.

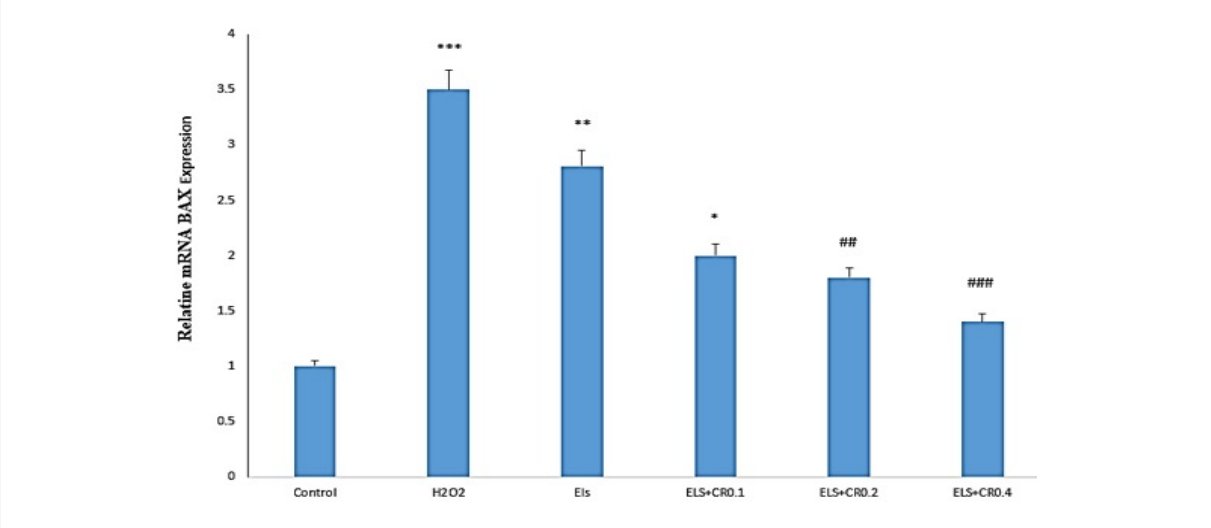


Figure 4. Comparison of BAX gene expression in different groups, including control, H_2O_2, Elastase (ELS), Elastase plus 0.1 mmol/L Carvacrol, Elastase plus 0.2 mmol/L Carvacrol and Elastase plus 0.4 mmol/L Carvacrol. The y-axis represents relative gene expression to GAPDH. Data are mean ± SEM (n = 3). One-way ANOVA: F (5,12) = 53.708, P < 0.001. Tukey's post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 Vs control group and ##p < 0.01, ###p < 0. 001 Vs ELS group.

Slika 4. Primerjava izražanja gena BAX v različnih skupinah, vključno s kontrolno skupino, H_2O_2, elastazo (ELS), elastazo plus 0,1 mmol/L karvakrol, elastazo plus 0,2 mmol/L karvakrol in elastazo plus 0,4 mmol/L karvakrol. Os y predstavlja relativno izražanje gena glede na GAPDH. Podatki so povprečje ± SEM (n = 3). Enosmerna ANOVA: F (5,12) = 53,708, P < 0,001. Tukeyjev post hoc test. *p < 0,05, **p < 0,01, ***p < 0,001 v primerjavi s kontrolno skupino in ##p < 0,01, ###p < 0,001 v primerjavi s skupino ELS.

Carvacrol increases BCL2 gene expression in A549 cells exposed to elastase

Figure 5 shows the expression level of the BCL2 (anti-apoptotic) gene across different groups. The control group exhibits the highest BCL2 gene expression, indicating a normal state and strong cellular protection. In the H_2O_2 group, the gene expression level significantly decreases, reflecting a substantial decline due to oxidative stress. The ELS group also showed a similar reduction to that of the control group. In the ELS+CR groups with different doses, a gradual increase in BCL2 gene expression is observed. The ELS+CR 0.1 group shows a relative increase. In the ELS+CR 0.2 group, BCL2 expression significantly increases, and in the ELS+CR 0.4 group, it reaches the highest level among all treatment groups.

These results indicate that oxidative stress leads to decreased BCL2 gene expression. However, treatment with CR in combination with ELS results in a dose-dependent, significant increase in the anti-apoptotic BCL2 gene expression and improved cellular protection.

Carvacrol improves the TNF-α concentration level in A549 cells exposed to elastase

Figure 6 shows the TNF-α concentration level in different groups. The control group had the lowest TNF-α level with a value of 50.17 pg/mL, which indicates a normal state and the absence of inflammation. In the H_2O_2 group, the TNF-α level increased to 208.11 pg/mL, which indicates a severe increase in inflammation caused by oxidative stress. The ELS group also showed a significant increase with a value of 191.26 pg/mL compared to the control group. In the groups treated with the combination of ELS and CR, a gradual decrease in TNF-α levels was observed. The ELS+CR 0.1 group showed a relative decrease with a value

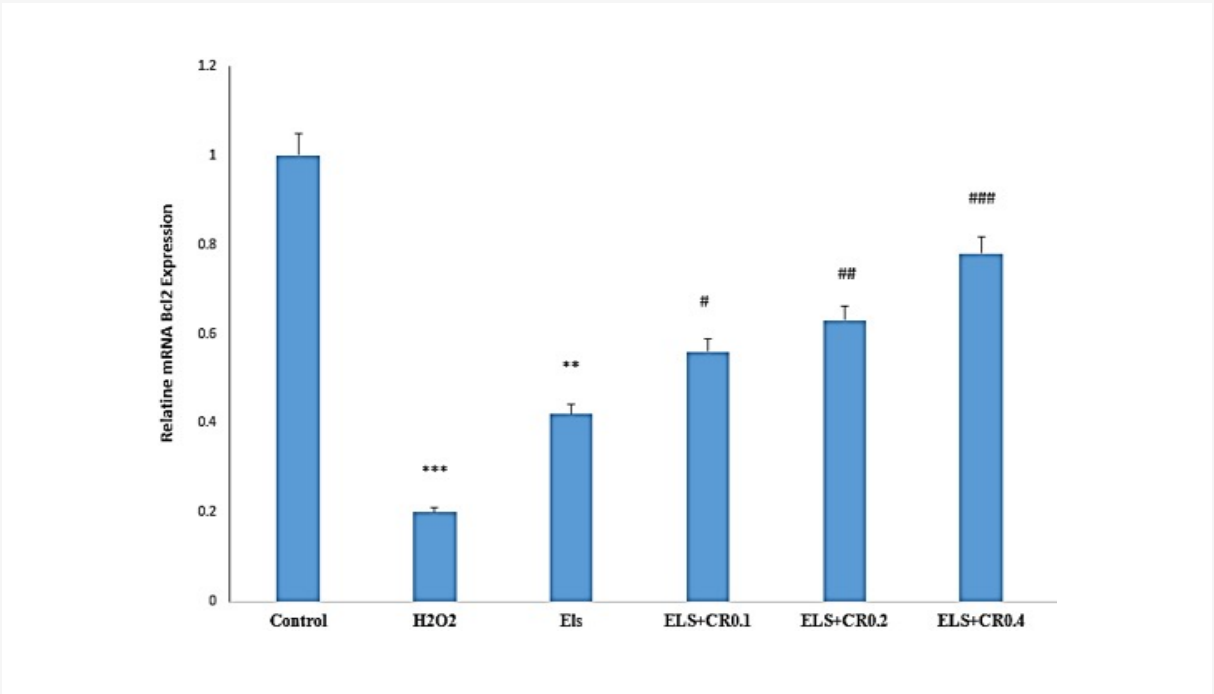


Figure 5. Expression level of the BCL2 gene in different groups, including control, H_2O_2, Elastase (ELS), Elastase plus 0.1 mmol/L Carvacrol, Elastase plus 0.2 mmol/L Carvacrol and Elastase plus 0.4 mmol/L Carvacrol. The y-axis represents relative gene expression to GAPDH. Data are mean ± SEM (n = 3). One-way ANOVA: F (5,12) = 31.938, P < 0.001. Tukey's post hoc test. **p < 0.01, ***p < 0.001 Vs control group and #p < 0.05, ##p < 0.01, ###p < 0. 001 Vs ELS group.

Slika 5. Raven izražanja gena BCL2 v različnih skupinah, vključno s kontrolno skupino, H_2O_2, elastazo (ELS), elastazo plus 0,1 mmol/L karvakrol, elastazo plus 0,2 mmol/L karvakrol in elastazo plus 0,4 mmol/L karvakrol. Os y predstavlja relativno izražanje gena glede na GAPDH. Podatki so povprečje ± SEM (n = 3). Enostranska ANOVA: F (5,12) = 31,938, P < 0,001. Tukeyjev post hoc test. **p < 0,01, ***p < 0,001 v primerjavi s kontrolno skupino in #p < 0,05, ##p < 0,01, ###p < 0,001 v primerjavi s skupino ELS.

of 143.18 pg/mL. In the ELS+CR 0.2 group, the TNF-α level decreased to 112.03 pg/mL, and in the ELS+CR 0.4 group, it reached 84.19 pg/mL, which was the lowest among the treatment groups. These results indicate that oxidative stress increases TNF-α gene expression and exacerbates inflammation, but treatment with CR in combination with ELS dose-dependently causes a significant decrease in TNF-α gene expression and improves the inflammatory status (Figure 6).

Correlation between TNF-α concentration and BAX/BCL2

Pearson's correlation coefficient results (Table 2) showed that TNF-α concentration was positively correlated with BAX gene expression level (r = 0.43, P = 0.002), while it was negatively correlated with BCL2 expression level (r = −0.62, P = 0.0031). Also, BAX expression had a negative correlation with BCL2 (r = −0.56, P = 0.007).

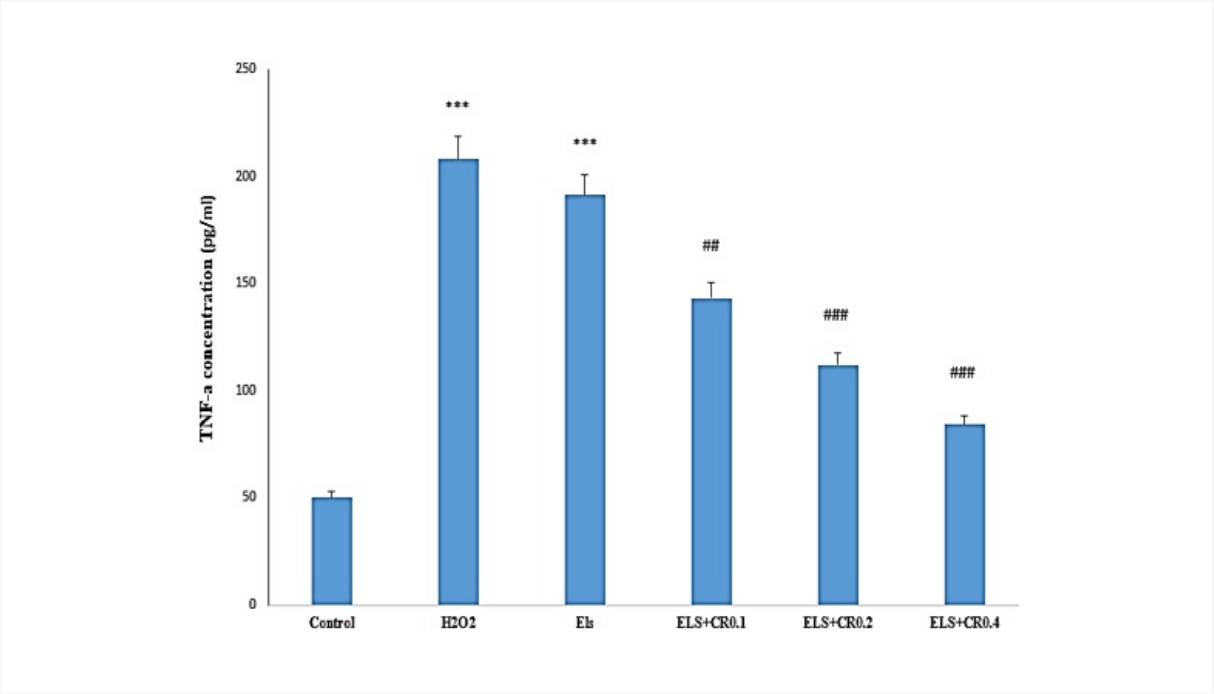


Figure 6. TNF-α concentration level in different groups, including control, H_2O_2, Elastase (ELS), Elastase plus 0.1 mmol/L carvacrol, Elastase plus 0.2 mmol/L Carvacrol and Elastase plus 0.4 mmol/L Carvacrol. The y-axis represents TNF-α (pg/mL). Data are mean ± SEM (n = 3). One-way ANOVA: F (5,12) = 51.742, P < 0.001. Tukey's post hoc test. ***p < 0.001 Vs control group and $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0. 001 Vs ELS group.

Slika 6. Koncentracija TNF-α v različnih skupinah, vključno s kontrolno skupino, H_2O_2, elastazo (ELS), elastazo plus 0,1 mmol/L karvakrol, elastazo plus 0,2 mmol/L karvakrol in elastazo plus 0,4 mmol/L karvakrol. Os y predstavlja TNF-α (pg/mL). Podatki so povprečje ± SEM (n = 3). Enosmerna ANOVA: F (5,12) = 51,742, P < 0,001. Tukeyjev post hoc test. ***p < 0,001 v primerjavi s kontrolno skupino in $^{\#\#}$p < 0,01, $^{\#\#\#}$p < 0,001 v primerjavi s skupino ELS.

Table 2. Relation between TNF-α, BAX, and BCL2 concentrations. Pearson's correlation coefficient test.

Tabela 2. Razmerje med koncentracijami TNF-α, BAX in BCL2. Pearsonov test korelacijskega koeficienta.

Factors	Value	Significance
BAX	0.56	0.007
BCL2	0.62	0.0031
TNF-α	0.43	0.002

Discussion

The present study demonstrates that Carvacrol exhibits a significant, dose-dependent protective effect against elastase- and H_2O_2-induced oxidative stress and apoptosis in A549 lung epithelial cells. Treatment with elastase led to a marked reduction in cell viability, increased levels of malondialdehyde (MDA), and altered apoptotic gene expression, with BAX expression upregulated and BCL2 downregulated. These findings are consistent with those of Hou et al. (2014), who reported that elastase induces apoptosis in A549 cells through activation of the JNK-P38 MAPK signalling pathway. The observed oxidative damage and imbalance in apoptotic signalling further support elastase's pro-apoptotic and cytotoxic role. Moreover, previous research by Koparal et al. (2004) on A549 cells reported that Carvacrol reduced cell numbers and altered cellular morphology, findings which appear to contrast with the protective effects observed in the present study. While our data show that Carvacrol mitigates elastase- and H_2O_2-induced oxidative damage and apoptosis, Koparal et al.'s results suggested a cytotoxic effect at certain concentrations or conditions. This discrepancy may be attributed to differences in experimental design, such as Carvacrol dosage, exposure duration, and the cellular stress model used. For instance, Koparal and Zeytinoglu examined Carvacrol effects on a non-small cell lung cancer (NSCLC) model under basal conditions, potentially reflecting its antiproliferative and pro-apoptotic properties in cancer cells (Koparal et al. 2003), whereas our study focused on its antioxidant and cytoprotective roles in oxidative stress-induced injury. These divergent findings highlight the dual nature of Carvacrol's biological activity, which may vary depending on cellular context, concentration, and pathological state.

Importantly, treatment with Carvacrol significantly counteracted these adverse effects in a concentration-dependent manner. Carvacrol improved cell viability, reduced MDA levels, increased total antioxidant capacity (TAC), and favorably regulated the expression of BAX and BCL2, restoring the balance between pro- and anti-apoptotic signals. The most notable protective effect was observed at 0.4 mmol/L, highlighting the compound's dose-related efficacy. These results are in line with previous reports by Yesildag et al. (2021), who showed that Carvacrol reduced MDA levels and increased antioxidant enzyme activity in cadmium-induced toxicity models (Yesildag et al. 2022). Similarly, Shoorei et al. (2019) demonstrated that Carvacrol decreased oxidative stress and apoptosis in testicular tissue by reducing BAX and increasing BCL2 expression and antioxidant enzyme activity.

The correlation analysis in our study revealed that TNF-α was positively associated with BAX expression and negatively associated with BCL2, indicating a central role of TNF-α in mediating apoptosis. This observation is supported by Moawadh et al. (2023), who reported similar correlations in blood cancers, and by Setayesh et al. (2021), who found that TNF-α inhibition downregulated BAX and upregulated BCL2, reducing oxidative stress and apoptosis. Additionally, Galeone et al. (2023) noted similar pro-apoptotic effects of TNF-α in the context of heart failure, although tissue-specific differences may influence the magnitude of response. Cruceriu et al. (2020) also confirmed the involvement of TNF-α in promoting apoptosis and therapeutic resistance in breast cancer. These findings collectively affirm TNF-α's broad pro-apoptotic role across various disease models, including our model of lung epithelial injury.

Interestingly, the pro-apoptotic effects of Carvacrol observed in other formulations contrast with the present findings. For example, Khan et al. (2018) reported that Carvacrol nano-emulsions induced reactive oxygen species (ROS) production and apoptosis, contrary to our findings of apoptosis reduction. This discrepancy could be attributed to the nano-formulation's enhanced cellular uptake and sustained release, which might lead to elevated intracellular concentrations and pro-oxidant activity.

The current findings are also in agreement with prior in vivo and in vitro studies investigating the cardioprotective and hepatoprotective effects of Carvacrol. Jamhiri et al. (2019) demonstrated reduced apoptosis and MDA levels along with increased antioxidant activity in heart tissue following Carvacrol treatment. Likewise, Sadeghzadeh et al. (2018) reported a decrease in pro-apoptotic gene expression and an increase in anti-apoptotic markers in a cardiac hypertrophy model, while Aristatile et al. (2009) showed that Carvacrol reduced oxidative damage in D-galactosamine-induced hepatotoxicity in rats. These studies corroborate our findings, underscoring Carvacrol's consistent antioxidant and anti-apoptotic effects across different organ systems and experimental models.

Toxicological considerations are essential when interpreting Carvacrol's therapeutic effects. While our study utilised concentrations within a safe and effective range,

prior research has identified potential cytotoxicity and genotoxicity at high doses. For example, a study using the Caco-2 intestinal cell line revealed DNA damage at 460 µM, although similar effects were not seen in other cell types (Nowak et al., 2020). Animal studies have reported species-specific median lethal doses (LD_{50}), ranging from 73 mg/kg intraperitoneally in rats to 2,700 mg/kg dermally in rabbits (Maul et al., 2018). These data suggest that while Carvacrol is relatively safe at lower concentrations, caution is warranted in translating in vitro findings to clinical applications.

Mechanistically, the results of this study support the hypothesis that Carvacrol exerts its protective effects by interfering with TNF-α-mediated apoptotic pathways and neutralising elastase-induced oxidative stress. TNF-α activates signalling cascades such as NF-κB and MAPK, promoting BAX expression and inhibiting BCL2, leading to mitochondrial dysfunction and activation of caspases (Liu et al. 2004; Aslan et al. 2020). Simultaneously, elastase, released from neutrophils, promotes cellular damage by degrading extracellular matrix components and increasing ROS through JNK-P38 MAPK pathway activation (Preston et al. 2002). Carvacrol appears to counteract both mechanisms: it reduces ROS generation, enhances TAC, and restores apoptotic homeostasis by downregulating BAX and upregulating BCL2.

The synergistic role of elastase and TNF-α in driving epithelial damage has been emphasised in earlier studies, where elastase was shown to stimulate TNF-α release, further amplifying cellular injury. Carvacrol effectively interrupts this deleterious feedback loop by modulating TNF-α signalling, reducing lipid peroxidation (MDA), and enhancing overall antioxidant defences. This highlights Carvacrol's potential as a multifunctional protective agent capable of modulating both oxidative and inflammatory pathways.

Although the concentrations of Carvacrol used in this study (0.1–0.4 mmol/L) may appear high, they are consistent with concentrations employed in prior in vitro investigations evaluating antioxidant and cytoprotective effects in epithelial and other mammalian cells (Yesildag et al. 2022; Shoorei et al., 2019). It is important to note that in vitro systems often require higher concentrations of bioactive compounds than what is needed in vivo due to limited bioavailability, lack of metabolism, and differences in cellular uptake dynamics. Furthermore, our findings demonstrated a clear dose–response relationship, with protective effects becoming more pronounced as the concentration increased. Nevertheless, future studies are warranted to explore the efficacy of Carvacrol in the low micromolar range and under physiologically relevant conditions, including using in vivo models to evaluate therapeutic safety and dosing thresholds.

A limitation of this study is the absence of a Carvacrol-alone control group, which would have enabled a more precise evaluation of Carvacrol's intrinsic effects on A549 cells under non-stressed conditions. Without this group, we cannot conclusively distinguish whether Carvacrol's observed protective effects in the elastase-treated groups result from direct antioxidant action or interactions specific to the oxidative stress environment. Future studies should include Carvacrol-alone groups to better clarify potential synergistic or antagonistic interactions with elastase.

In conclusion, Carvacrol exhibits potent antioxidant and anti-apoptotic properties in A549 lung epithelial cells exposed to elastase and H_2O_2. By attenuating TNF-α-mediated apoptosis and suppressing oxidative stress, Carvacrol may hold therapeutic promise in the management of inflammatory lung diseases characterised by excessive neutrophil activity and oxidative damage. Future studies should investigate the clinical relevance of these findings in in vivo models and explore optimal delivery strategies to enhance Carvacrol's therapeutic efficacy.

Conclusion

In this study, Carvacrol, as an antioxidant and anti-inflammatory compound, was able to ameliorate the damaging effects of elastase and H_2O_2 on A549 lung epithelial cells. Carvacrol dose-dependently increased cell viability and reduced oxidative stress. This compound restored the balance between pro-apoptotic and anti-apoptotic pathways by modulating the expression of apoptosis genes. The positive association of TNF-α with BAX and its negative association with BCL2 indicated the key role of these pathways in cell damage. The findings confirm the effectiveness of Carvacrol in reducing elastase-induced damage and regulating inflammatory and apoptotic pathways. While our in vitro results suggest potential mechanisms that could be targeted in COPD, we acknowledge that these findings are preliminary. Further studies involving in vivo models and clinical trials are essential to validate the therapeutic relevance of these observations. Therefore, our current data serve as a foundation for future research rather than direct evidence for clinical application.

Author Contributions

Conceptualization, K.H. and M.R.; methodology, F.N.D.; software, S.M.N.M.; validation, M.R.; formal analysis, S.M.N.M.; investigation, S.M.N.M.; resources, K.H.; data curation, M.R.; writing—original draft preparation, K.H.; writing—review and editing, M.R.; visualization, F.N.D.; supervision, K.H. and M.R.; project administration, M.R.; funding acquisition, M.R.. All authors have read and agreed to the published version of the manuscript.

Acknowledgment

This article is a part of MD thesis of Sabah Mahmoudnejad Monfared (Etical Code: IR.AJUMS.MEDICINE.REC.1402.018). The authors of current manuscript appreciate the financial support of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Grant NO: CMRC-0209).

Conflict of interest

The authors declare that there is no conflict of interest.

References

Andrade Júnior, D.R., Souza, R.B., Santos, S.A., Andrade, D.R., 2005. Oxygen free radicals and pulmonary disease. Jornal Brasileiro De Pneumologia, 31, 60-68. https://doi.org/10.1590/S1806-37132005000100011.

Aristatile, B., Al-Numair, K.S., Veeramani, C., Pugalendi, K.V., 2009. Effect of carvacrol on hepatic marker enzymes and antioxidant status in d-galactosamine-induced hepatotoxicity in rats. Fundamental & clinical pharmacology, 23(6), 757-765. https://doi.org/10.1111/j.1472-8206.2009.00721.x

Aslan, A., Beyaz, S., Gok, O., Erman, O., 2020. The effect of ellagic acid on caspase-3/bcl-2/Nrf-2/NF-kB/TNF-α/COX-2 gene expression product apoptosis pathway: a new approach for muscle damage therapy. Molecular biology reports, 47(4), 2573-2582. https://doi.org/10.1007/s11033-020-05340-7.

Barnes, P.J., 2022. Oxidative stress in chronic obstructive pulmonary disease. Antioxidants, 11(5), 965. https://doi.org/10.3390/antiox11050965.

Chakraborty, K., Bhattacharyya, A., 2013. Role of Proteases in Inflammatory Lung Diseases. In: Chakraborti, S., Dhalla, N.S. (Eds.) Proteases in Health and Disease, Springer Verlag, pp. 361-385.

Cruceriu, D., Baldasici, O., Balacescu, O., Berindan-Neagoe, I., 2020. The dual role of tumor necrosis factor-alpha (TNF-α) in breast cancer: molecular insights and therapeutic approaches. Cellular Oncology, 43(1), 1-8. https://doi.org/10.1007/s13402-019-00489-1.

Galeone, A., Grano, M., Brunetti, G., 2023. Tumor necrosis factor family members and myocardial ischemia-reperfusion injury: state of the art and therapeutic implications. International journal of molecular sciences, 24(5), 4606. https://doi.org/10.3390/ijms24054606.

Greenlee, K.J., Werb, Z., Kheradmand, F., 2007. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiological reviews, 87(1), 69-98. https://doi.org/10.1152/physrev.00022.2006.

Hou, H.H., Cheng, S.L., Chung, K.P., Kuo, M.Y., Yeh, C.C., Chang, B.E., Lu, H.H., Wang, H.C., Yu, C.J., 2014. Elastase induces lung epithelial cell autophagy through placental growth factor: a new insight of emphysema pathogenesis. Autophagy, 10(9), 1509-1521. https://doi.org/10.4161/auto.29190.

Hou, H.H., Cheng, S.L., Chung, K.P., Wei, S.C., Tsao, P.N., Lu, H.H., Wang, H.C., Yu, C.J., 2014. PlGF mediates neutrophil elastase-induced airway epithelial cell apoptosis and emphysema. Respiratory research, 15(1), 106. https://doi.org/10.1186/s12931-014-0106-1.

Imran, M., Aslam, M., Alsagaby, S.A., Saeed, F., Ahmad, I., Afzaal, M., Arshad, M.U., Abdelgawad, M.A., El-Ghorab, A.H., Khames, A., Shariati, M.A., 2022. Therapeutic application of carvacrol: A comprehensive review. Food science & nutrition, 10(11), 3544-3561. https://doi.org/10.1002/fsn3.2994.

Jamhiri, M., Dahaj, F.S., Astani, A., Hejazian, S.H., Hafizibarjin, Z., Ghobadi, M., Moradi, A., Khoradmehr, A., Safari, F., 2019. Carvacrol ameliorates pathological cardiac hypertrophy in both in-vivo and in-vitro models. Iranian Journal of Pharmaceutical Research: IJPR, 18(3), 1380. https://doi.org/10.22037/ijpr.2019.1100766.

Khan, I., Bahuguna, A., Kumar, P., Bajpai, V.K., Kang, S.C., 2018. In vitro and in vivo antitumor potential of carvacrol nanoemulsion against human lung adenocarcinoma A549 cells via mitochondrial mediated apoptosis. Scientific reports, 8(1), 144. https://doi.org/10.1038/s41598-017-18644-9.

Koparal, A.T., Zeytinoglu, M., 2003. Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line, A549. Cytotechnology, 43(1), 149-154. https://doi.org/10.1023/b:cyto.0000039917.60348.45.

Li, J., Ye, Z., 2020. The potential role and regulatory mechanisms of MUC5AC in chronic obstructive pulmonary disease. Molecules, 25(19), 4437. https://doi.org/10.3390/molecules25194437.

Liu, H., Ma, Y., Pagliari, L.J., Perlman, H., Yu, C., Lin, A., Pope, R.M., 2004. TNF-α-induced apoptosis of macrophages following inhibition of NF-κB: a central role for disruption of mitochondria. The Journal of Immunology, 172(3), 1907-1915. https://doi.org/10.4049/jimmunol.172.3.1907.

Maul, J.D., Blackstock, C., Brain, R.A., 2018. Derivation of avian dermal LD50 values for dermal exposure models using in vitro percutaneous absorption of [14C]-atrazine through rat, mallard, and northern bobwhite full thickness skin. Science of the Total Environment, 630, 517-525. https://doi.org/10.1016/j.scitotenv.2018.02.206.

McKelvey, M.C., Brown, R., Ryan, S., Mall, M.A., Weldon, S., Taggart, C.C., 2021. Proteases, mucus, and mucosal immunity in chronic lung disease. International journal of molecular sciences, 22(9), 5018. https://doi:10.3390/ijms22095018.

Moawadh, M.S., Mir, R., Tayeb, F.J., Asim, O., Ullah, M.F., 2023. Molecular evaluation of the impact of polymorphic variants in apoptotic (Bcl-2/Bax) and proinflammatory cytokine (TNF-α/IL-8) genes on the susceptibility and progression of myeloproliferative neoplasms: A case-control biomarker study. Current Issues in Molecular Biology, 45(5), 3933-3952. https://doi.org/10.3390/cimb45050251.

Naghdi Badi, H.A., Abdollahi, M., Mehrafarin, A., Ghorbanpour, M., Tolyat, S.M., Qaderi, A., Ghiaci Yekta, M., 2017. An overview on two valuable natural and bioactive compounds, thymol and carvacrol, in medicinal plants. Journal of Medicinal Plants, 16(63), 1-32. http://jmp.ir/article-1-1370-en.html.

Nowak, A., Zakłos-Szyda, M., Żyżelewicz, D., Koszucka, A., Motyl, I., 2020. Acrylamide decreases cell viability, and provides oxidative stress, DNA damage, and apoptosis in human colon adenocarcinoma cell line Caco-2. Molecules, 25(2), 368. https://doi.org/10.3390/molecules25020368.

Preston, G.A., Zarella, C.S., Pendergraft, III W.F., Rudolph, E.H., Yang, J.J., Sekura, S.B., Jennette, J.C., Falk, R.J., 2002. Novel effects of neutrophil-derived proteinase 3 and elastase on the vascular endothelium involve in vivo cleavage of NF-κB and proapoptotic changes in JNK, ERK, and p38 MAPK signaling pathways. Journal of the American Society of Nephrology, 13(12), 2840-2849. https://doi.org/10.1097/01.asn.0000034911.03334.c3.

Rana, A., Samtiya, M., Dhewa, T., Mishra, V., Aluko, R.E., 2022. Health benefits of polyphenols: A concise review. Journal of Food Biochemistry, 46(10), e14264. https://doi.org/10.1111/jfbc.14264.

Sadeghzadeh, S., Hejazian, S.H., Jamhiri, M., Hafizibarjin, Z., Sadeghzadeh, S., Safari, F., 2018. The effect of carvacrol on transcription levels of Bcl-2 family proteins in hypertrophied heart of rats. Physiology and Pharmacology, 22(1), 54-62.

Setayesh-Mehr, Z., Asoodeh, A., Poorsargol, M., 2021. Upregulation of Bax, TNF-α and down-regulation of Bcl-2 in liver cancer cells treated with HL-7 and HL-10 peptides. Biologia, 76(9), 2735-2743. https://doi.org/10.1007/s11756-021-00800-2.

Shao, M.X., Nadel, J.A., 2005. Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-α-converting enzyme. The Journal of Immunology, 175(6), 4009-4016. https://doi.org/10.4049/jimmunol.175.6.4009.

Shoorei, H., Khaki, A., Khaki, A.A., Hemmati, A.A., Moghimian, M., Shokoohi, M., 2019. The ameliorative effect of carvacrol on oxidative stress and germ cell apoptosis in testicular tissue of adult diabetic rats. Biomedicine & Pharmacotherapy, 111, 568-578. https://doi.org/10.1016/j.biopha.2018.12.054.

Suntres, Z.E., Coccimiglio, J., Alipour, M., 2015. The bioactivity and toxicological actions of carvacrol. Critical reviews in food science and nutrition, 55(3), 304-318. https://doi.org/10.1080/10408398.2011.653458.

Vilema-Enríquez, G., Arroyo, A., Grijalva, M., Amador-Zafra, R.I., Camacho, J., 2016. Molecular and cellular effects of hydrogen peroxide on human lung cancer cells: potential therapeutic implications. Oxidative Medicine and Cellular Longevity 2016(1), 1908164. https://doi.org/10.1155/2016/1908164.

Voynow, J.A., Shinbashi, M., 2021. Neutrophil elastase and chronic lung disease. Biomolecules, 11(8), 1065. https://doi.org/10.3390/biom11081065.

Yesildag, K., Gur, C., Ileriturk, M., Kandemir, F.M., 2022. Evaluation of oxidative stress, inflammation, apoptosis, oxidative DNA damage and metalloproteinases in the lungs of rats treated with cadmium and carvacrol. Molecular biology reports, 49(2), 1201-1211. https://doi.org/10.1007/s11033-021-06948-z.

Yin, Q.H., Yan, F.X., Zu, X.Y., Wu, Y.H., Wu, X.P., Liao, M.C., Deng, S.W., Yin, L.L., Zhuang, Y.Z., 2012. Anti-proliferative and pro-apoptotic effect of Carvacrol on human hepatocellular carcinoma cell line HepG-2. Cytotechnology, 64(1), 43-51. https://doi:10.1007/s10616-011-9389-y.

ABS

Original Research

Significant records of plants, algae, fungi, and animals in SE Europe and adjacent regions, 4

Aljaž Curk [1], Maj Kastelic [1], Aljaž Jakob [2], Alenka Mihorič [3], Predrag Jakšić [4], Tina Klenovšek [5]
Editors: Simona Strgulc Krajšek [1] and Tina Klenovšek [5]

Abstract

In this article, we present the first records of the bryophyte *Schistostega pennata* in Central Slovenia. In addition to its typical occurrence on rocky surfaces, *Schistostega pennata* was observed on bare loose soil. We also report a rare natural aberration of the male meadow brown butterfly, *Maniola jurtina subtus-albida*. This represents the first record of this aberration for the Balkan Peninsula.

Keywords

Schistostega pennata; bryophytes; mosses; *Maniola jurtina* ab. *subtus-albida*; Nymphalidae; Slovenia; Montenegro

1 University of Ljubljana, Biotechnical Faculty, Jamnikarjeva ulica 101, 1000 Ljubljana, Slovenia

2 ZRC SAZU, Jovan Hadži Institute of Biology, Novi trg 2, 1000 Ljubljana, Slovenia

3 Linhartova ul. 3, 1233 Dob, Slovenia

4 Čingrijina 14/25, 11000 Beograd, Serbia

5 University of Maribor, Faculty of Natural Sciences and Mathematics, Koroška c. 160, 2000 Maribor, Slovenia

All authors contributed equally to this work. For correspondence, see the individual chapters.

Citation: Curk, A., Kastelic, M., Jakob, A., Mihorič, A., Jakšić, P., Klenovšek, T., (2025). Significant records of plants, algae, fungi, and animals in SE Europe and adjacent regions, 4. Acta Biologica Slovenica 68 (4)

https://doi.org/10.14720/abs.68.4.24300

Pomembne zabeležke rastlin, alg, gliv in živali za JV Evropo in sosednje regije, 4

Izvleček

V tem članku predstavljamo prve zapise o mahovcu *Schistostega pennata* v osrednji Sloveniji. Poleg tipičnega pojavljanja na skalnatih površinah je bil *Schistostega pennata* opažen tudi na goli, rahli prsti. Poročamo tudi o redki naravni aberaciji samca metulja *Maniola jurtina subtus-albida*. To je prvi zapis te aberacije na Balkanskem polotoku.

Ključne besede

Schistostega pennata; briofiti; mahovi; *Maniola jurtina* ab. *subtus-albida*; Nymphalidae; Slovenija; Črna gora

Schistostega pennata, (Hedw.) F. Weber & D. Mohr fam. Schistostegaceae

Author(s)	Aljaž Curk, Maj Kastelic, Aljaž Jakob, Alenka Mihorič
Corresponding author	Aljaž Jakob (aljaz.jakob@zrc-sazu.si)
Leg.	Aljaž Curk, Maj Kastelic, Aljaž Jakob, Alenka Mihorič
Country	Slovenia
Statement of significance	First finds of the species in Central Slovenia
Locality description	Slovenia, Ljubljana, Golovec, along a forestry road between the observatory and the new Codelli bridge, 200 m NW of the observatory, 370 m a. s. l. Slovenia, Ljubljana, Tivoli, Rožnik and Šiška Hill Landscape Park, near a trail in the forest, 200 m SW of the Petra Držaja hospital, 305 m a. s. l. Slovenia, Ljubljana, Rožnik, along a walking path from the parking lot of ZOO Ljubljana to Cankarjev vrh, 140 m NE of the main entrance to the ZOO, 316 m. a. s. l. Slovenia, Gorenjska, Lukovica, Mali Jelnik, shale hollow next to a forest path, 490 m a. s. l.
Habitat	A small depression in the clay on the bank of the forestry road A crevice cut horizontally into the slope of the hill, comprised of loose soil and plant roots Loose soil in a horizontal crevice under an overhang of clay, caused by erosion On shale between roots in the road cut bank in a mixed forest, SE exposition
Date of observation	2024-04-26 2024-03-16 2024-10-24 2024-11-01
Geographical coordinates	N 46.0454291°, E 14.53018952° N 46.066693°, E 14.485182° N 46.0536539°, E 14.4733961° N 46.17844°, E 14.80125°
Voucher	Photo documented

On site no. 1 *Schistostega pennata* grew in small depressions in a steep bank above a forestry road. Only protonema was present. On site no. 2, it was located in a crevice cut horizontally into the slope, where it grew on unconsolidated soil sediment intertwined with roots. The habitat was shaded, protonema inhabited the bottom of this crevice. On site no. 3 the plant grew above a walking path in a few metres-long horizontal crevice under a soil overhang. The habitat was shaded; other moss species grew in lighter parts of the crevice. Protonema and gametophores were

present (Figure 1). On site no. 4 the plant was growing on shale in the roadcut directly on the rock, exposed to the morning sun. Protonema and gametophytes were present (Figure 2).

The moss is recognisable by its reflective protonema. This is caused by enlarged vacuoles of protonemal cells, acting like lenses (Atherton et al., 2010; Robla et al., 2023). Shoots are 1.5cm tall, with short, nerveless and decurrent green leaves arranged in 2 ranks. It is found in Europe from Spain to Russia (Ignatov & Ignatova, 2001; Hodgetts & Lockhart, 2020). It is considered vulnerable in Finland and Spain and near-threatened in Portugal, but least-concern on the Slovenian and European Red Lists (Hodgetts & Lockhart, 2020; Martinčič 2023). In Slovenia, the recent finds are in the Alpine parts of the country in the Julian Alps, Karawanks, Pohorje, Kozjak-mountain and Meža-Mislinja valley, and additionally before 1959 in the Kamnik-Savinja Alps. The observations published in this paper are the first from Central Slovenia, in the pre-Alpine part of the country.

Schistostega pennata grows on rocky surfaces such as siliceous and other non-calcareous rocks, overhangs and walls of excavations (Ochyra et. al., 1988). An example of such a site is depicted in Figure 2. Similar habitats are described from Poland (Ochyra et. al., 1988), Norway (Lye, 1972), Japan (Kanda, 1971), North America (Crum & Anderson, 1981) and Russia (Ignatov & Ignatova, 2001). It does not tolerate competition from other plants (Atherton et al., 2010). Habitats that we found in Central Slovenia on localities 1-3 differ from the habitats described above; here, it is found on bare loose soil (Figure 1). Similar habitats are reported from central Russia (Ignatov & Ignatova 2001) and Poland (Ochyra et. al., 1988), where the plant is found on bare soil on the upturnings of fallen trees. In our cases, the soil is exposed by roadcuts and erosion. The synanthropic nature of all our new localities suggests that this species may benefit from human activity in an area where natural processes providing appropriate habitat are largely absent.

Funding

This research was funded by the Slovenian Research and Innovation Agency (ARIS), grant numbers MR-55879 and P1-0236.

Figure 1. Gametophytes of *Schistostega pennata* growing on soil on site no. 3. (photo: M. Kastelic).

Slika 1. Gametofiti *Schistostega pennata*, ki rastejo na tleh na lokaciji št. 3. (foto: M. Kastelic).

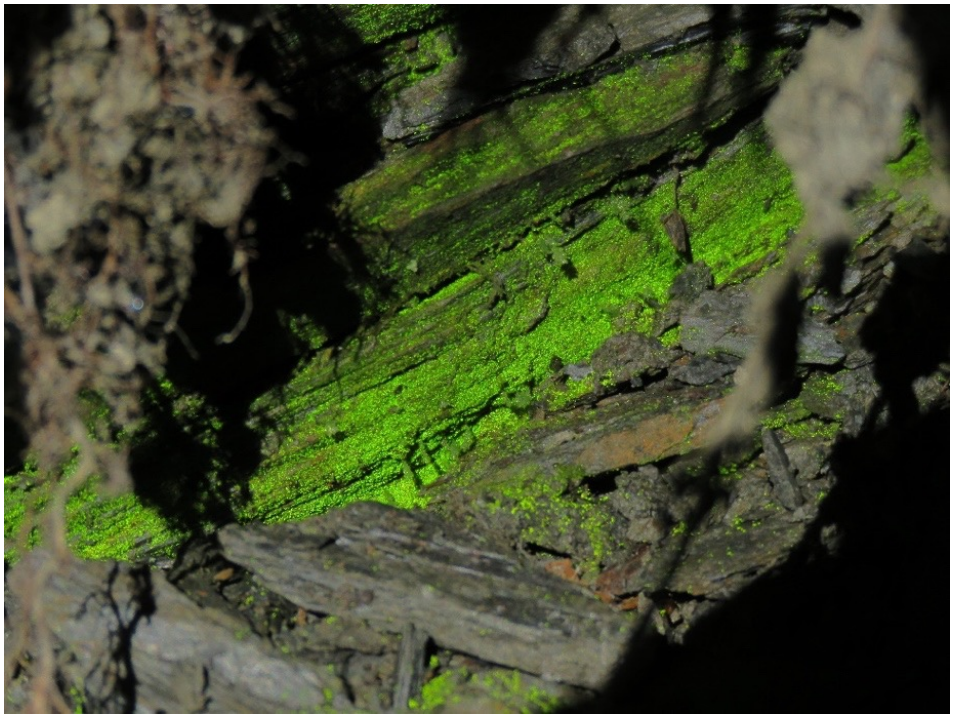


Figure 2. Reflection of green light from the protonema of *Schistostega pennata* on site no. 4. (photo: A. Mihorič).

Slika 2. Odboj zelene svetlobe od protoneme *Schistostega pennata* na lokaciji št. 4. (foto: A. Mihorič).

Maniola jurtina (Linnaeus, 1758) ab. *subtus-albida* Silbernagel, 1943, fam. Nymphalidae (animal)

Author(s)	Predrag Jakšić, Tina Klenovšek
Corresponding author	Predrag Jakšić (jaksicpredrag@gmail.com)
Leg.	Predrag Jakšić
Country	Montenegro
Statement of significance	Rare natural aberration of the meadow brown, *Maniola jurtina*. First record for the Balkan Peninsula.
Locality description	Montenegro, Čakor, Karamanov krš, 1134 m a. s. l.
Habitat	Rocky, limestone-dominated grassland with shallow soil and xerophytic grassland species.
Date of observation	2022-07-05
Geographical coordinates	N 42.68056°, E 19.92417°
Voucher	Author's (P.J.) personal collection

The meadow brown, *Maniola jurtina* (Linnaeus, 1758), is a butterfly from the family Nymphalidae distributed across the Palearctic region. Its habitats include grasslands, forest edges, shrublands, and woodland clearings. Its altitudinal range spans from sea level up to approximately 2000 m, and it is active from May to September (Scott, 1990). The wings of *Maniola jurtina* are dark brown, with a distinctive large eyespot located in the upper third of the forewings. The underside of the hindwings displays a pattern of small eyespots. The number of the small eyespots is variable, typically numbering two, though the total can range from zero to six (Scott, 1990).

An aberration is an unusual variation in the wing pattern, shape or colouration, or other body structures, within a particular species. Variations of the usual form of a species can result from genetic (Rivera-Colón et al., 2020) or environmental factors (Mowbray et al., 2024) or a combination of both. Some aberrant forms, although rare, can occur on a relatively regular basis and were, in the past, documented as new forms of a butterfly species. Many forms, aberrations, and varieties were also described in *M. jurtina* (Spuler, 1908; Rebel & Zerny, 1931; Russwurm, 1978). Nowadays, they no longer hold taxonomic significance.

The aberration of male *M. jurtina subtus-albida* was first described by Silbernagel (1943). The underside of the forewings of which, in typical individuals, is rich ochre yellow with a wide dark border, in this form is whitish with a faint yellowish tinge. The dark border is preserved. The upperside of both wings is normal. Only the dusting of the forewings is slightly translucent due to the light background. This aberration has only been documented in specimens collected in the Czech Republic (Silbernagel, 1943) and Great Britain (Russwurm, 1978; Barrington, 1987, 1991). Now it is also reported for Montenegro (Figure 3), where it was collected by the first author (P.J.). Compared to the specimen presented in Russwurm (1978), the black eyespots on the underside of the forewings of the specimen collected in Montenegro are not centred with a white spot (pupilled).

Figure 3. *Maniola jurtina* ab. *subtus-albida*. Left: Rare natural aberration of a male meadow brown, *Maniola jurtina* ab. *subtus-albida* collected on Karamanov krš in Montenegro (underside view) (photo: Predrag Jakšić). Right: Eyespot on the underside of the right forewing (photo: Miloš Jović).

Slika 3. *Maniola jurtina* ab. *subtus-albida*. Levo: Redka naravna aberacija samca navadnega lešnikarja, *Maniola jurtina* ab. *subtus-albida*, najdena na Karamanovem kršu v Črni gori (pogled od spodaj) (foto: Predrag Jakšić). Desno: Očesna lisa na spodnji strani desnega sprednjega krila (foto: Miloš Jović).

Acknowledgement

We thank Miloš Jović for the photos of the eyespot.

Funding

T.K. was supported by the Slovenian Research and Innovation Agency (ARIS) and research programme Computationally Intensive Complex Systems (P1-0403).

References

Atherton, I., Bosanquet, S.D., Lawley, M., 2010. Mosses and liverworts of Britain and Ireland: a field guide. British Bryological Society, Plymouth, Great Britain, p. 419.

Barrington, R.D.G., 1987. Further notes on variation in a north Dorset colony of the meadow brown butterfly *Maniola jurtina* L. Entomologist's Record and Journal of Variation, 99, 97–102.

Barrington, R.D.G., 1991. Continued notes on a north Dorset colony of the meadow brown butterfly *Maniola jurtina* L. Entomologist's Record and Journal of Variation, 103, 7–15.

Crum, H.A., Anderson, L.E., 1981. Mosses of the Eastern North America. Columbia Univ. Press, New York, USA, p. 1328.

Hodgetts, N., Lockhart, N., 2020. Checklist and country status of European bryophytes - update 2020. Irish Wildlife Manuals, No. 123. National Parks and Wildlife Service, Department of Culture, Heritage and the Gaeltacht, Ireland.

Ignatov, M., Ignatova, E., 2001. On the zoochory of *Schistostega pennata* (Schistostegaceae, Musci). Arctoa, 10, 83–96.

Kanda, H., 1971. *Schistostega pennata* Hedw. in Hokkaido: its ecology and germination. Hikobia, 6(1-2), 60–75.

Lye, K.A., 1972. Studies in Norwegian bryophytes. 1. The family Schistostegaceae. Lindbergia, 1, 205–213.

Martinčič, A., 2024. New checklist and the red list of the mosses (Bryophyta) of Slovenia. Hacquetia, 23 (1), 69–118. https://doi.org/10.2478/hacq-2023-0006

Mowbray, S., Bennie, J., Rhodes, M.W., Smith, D.A.S., ffrench-Constant, R.H., 2024. Eyespot variation and field temperature in the Meadow Brown butterfly. Ecology and Evolution, 14, e10842. https://doi.org/10.1002/ece3.10842

Ochyra, R., Szmajda, P., Bochenski, W., Karczarz, K., 1988. *Schistostega pannata* (Hedw.) Web. & Mohr. In: Tobolewski, Z. & Wojterski, T. (eds.) Atlas Rozmieszczenia Roslin Zarodnikowich w Polsce. Ser. 5 Mchy (Musci), 4, 15–17.

Rebel, H., Zerny, H., 1931. Die Lepidopterenfauna Albaniens. Denkschriften der Akademie der Wissenschaften in Wien. Math. Nat. Klasse. Vienna.

Rivera-Colón, A.G., Westerman, E.L., Van Belleghem, S.M., Monteiro, A., Papa, R., 2020. Multiple loci control eyespot number variation on the hindwings of *Bicyclus anynana* butterflies. Genetics, 214 (4), 1059–1078. https://doi.org/10.1534/genetics.120.303059

Robla, J., González-García, V., Santamarina, S., Artazkoz, M., 2023. Unravelling the ecological drivers of *Schistostega pennata* (Hedw.) F. Weber & D. Mohr on the Iberian Peninsula: distribution and conservation. Journal of Bryology, 45(3), 192–207.

Russwurm, A.D.A., 1978. Aberrations of British Butterflies. EW Classey, Farrington.

Scott J., 1990. Adult structure and function. In: Kudrna O. (ed.). Butterflies of Europe, Volume 2 Introduction to Lepidopterology. AULA–Verlag, 108–151.

Silbernagel, A., 1943. Nekolik novych forem macrolepidopter z Čech. Časopis české společnosti entomologické, 40, 1–7.

Spuler A., 1908. Die Schmetterlinge Europas. E. Schweizerbartsche Verlagsbuchhandlung, Stuttgart.

ABS

Original Research

Study of the Effect of Autotrophic and Mixotrophic Culture Media on Heavy Metal Uptake by *Fischerella* sp. and *Desmonostoc alborizicum*

Bahareh Nowruzi [1,*], Hassan Beiranvand [2], Roya Rahmani [2], Seyedeh Sajedeh Al-Tayeb [2], Amir Aala Zakeralhosseini Khafri [2]

Abstract

Heavy metals, even at low concentrations, can have detrimental effects on human health and the environment. The use of biological methods for heavy metal removal is considered a suitable alternative to conventional physicochemical methods due to lower costs and environmental compatibility. Among these methods, exopolysaccharides produced by cyanobacteria play a significant role in the adsorption of metal ions. This study aimed to evaluate the capability of two native Iranian cyanobacteria, *Fischerella* sp. and *Desmonostoc alborizicum*, in removing the heavy metals chromium, nickel, and copper. The cyanobacterial strains *Fischerella* sp. and *Desmonostoc alborizicum* were cultured in mixotrophic media containing glucose, maltose, lactose, and sucrose (10 g/L), as well as in a control medium (carbohydrate-free). Dry cell weight (6–48 hours), exopolysaccharide content (at 48 hours), and heavy metal removal (copper, chromium, and nickel, 10–90 minutes) were measured. Carbohydrate and protein contents were determined using the phenol-sulfuric acid method and the Lowry method, respectively. Additionally, volatile compounds produced by *Fischerella* sp. in the presence of copper were analysed using gas chromatography-mass spectrometry (GC-MS). The results demonstrated that *Fischerella* sp. exhibited superior performance in maltose-enriched culture media compared to *Desmonostoc alborizicum*. This strain led to a 1.3-fold increase in exopolysaccharide production, a 1.02-fold increase in copper removal, and higher dry cell weight relative to *Desmonostoc alborizicum*. The removal efficiency of chromium and nickel ranked second and third, respectively, after copper in maltose-enriched media. Quantitative comparison of the compounds identified by GC-MS in *Fischerella* sp. under control conditions and in the presence of maltose and copper revealed an increase in the levels of 3-methylbutane, 2-methylbutane, methyl acetate, 2-butanone, acetic acid,

1 Department of Biology, SR.C., Islamic Azad University, Tehran, Iran

2 Department of Biology, SR.C., Islamic Azad University, Tehran, Iran

* **Corresponding author:**
E-mail address: baharehnowruzi77@iau.ac.ir

Citation: Nowruzi, B., Beiranvand, H., Rahmani, R., Al-Tayeb, S. S., Zakeralhosseini Khafri, A. A., (2025). Study of the Effect of Autotrophic and Mixotrophic Culture Media on Heavy Metal Uptake by *Fischerella* sp. and *Desmonostoc alborizicum*. Acta Biologica Slovenica 68 (4)

Received: 10.06.2025 / **Accepted:** 16.09.2025 / **Published:** 07.10.2025

https://doi.org/10.14720/abs.68.4.23013

3-methylbutyl acetate, 1-butanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone, 2-phenylethanol, and 3-octen-2-ol. This study demonstrated that *Fischerella* sp. exhibited high efficiency in heavy metal removal, exopolysaccharide, and protein production in maltose-enriched media. Therefore, this strain can be considered an eco-friendly candidate for reducing environmental pollution.

Keywords

Heavy metal removal, Cyanobacteria, Exopolysaccharides, Mixotrophic culture medium, Autotrophic culture medium

Študija vpliva avtotrofnih in miksotrofnih gojišč na absorpcijo težkih kovin s strani *Fischerella* sp. in *Desmonostoc alborizicum*

Izvleček

Težke kovine lahko že v majhnih koncentracijah škodljivo vplivajo na zdravje ljudi in okolje. Uporaba bioloških metod za odstranjevanje težkih kovin se šteje za ustrezno alternativo konvencionalnim fizikalno-kemijskim metodam zaradi nižjih stroškov in okoljske združljivosti. Med temi metodami imajo eksopolisaharidi, ki jih proizvajajo cianobakterije, pomembno vlogo pri adsorpciji kovinskih ionov. Cilj te študije je bil oceniti sposobnost dveh avtohtonih iranskih cianobakterij, *Fischerella* sp. in *Desmonostoc alborizicum*, za odstranjevanje težkih kovin kroma, niklja in bakra. Cianobakterijski sevi *Fischerella* sp. in *Desmonostoc alborizicum* so bili gojeni v mixotrofnih medijih, ki so vsebovali glukozo, maltozo, laktozo in saharozo (10 g/l), ter v kontrolnem mediju (brez ogljikovih hidratov). Merili so suho maso celic (6–48 ur), vsebnost eksopolisaharidov (po 48 urah) in odstranjevanje težkih kovin (baker, krom in nikelj, 10–90 minut). Vsebnost ogljikovih hidratov in beljakovin je bila določena z metodo fenol-žveplove kisline oziroma metodo Lowry. Poleg tega so bile hlapne spojine, ki jih je proizvedla *Fischerella* sp. v prisotnosti bakra, analizirane z uporabo plinske kromatografije-masne spektrometrije (GC-MS). Rezultati so pokazali, da je *Fischerella* sp. v primerjavi z *Desmonostoc alborizicum* pokazala boljšo učinkovitost v gojiščih, obogatenih z maltozo. Ta sev je v primerjavi z *Desmonostoc alborizicum* povzročil 1,3-kratno povečanje proizvodnje eksopolisaharidov, 1,02-kratno povečanje odstranjevanja bakra in višjo suho maso celic. Učinkovitost odstranjevanja kroma in niklja je bila na drugem in tretjem mestu, takoj za bakrom v mediju, obogatenem z maltozo. Kvantitativna primerjava spojin, identificiranih z GC-MS v *Fischerella* sp. v kontrolnih pogojih in v prisotnosti maltoze in bakra, je pokazala povečanje ravni 3-metilbutana, 2-metilbutana, metilacetata, 2-butanona, ocetne kisline, 3-metilbutilacetata, 1-butanola, 3-metil-1-butanola, 3-hidroksi-2-butanona, 2-feniletanola in 3-okten-2-ola. Ta študija je pokazala, da je *Fischerella* sp. pokazala visoko učinkovitost pri odstranjevanju težkih kovin, proizvodnji eksopolisaharidov in beljakovin v mediju, obogatenem z maltozo. Zato se ta sev lahko šteje za okolju prijazen kandidat za zmanjšanje onesnaževanja okolja.

Ključne besede

Odstranjevanje težkih kovin, Cianobakterije, Eksopolisaharidi, Mešano trofično gojišče, Avtotrofno gojišče

Introduction

Heavy metals, even in extremely low concentrations, can be hazardous to many biological processes. Due to the large-scale industrial applications of pharmaceuticals, pesticides, plastics, slaughterhouses, rubber, organic chemicals, and wood products, the free forms of heavy metals in soil and aquatic systems are continuously increasing. Furthermore, the non-degradable nature of heavy metals prolongs their persistence in the environment. Their high solubility in water leads to bioaccumulation, ultimately resulting in severe and irreversible damage, including potential carcinogenicity, even at very low concentrations of approximately 1 mg/L (Pierre et al., 2016). Reducing the bioavailability or mobility of heavy metal ions is crucial to mitigate the risks associated with their absorption and accumulation. Therefore, removing these contaminants from wastewater before their release into the environment is imperative (Pereira et al., 2013).

Various physicochemical methods, such as adsorption, chemical precipitation, solvent extraction, and ion exchange techniques, may be employed for heavy metal removal. However, these methods have notable disadvantages, including incomplete metal removal, the requirement for expensive equipment and monitoring systems, high energy consumption, and the generation of contaminated residues that must be disposed of properly (Pereira et al., 2016). Additionally, these methods are often ineffective when metal ion concentrations range between 10 and 100 mg/L. These limitations have increased interest in developing environmentally friendly and cost-effective biological treatment methods. Initially, it was believed that metals only exert toxic effects on microbial systems; however, it was later discovered that microorganisms can develop metal resistance mechanisms. Microorganisms actively bind to heavy metal ions, thereby reducing their toxic impact on the environment.

For many years, the isolation and identification of cyanobacterial strains with potential bioremediation activity have highlighted their significance as valuable organisms in the wastewater treatment industry. Cyanobacteria are particularly promising candidates for heavy metal removal, as they eliminate metal ions through biosorption or bioaccumulation, and in many cases, via both mechanisms. The advantages of using microorganisms in heavy metal bioremediation include their reliance on natural and renewable resources, cost-effectiveness, rapid metal removal efficiency, the ability to eliminate metal ions even at low concentrations, autonomous purification of contaminated water containing various metal ions, and the recovery of valuable metals through biosorption (Liu et al., 2003; Knowles and Castenholz, 2008). The exopolysaccharides extracted from cyanobacteria provide protection against extreme conditions, including high pH and temperature. The net anionic charge of exopolysaccharides facilitates the adsorption of positively charged heavy metal ions, while the presence of negatively charged uronic acids plays a crucial role in cation binding. Consequently, the exopolysaccharides produced by cyanobacteria could serve as a viable alternative to conventional chemical and physicochemical methods for removing metal cations from contaminated water sources (Pierre et al., 2016; Pereira et al., 2009).

Additionally, enriching the culture medium and increasing exopolysaccharide production can further highlight the adaptability of cyanobacteria to mixotrophic environments. In fact, optimising the culture medium enhances biomass and exopolysaccharide production. The chemical characteristics and abundance of extracellular polysaccharide structures depend on the specific cyanobacterial strain and the environmental or culture conditions. Therefore, in this study, mixotrophic media enriched with glucose, sucrose, maltose, and lactose were utilised (Ghorbani et al., 2022).

The freshwater wetlands and qanats of Golestan Province serve as active ecosystems, providing habitats for various aquatic species and native birds. Given the presence of environmental pollutants and the significant influx of wastewater into these areas, as well as the seepage of contaminants into wetlands and freshwater qanats, this study aims to evaluate and compare the heavy metal removal capabilities of two native Iranian cyanobacterial strains, *Fischerella* sp. and *Desmonostoc alborizicum*, isolated from the wetlands and qanats of Golestan Province. By investigating their biosorption effectiveness under mixotrophic conditions, this research seeks to identify the most efficient strain for bioremediation and to contribute novel insights into the application of native cyanobacteria for environmentally sustainable heavy metal removal in polluted aquatic ecosystems.

Materials and methods

Cultivation of cyanobacterial strains

The cyanobacterial strains *Fischerella* sp. and *Desmonostoc alborizicum* used in this study were obtained from the cyanobacteria culture collection of the Alborz Herbarium,

Islamic Azad University, Science and Research Branch. These strains were selected due to their native origin from wetlands and qanats of Golestan Province in Iran, representing locally adapted biota with potential environmental significance. The strains were cultured in standard Z8 liquid medium and maintained in a growth chamber at 28°C under continuous fluorescent illumination at an intensity of 300 µE/m^2/s for 30 days, following established protocols (Nowruzi and Lorenzi, 2021; Nowruzi et al., 2023).

Design and preparation of different culture media

To evaluate the effects of carbon sources on growth and heavy metal uptake, four mixotrophic culture media were prepared by supplementing Z8 medium with 10 g/L of one of the following carbohydrates: glucose, maltose, lactose, or sucrose. A control medium consisted of Z8 without any added carbohydrates to represent autotrophic conditions. All media were sterilised, cooled, and the pH was adjusted to approximately 7.2 prior to inoculation with cyanobacterial strains (Figure 1) (Ghorbani et al., 2022). This cultivation approach is widely used in cyanobacterial research to enhance biomass and metabolite production, facilitating subsequent bioremediation assessments.

Determination of dry cell weight

To determine the dry cell weight, samples were collected at 6, 12, 24, and 48 hours and dried at 100°C (Ghorbani et al., 2022).

Determination of extracellular polysaccharides

Extraction of exopolysaccharides (EPS) was performed using a modified method of Ozturk and Aslim. Cells were separated by centrifugation at room temperature, and the resulting pellet was boiled in distilled water. The final solution was washed with ethanol and freeze-dried to obtain purified EPS (Ozturk and Aslim, 2010).

Estimation of heavy metal removal

Dialysis bags containing the culture medium were treated with HCl to remove metal ions and subsequently dialysed with water. The treated cultures were exposed to a metal solution containing Cu(II), Cr(III), and Ni(II) at 30°C for 24 hours. Biomass was separated from the solution by centrifugation and filtration. The final metal content in the supernatant was measured using atomic absorption spectrophotometry at

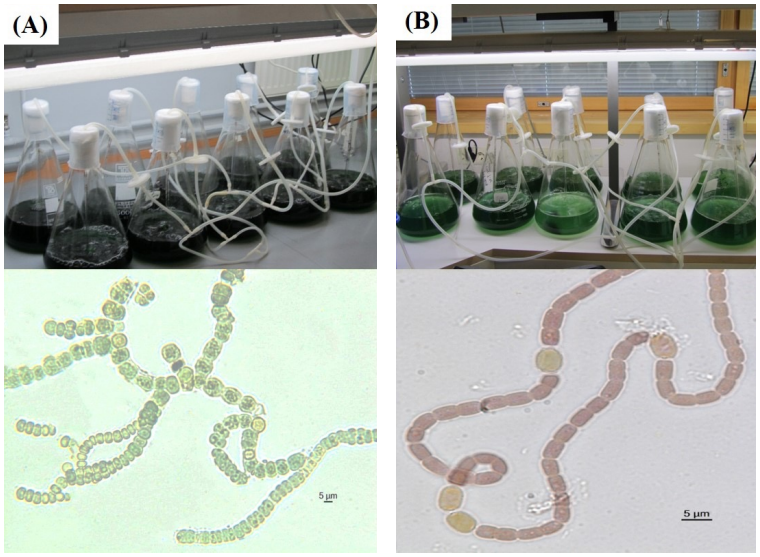


Figure 1. Cultivation of (A) *Fischerella* sp., and a microscopic image. (B) *Desmonostoc alborizicum*, and a microscopic image.

Slika 1. Gojenje (A) *Fischerella* sp. in mikroskopska slika. (B) *Desmonostoc alborizicum* in mikroskopska slika.

wavelengths of 232 nm for copper, 359.9 nm for chromium, and 324.7 nm for nickel. The metal removal efficiency was determined by measuring the difference in metal concentration before and after exposure to the cyanobacterial culture and was compared to a blank control. All experiments were performed in triplicate, and data were analysed as mean ± standard deviation. The amount of metal removed (q) was expressed as milligrams of metal removed per gram of dry weight using the equation:

$q\ (mg\ g^{-1}) = V\ (C_1 - C_2) / m$
Where:
V = sample volume (L),
C_1 = initial metal concentration (mg/L),
C_2 = final metal concentration (mg/L),
m = dry biomass weight (g).

The dry biomass weight (g/L) was determined by filtering the dialysed cultures and drying the filters at 100°C (Rossi and De Philippis, 2015).

Quality assessment methods

Carbohydrates were quantified using the phenol-sulfuric acid method, with glucose as the standard, at an absorbance wavelength of 490 nm. Polysaccharide solutions were mixed with sulfuric acid and phenol, incubated at 25°C, and their absorbance was measured at 490 nm. A standard glucose curve was prepared using diluted glucose solutions, and absorbance values were recorded with a spectrophotometer. Proteins were quantified using a modified Lowry method at a wavelength of 750 nm (DuBois et al., 1956; Olson and Markwell, 2007).

Identification of volatile compounds using GC-MS

First, 50 mL of the sample was centrifuged, and the supernatant was mixed with a methanolic solution of 2M HCl for 4 hours at 100°C. The solution was then centrifuged at 8000 rpm for 10 minutes, and the supernatant was transferred to a round-bottom flask connected to a rotary evaporator. After rotary evaporation, 2 mL of hexane was added to the flask contents. The mixture was filtered through a PTFE membrane filter with a pore size of 0.45 µm. The resulting solution was centrifuged again at 8000 rpm for 10 minutes, and the supernatant was collected for GC-MS analysis. A 2-µL aliquot of the supernatant was injected into the GC-MS instrument using a specialised syringe, and the spectrum was obtained (Guo et al., 2024).

Methods and data analysis tools

The experimental data were analysed using one-way analysis of variance (One-way ANOVA). Statistical tests were conducted using SPSS software, version 26. A significance level of $p \leq 0.05$ was considered for all data comparisons (Parikh and Madamwar, 2006).

Results

Results of dry cell weight measurement

The results of dry cell weight measurements (g/l) in culture media containing maltose, lactose, glucose, sucrose, and control showed that this value was significantly higher in the *Fischerella* sp. strain compared to the *Desmonostoc alborizicum* strain. In both strains, the dry cell weight significantly increased until the end of 48 hours (Figure 2).

Results of extracellular polysaccharide measurement

The results of exopolysaccharide measurements in culture media containing maltose, lactose, glucose, sucrose, and control indicated that the extracellular exopolysaccharide produced by the *Fischerella* sp. strain in the maltose-containing medium was approximately 1.30 times higher than that of the *Desmonostoc alborizicum* strain (Figure 3).

Results of heavy metal removal

The results of heavy metal removal measurements in the control culture medium showed no significant difference in heavy metal removal between the two strains. However, the analysis of heavy metal removal in media containing maltose, lactose, glucose, and sucrose demonstrated that the highest heavy metal removal occurred in the *Fischerella* sp. strain in the maltose-containing medium with copper. At time T10 (the first 10 minutes), the amount was 360 mg/g, which was 1.02 times higher than that of the *Desmonostoc alborizicum* strain. Following copper, the highest levels of heavy metal removal were observed for chromium and nickel (Figure 4).

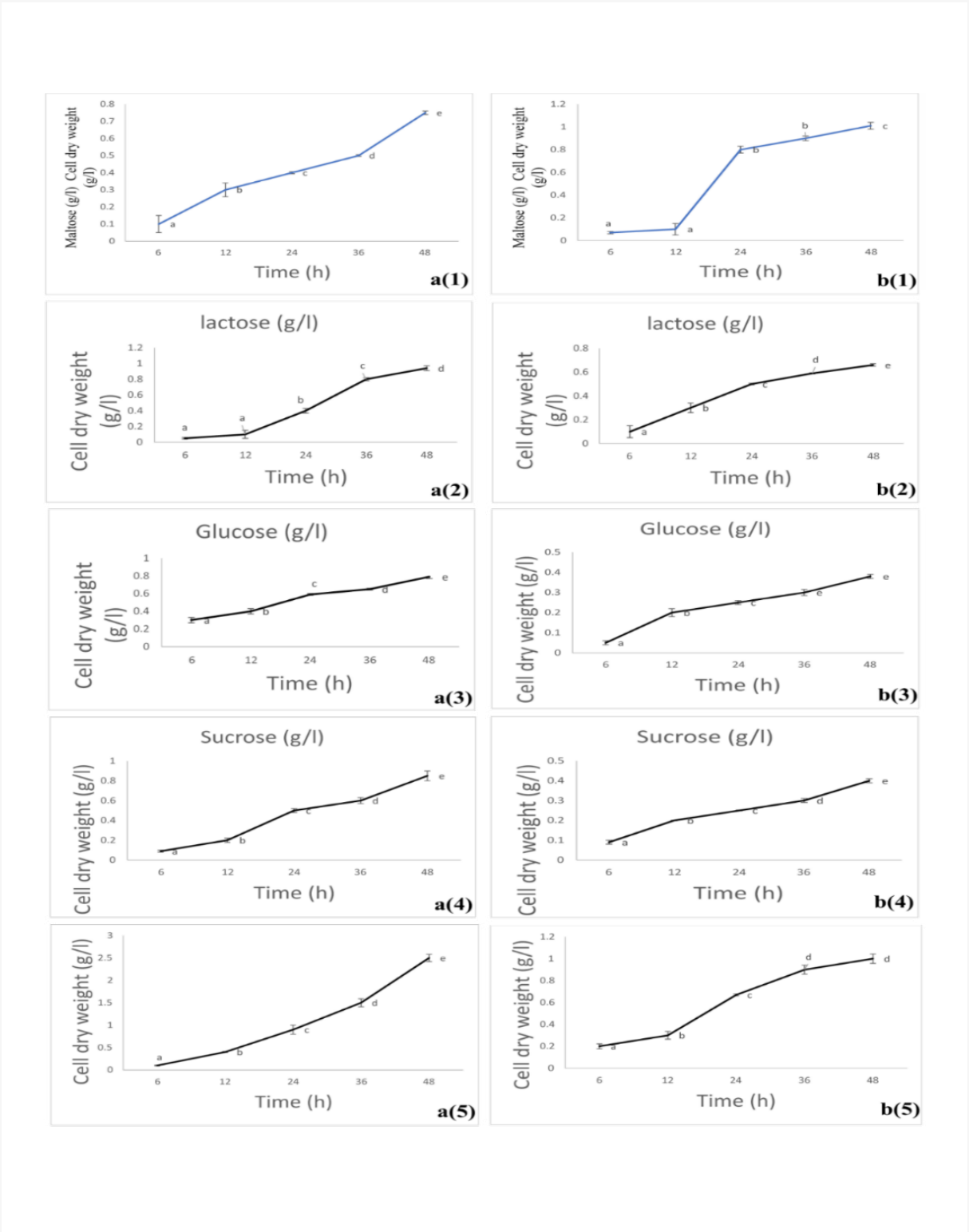


Figure 2. Results of dry cell weight measurements (g/l) in cyanobacterial strains (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* in culture media containing (1) maltose, (2) lactose, (3) glucose, (4) sucrose, and (5) control.

Slika 2. Rezultati meritev suhe teže celic (g/l) v sevih cianobakterij (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* v gojiščih, ki vsebujejo (1) maltozo, (2) laktozo, (3) glukozo, (4) saharozo in (5) kontrolno.

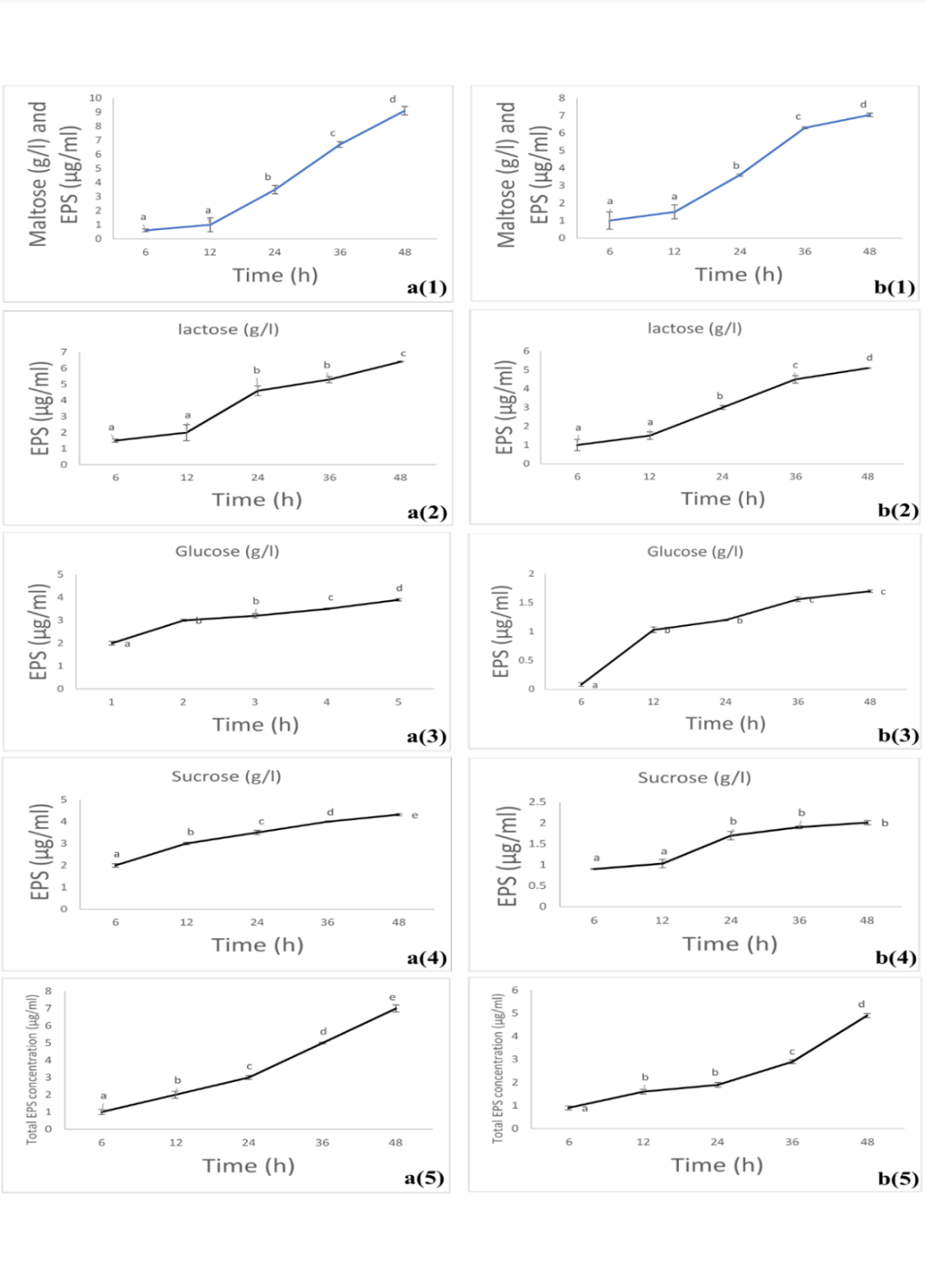


Figure 3. Results of extracellular polysaccharide (µg/ml) measurements in cyanobacterial strains (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* in culture media containing (1) maltose, (2) lactose, (3) glucose, (4) sucrose, and (5) control.

Slika 3. Rezultati meritev ekstracelularnih polisaharidov (µg/ml) v sevih cianobakterij (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* v gojiščih, ki vsebujejo (1) maltozo, (2) laktozo, (3) glukozo, (4) saharozo in (5) kontrolno.

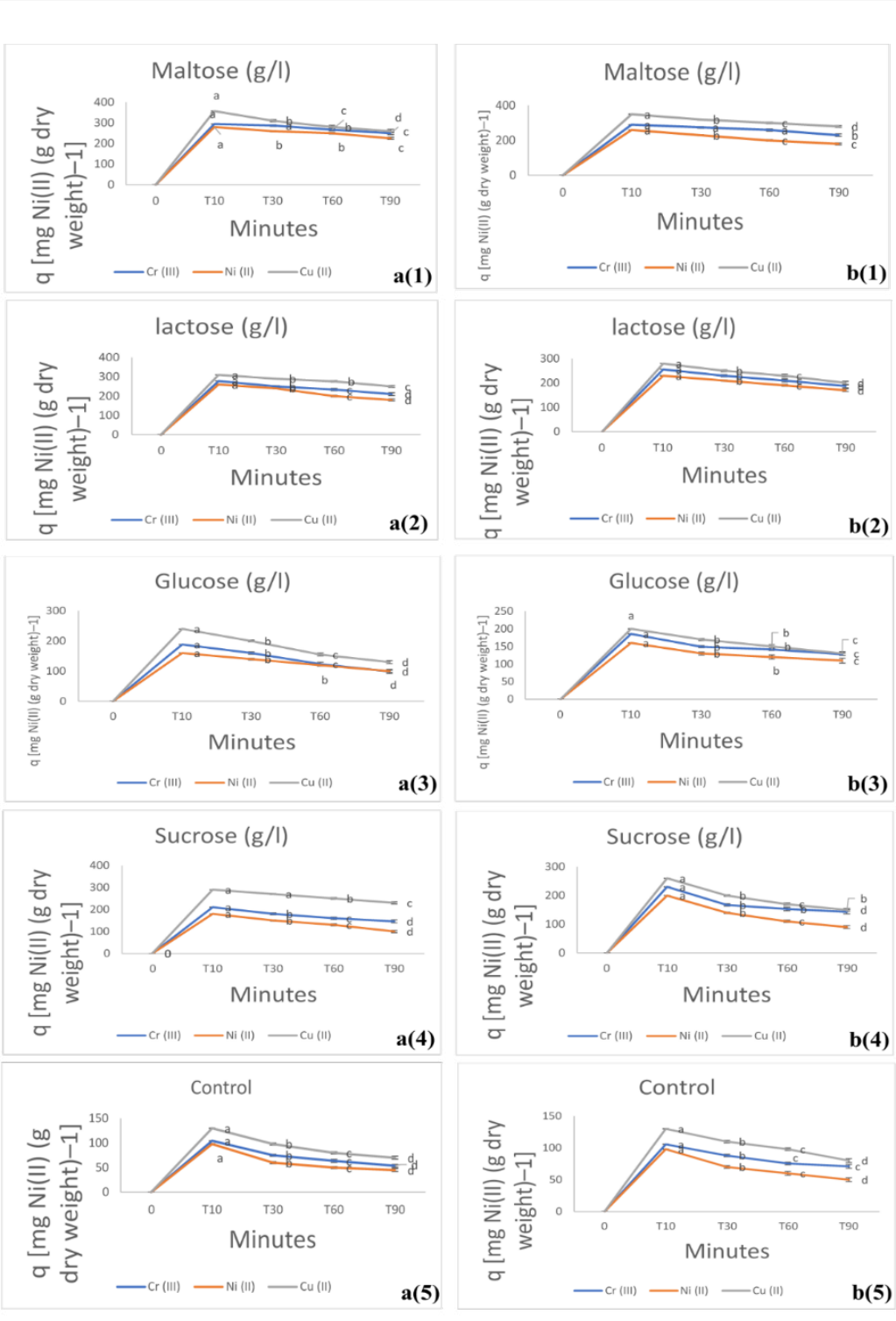


Figure 4. Results of heavy metal removal measurements in cyanobacterial strains (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* in culture media containing (1) maltose, (2) lactose, (3) glucose, (4) sucrose, and (5) control.

Slika 4. Rezultati meritev odstranjevanja težkih kovin v sevih cianobakterij (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* v gojiščih, ki vsebujejo (1) maltozo, (2) laktozo, (3) glukozo, (4) saharozo in (5) kontrolno skupino.

Results of exopolysaccharide measurement in the presence of heavy metals

The results of exopolysaccharide measurements (µg/l) showed that the highest exopolysaccharide levels were produced by the *Fischerella* sp. strain in media containing copper and maltose (14 µg/L), which was 1.16 times higher than that of the *Desmonostoc alborizicum* strain (Figure 5).

Results of cellular protein measurement

The results of cellular protein measurements in the control culture medium showed that the protein level in the *Fischerella* sp. strain was significantly higher than in the *Desmonostoc alborizicum* strain. In both strains, the cellular protein content significantly increased until the end of 48 hours. However, the protein levels produced by the *Fischerella* sp. strain in the control, maltose, and lactose-containing media were approximately 8.88, 1.08, and 1.03 times higher, respectively, than those in the *Desmonostoc alborizicum* strain.

Additionally, the results of cellular protein measurements in glucose- and sucrose-containing media indicated that in both strains, protein levels significantly decreased until the end of 48 hours, with no significant difference observed between the two strains (Figure 6).

Identification of volatile compounds using GC-MS

Since the results of heavy metal removal demonstrated that *Fischerella* sp. exhibited the highest efficiency in removing copper in the maltose-containing culture medium, the analysis of volatile compounds in this medium was conducted using *Fischerella* sp. The analysis of volatile compounds in *Fischerella* sp. under control conditions using GC-MS revealed the presence of 18 compounds, including furan, aldehyde, ester, ketone, benzene, acid, alkyne alcohol, and alcohol. According to the results, the most abundant compounds were 3-methylbutanal and 2-methylbutanal (12.210%), followed by 2-methyl-L-propanol (11.056%). The least abundant compound identified was 2-phenylethanol (2.003%). The analysis of volatile compounds in *Fischerella* sp. cultured in maltose-containing medium with copper using GC-MS identified similar classes of compounds, including furan, aldehyde, ester, ketone, benzene, acid, alkyne alcohol, and alcohol. The most abundant compounds were 3-methylbutanal and 2-methylbutanal (18.718%), followed by 3-methyl-1-propanol (12.566%). Ethanol (1.763%) was the least abundant compound. A quantitative comparison of volatile compounds in *Fischerella* sp. under control conditions and in the maltose-containing medium with copper revealed a decrease in 2-methylfuran, ethyl acetate, 2-pentanone, ethanol, 1,3-dimethylbenzene, 2-methyl-L-propanol, acetic acid, benzaldehyde, and 1-octen-3-ol compared to the control. Conversely, an increase was observed in 3-methylbutane, 2-methylbutane, methyl acetate, 2-butanone, acetic acid, 3-methylbutyl ester, 1-butanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone, 2-phenylethanol, and 3-octen-2-ol (Table 1).

Discussion

Heavy metal contamination is one of the major environmental challenges, particularly in aquatic ecosystems, where it has extensive negative impacts. The use of biological methods for removing these pollutants, including the application of cyanobacteria as natural and efficient agents, has garnered significant attention. Cyanobacteria are capable of adsorbing and removing heavy metals from aquatic environments, with the production of EPS playing a key role in this process. In this context, the present study investigates the impact of autotrophic and mixotrophic culture conditions on heavy metal adsorption by two cyanobacterial species, *Fischerella* sp. and *Desmonostoc alborizicum*, isolated from wetlands and qanats in Golestan Province. This study represents a novel approach aimed at optimising heavy metal removal and enhancing the bioremediation efficiency of these species.

The findings of this study indicated that *Fischerella* sp. exhibited a greater ability to produce dry cell biomass compared to *Desmonostoc alborizicum*. This advantage may be attributed to factors such as higher efficiency in nutrient uptake and metabolism, particularly in sugar utilisation, in *Fischerella* sp. The significant increase in dry cell weight in both strains up to 48 hours suggests their adaptation to the culture conditions and effective utilisation of available resources, particularly maltose as a carbon and energy source for growth and proliferation. A study by Xie et al. (2011) examined the effect of glucose on the growth of the cyanobacterium *Synechocystis* PCC 6714. Their results showed that glucose supplementation not only enhanced growth but also led to glucose consumption and a reduc-

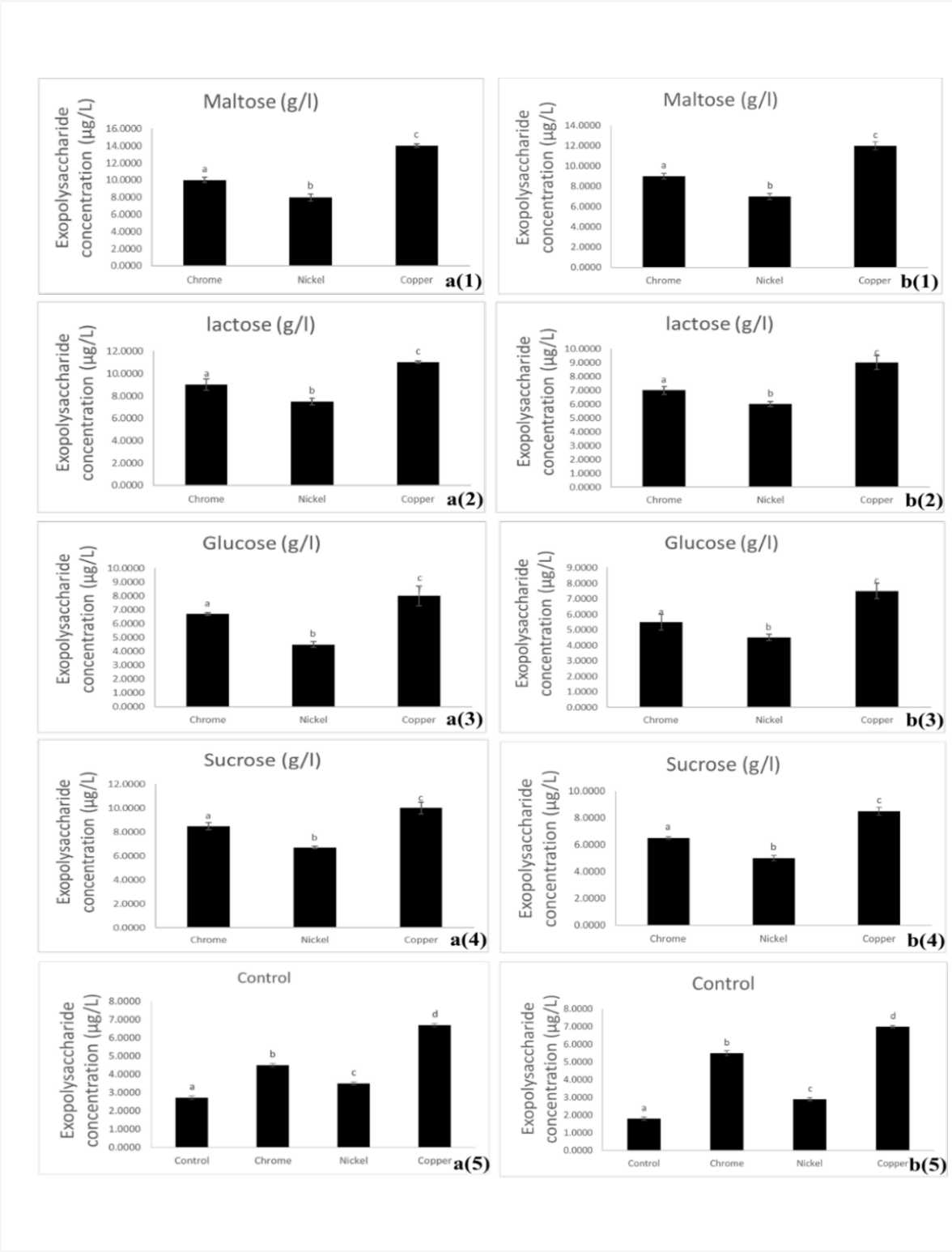


Figure 5. Results of exopolysaccharide measurements in cyanobacterial strains (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* in culture media containing heavy metals and (1) maltose, (2) lactose, (3) glucose, (4) sucrose, and (5) control.

Slika 5. Rezultati meritev eksopolisaharidov v cianobakterijskih sevih (a) *Fischerella* sp., (b) *Desmonostoc alborizicum* v gojiščih, ki vsebujejo težke kovine in (1) maltozo, (2) laktozo, (3) glukozo, (4) saharozo in (5) kontrolno skupino.

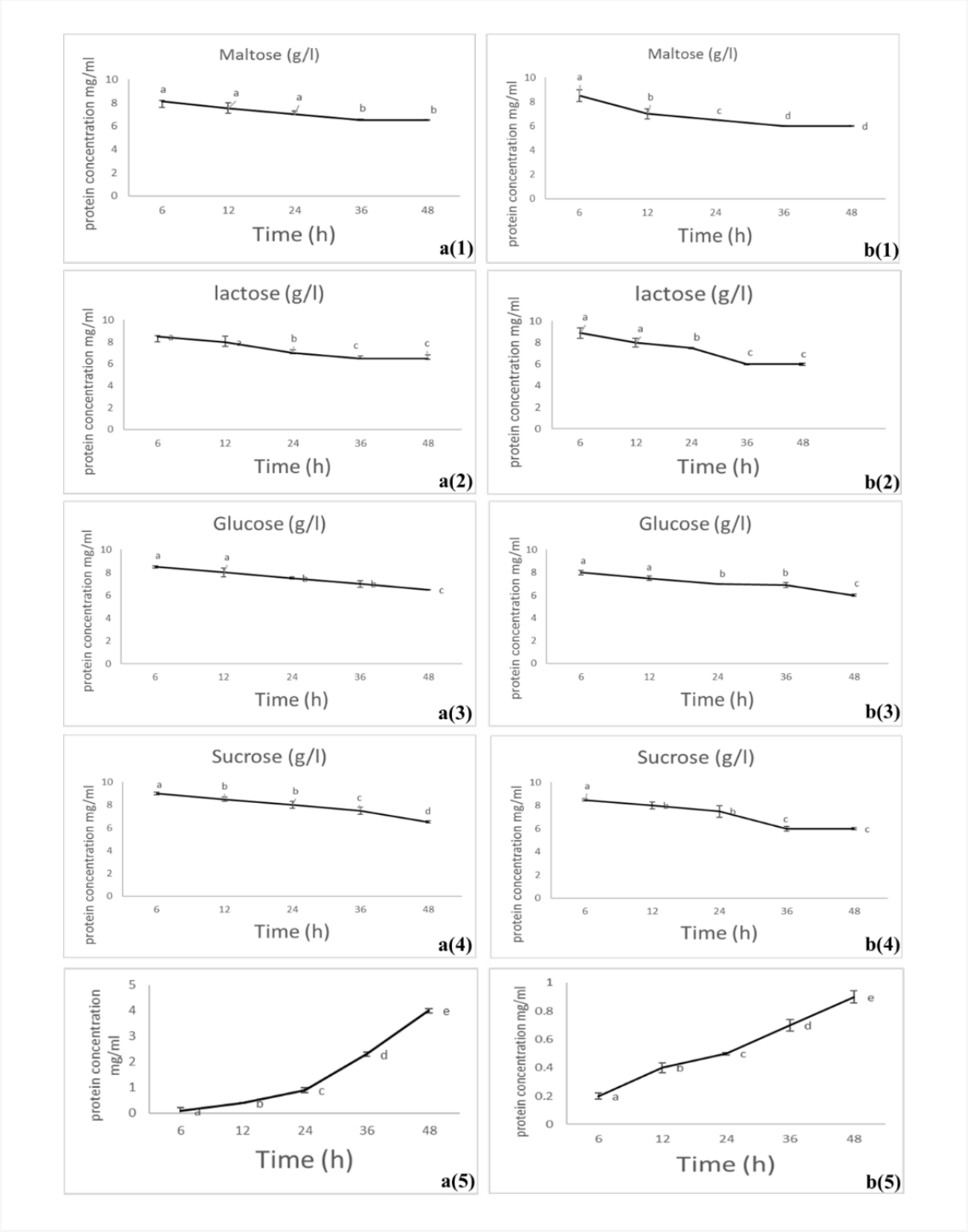


Figure 6. presents the results of protein content measurements (mg/ml) in the cyanobacterial strains (a) *Fischerella* sp. and (b) *Desmonostoc alborizicum* in culture media containing (1) maltose, (2) lactose, (3) glucose, (4) sucrose, and (5) control.

Slika 6. prikazuje rezultate meritev vsebnosti beljakovin (mg/ml) v cianobakterijskih sevih (a) *Fischerella* sp. in (b) *Desmonostoc alborizicum* v gojiščih, ki vsebujejo (1) maltozo, (2) laktozo, (3) glukozo, (4) saharozo in (5) kontrolno skupino.

Table 1. Comparison of Volatile Compound Analysis in *Fischerella* sp. Cultured in Maltose-Containing Medium with Copper and Control Medium.
Tabela 1. Primerjava analize hlapnih spojin v *Fischerella* sp., gojeni v gojišču, ki vsebuje maltozo, s bakrom in kontrolnem gojišču.

Peak IDs	Molecular Formula	RT (time)	Nature of Compounds	Abandnce (%) 16 h	24 h	Increase / Decrease
2-Methylfuran	C5H6O	4.57	Furan	7.231	5.181	1.39 % Decrease
3-Methylbutanal, 2-methylbutanal	C5H10O	5.14	Aldehyde	12.210	18.718	1.53 % Increase
Acetic acid methyl ester	C3H6O2	7.02	Eester	5.742	6.518	1.13 % Increase
Acetic acid ethyl ester	CH3COOC2H5	7.41	Eester	6.574	2.551	2.57 % Decrease
2-Butanone	C4H8O	8.33	ketone	4.727	4.838	1.02 % Increase
2-Pentanone	C5H10O	8.87	ketone	3.937	3.577	1.10 % Decrease
Ethanol	CH_3COOH, C9H18O2	9.24	Ester	3.648	1.763	2.06% Decrease
Acetic acid, 3-methylbutyl ester	CH3(CH2)3OH	13.33	Alcohol	4.141	4.504	1.08 % Increase
1-butanol	C6H4(CH3)2	13.82	benzene	2.729	3.024	1.11 % Increase
1,3-dimethylbenzene	C4H9OH	14.68	Alcohol	3.982	3.744	1.06 % Decrease
2-Methyl-l-propanol	C5H12O	15.07	Alcohol	11.056	7.939	1.39 % Decrease
3-Methyl-1-butanol	CH_3COOH, C9H18O2	15.61	Ester	7.986	12.566	1.57% Increase
3-Hydroxy-2-butanone	C8H16O4	17.26	ketone	3.105	4.546	1.46% Increase
Acetic acid	CH3COOH	20.83	Acid	3.615	2.510	1.44% Decrease
Benzaldehyde	C7H6O	22.85	Aldehyde	8.617	7.477	1.15 % Decrease
2-Phenylethanol	C8H10O	28.41	Alcohol	2.003	2.542	1.26% Increase
1-Octen-3-ol	C8H16O	28.77	alkenyl alcohol	5.255	4.490	1.17 % Decrease
3-Octen-2-ol	C8H16O	29.53	alkenyl alcohol	3.441	3.511	1.02 % Increase

tion in its concentration in the culture medium. These findings align with those of the present study, highlighting the role of sugars as accessible carbon sources that promote cyanobacterial growth and biomass production. However, it is important to note that Xie et al.'s study utilised glucose, whereas the present research focused on maltose. Therefore, it can be inferred that the cyanobacterial strains examined in both studies are capable of utilising glucose, either directly or as part of maltose, for their growth (Xie et al., 2011).

The results of EPS measurements in this study revealed that *Fischerella* sp. produced approximately 1.3 times more exopolysaccharides in the maltose-containing medium compared to *Desmonostoc alborizicum*. This finding suggests that maltose, as a carbon source, has a significant impact on EPS production in these strains, particularly in *Fischerella* sp. In this regard, Yu et al. (2010) also observed that maltose had the greatest influence on exopolysaccharide production in *Nostoc flagelliforme* (Jia et al., 2010). Yu et al. (2012) demonstrated that adding sugars such as glucose, sucrose, and glycerol to the mixotrophic culture medium of *Nostoc flagelliforme* leads to an increase in EPS production and biomass. Other studies have also examined the role of different carbon sources in EPS production in cyanobacteria (Yu et al., 2012). Hassan et al. (2012) showed that a maltose-containing medium is suitable for the growth of cyanobacterial species, and treating the strains with sugars such as glucose, sucrose, maltose, and lactose enhances extracellular polysaccharide production, protein content, and biomass (Hassan et al., 2012). Nieves-Morión and Flores (2018) also reported increased growth of *Anabaena* sp. in the presence of sucrose, fructose, and glucose. Although these studies emphasise the effects of various sugars, the findings of the present research indicate that maltose has a more pronounced impact on EPS production in the studied strains compared to other

examined sugars. In addition to the influence of carbon sources, other factors also play a role in EPS production (Nieves-Morión and Flores, 2018).

Pereira et al. (2009), in their review study, highlighted the role of available energy parameters (provided by sugar sources) and the carbon-to-nitrogen (C:N) ratio in EPS production (Pereira et al., 2009). Finally, the study by Zhang et al. (2019) emphasised the role of maltose in exopolysaccharide production in *Lactobacillus sanfranciscensis*, showing that maltose can serve as an effective carbon source for EPS production in various microorganisms (Zhang et al., 2019). In this study, the results of heavy metal removal indicated that in *Fischerella* sp., copper removal was higher than that of nickel and chromium. The highest removal efficiency for all three heavy metals was recorded at 10 minutes, after which it decreased at 30 minutes and then reached a relatively stable level at 60 to 90 minutes. Similar results, though with lower efficiency, were observed for the strain *Desmonostoc alborizicum*. A comparison of heavy metal removal efficiency between *Fischerella* sp. and *Desmonostoc alborizicum* revealed that the strain isolated from the wetlands of Golestan Province exhibited a greater ability to remove heavy metals from the environment within a fixed time period. Mota et al. (2016) conducted an experiment on the unicellular cyanobacterium *Cyanothece* sp. CCY 0110 to assess the efficiency of removing three pollutants—copper, cadmium, and lead—individually. They reported that released polysaccharides (RPS) from *Cyanothece* sp. CCY 0110 exhibited higher biosorption efficiency for heavy metal removal (Mota et al., 2016). These studies have demonstrated that cyanobacterial exopolysaccharides can facilitate heavy metal removal from the environment, which aligns with the findings of the present study. Pereira et al. (2011) used exopolysaccharide-producing cyanobacteria for heavy metal removal. In their research, the cyanobacterial species *Gloeothece* sp. PCC 6909 was employed to study the removal of copper and lead (Pereira et al., 2011). The results showed that both this cyanobacterial species and its mutant strain lacking exopolysaccharides were capable of removing copper and lead from the environment. Furthermore, this research group demonstrated that structural changes occurred in both the wild-type and mutant strains when exposed to copper. This study observed that lead was removed more efficiently from the environment than copper. Mohite et al. (2018) investigated heavy metal stress and its role in exopolysaccharide production in *Pantoea agglomerans*. In this study, the heavy metals mercury, copper, silver, arsenic, lead, chromium, and cadmium were examined. The results indicated that metal-induced stress reduces total protein content (Mohite et al., 2018). Surosz and Palinska (2016) demonstrated that heavy metals such as copper and cadmium significantly decrease total cellular protein content in the cyanobacterium *Anabaena flos-aquae*. Additionally, this study observed that copper had a greater effect than cadmium in reducing total cellular protein concentration (Surosz and Palinska, 2016). Okmen et al. (2011) evaluated the impact of zinc on chlorophyll a, total carbohydrates, total protein, and biomass. They observed that at a concentration of 10 mg/L (a high concentration of this metal), biomass, total protein, and chlorophyll a levels significantly decreased (Okmen et al., 2011).

Shukla et al. (2009) showed that treating the cyanobacterial species *Anabaena doliolum* with nickel at concentrations of 10 and 50 µmol/L led to an increase in total cellular protein concentration, but increasing the concentration to 100 µmol/L reduced protein content, indicating the toxic effects of this metal at higher concentrations (Shukla et al., 2009). Elsalhin et al. (2016) evaluated the effect of cobalt on total protein and total carbohydrates in the cyanobacterium *Spirulina platensis*. The results of this study demonstrated that at low concentrations of cobalt, total protein and carbohydrate concentrations increased; however, with increasing concentrations of this heavy metal, these parameters significantly decreased, indicating its toxic effects (Elsalhin et al., 2016). Martínez-Ruiz et al. (2016) investigated the effect of the heavy metal nickel on the total cellular protein, total carbohydrate, and lipid content of the bacterium *Microcystis aeruginosa*. The results of this study demonstrated that increasing the concentration of nickel up to 1.64 µg/L led to an increase in total cellular protein, carbohydrate, and lipid concentrations. However, at a concentration of 3.7 µg/L, the total carbohydrate content decreased, whereas the total protein and lipid concentrations continued to increase. The findings of previous studies confirm the results of the present study, as we also observed that treatment of *Fischerella* sp. and *Desmonostoc alborizicum* species with the heavy metals copper, chromium, and nickel resulted in an increase in total cellular carbohydrate content, with nickel playing a more significant role in enhancing carbohydrate levels (Martínez-Ruiz et al., 2016).

In this study, GC-MS was used to analyse the chemical compounds and their relative abundances in both the control sample and samples treated with the heavy

metal elements chromium, nickel, and copper. The results indicated that treatment with copper led to an increased percentage of the following compounds: 3-methylbutane, 2-methylbutane, methyl acetate ester, 2-butanone, acetic acid, 3-methylbutyl ester, 1-butanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone, 2-phenylethanol, and 3-octan-2-ol. In 2013, researchers demonstrated that the primary fatty acid components in *Nostoc* and *Scenedesmus* sp. treated with the heavy metals lead, zinc, copper, cadmium, manganese, chromium, and nickel included methyl palmitate, methyl oleate, methyl linoleate, and methyl linolenate. Gheda et al. (2020) reported that the compound cyclotrisiloxane in *Oscillatoria acuminata*, *Oscillatoria amphigranulata*, and *Spirulina platensis* exhibited antioxidant activity and mitigated the toxic effects of heavy metals (Gheda et al., 2020). Babu et al. (2010) studied the biodegradation of phthalate esters by cyanobacteria. In this study, the cyanobacterial species *Microcystis aeruginosa* and *Anabaena flos-aquae* G. S. West were examined, and GC-MS results revealed a significant presence of benzenedicarboxylic acid 1,2-, which is consistent with the findings of this study (Babu and Wu, 2010).

While this study provides valuable insights into the heavy metal bioremediation potential of *Fischerella* sp. and *Desmonostoc alborizicum* under autotrophic and mixotrophic culture conditions, several limitations should be considered. The experiments were conducted under controlled laboratory conditions, which may not fully represent the complex and dynamic conditions of natural ecosystems. Additionally, only a limited range of heavy metals was evaluated, and the long-term stability, regeneration, and reusability of cyanobacterial biomass for practical wastewater treatment were not assessed. Future research should focus on applying these strains in pilot-scale or field studies to validate their heavy metal removal efficiency in real contaminated environments. Furthermore, deeper exploration of the molecular mechanisms involved in exopolysaccharide biosynthesis and heavy metal binding, along with optimisation of culture parameters for scale-up and continuous operation, would facilitate the practical and sustainable application of these native cyanobacterial strains in environmental bioremediation.

Conclusion

This research demonstrated that native Iranian cyanobacteria, particularly the *Fischerella* sp. strain, can serve as effective candidates for the removal of heavy metals from aquatic ecosystems. The increased production of exopolysaccharides in culture media enriched with carbon sources such as maltose played a key role in enhancing metal removal efficiency. Additionally, volatile compound analysis indicated positive metabolic changes under heavy metal treatment. These findings not only introduce high-potential cyanobacterial strains for heavy metal removal but also propose an environmentally friendly approach to mitigating metal pollution in water resources. By optimising culture conditions and further investigating the underlying biochemical mechanisms, this study lays a foundation for developing sustainable and scalable bioremediation technologies with practical application potential in wastewater treatment and environmental restoration.

Author Contributions

Conceptualization, B. N.; methodology, all authors; software, B.N.; validation, B.N; formal analysis, B.N.; investigation, H.B.; writing—original draft preparation, H.B; writing—review and editing, B.N. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

Not applicable

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The authors declare that there is no conflict of interest.

References

Babu, B., Wu, J.T., 2010. Biodegradation of phthalate esters by cyanobacteria. Journal of phycology, 46(6), 1106-1113.

Delattre, C., Pierre, G., Laroche, C., Michaud, P., 2016. Production, extraction and characterization of microalgal and cyanobacterial exopolysaccharides. Biotechnology advances, 34(7), 1159-1179.

DuBois, M., Gilles, K.A., Hamilton, J. K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28(3), 350-356.

Elsalhin, H.E., Abobaker, H.M., Ali, M.S., 2016. Toxicity effect of Cobalt on total protein and carbohydrate of cyanobacteria *Spirulina platensis*. IOSR Journal of Environmental Science, Toxicology and Food Technology, 10(9), 114-120.

Gheda, S.F., Ismail, G.A., 2020. Natural products from some soil cyanobacterial extracts with potent antimicrobial, antioxidant and cytotoxic activities. Anais da Academia Brasileira de Ciências, 92(2), e20190934.

Ghorbani, E., Nowruzi, B., Nezhadali, M., Hekmat, A., 2022. Metal removal capability of two cyanobacterial species in autotrophic and mixotrophic mode of nutrition. BMC microbiology, 22(1), 58.

Giner-Lamia, J., Pereira, S. B., Bovea-Marco, M., Futschik, M. E., Tamagnini, P., Oliveira, P., 2016. Extracellular proteins: novel key components of metal resistance in cyanobacteria? Frontiers in microbiology, 7, 878.

Guo, Q., Meng, Q., Wang, L., Yu, J., Chen, X., Liu, D., Li, D., Wang, Ch., Liang, F., Ding, C., 2024. Identification of odor-causing compounds in six species of odor-producing microalgae separated from drinking water source with distinct fishy odor: Insight into microalgae growth and odor characteristics. Chemosphere, 350, 141-243.

Hassan, S.H., Hameed, M.S.A., Hammouda, O.E., Ghazal, F.M., Hamed, S.M., 2012. Effect of different growth conditions on certain biochemical parameters of different cyanobacterial strains. Malaysian Journal of Microbiology, 8(4), 266-272.

Hu, C., Liu, Y., Paulsen, B.S., Petersen, D., Klaveness, D., 2003. Extracellular carbohydrate polymers from five desert soil algae with different cohesion in the stabilization of fine sand grain. Carbohydrate polymers, 54(1), 33-42.

Knowles, E. J., Castenholz, R. W., 2008. Effect of exogenous extracellular polysaccharides on the desiccation and freezing tolerance of rock-inhabiting phototrophic microorganisms. FEMS microbiology ecology, 66(2), 261-270.

Martínez-Ruiz, E. B., Martinez-Jeronimo, F., 2016. How do toxic metals affect harmful cyanobacteria? An integrative study with a toxigenic strain of *Microcystis aeruginosa* exposed to nickel stress. Ecotoxicology and Environmental Safety, 133, 36-46.

Mohite, B. V., Koli, S. H., Patil, S. V., 2018. Heavy metal stress and its consequences on exopolysaccharide (EPS)-producing *Pantoea agglomerans*. Applied biochemistry and biotechnology, 186(1), 199-216.

Mota, R., Rossi, F., Andrenelli, L., Pereira, S. B., De Philippis, R., Tamagnini, P., 2016. Released polysaccharides (RPS) from *Cyanothece* sp. CCY 0110 as biosorbent for heavy metals bioremediation: interactions between metals and RPS binding sites. Applied microbiology and biotechnology, 100(17), 7765-7775.

Nieves-Morión, M., Flores, E., 2018. Multiple ABC glucoside transporters mediate sugar-stimulated growth in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. Environmental Microbiology Reports, 10(1), 40-48.

Nowruzi, B., Becerra-Absalón, I., Metcalf, J. S., 2023. A Novel Microcystin-Producing Cyanobacterial Species from the Genus *Desmonostoc*, *Desmonostoc alborizicum* sp. nov., Isolated from a Water Supply System of Iran: B. Nowruzi. Current Microbiology, 80(1), 49.

Nowruzi, B., Lorenzi, A. S., 2021. Characterization of a potentially microcystin-producing *Fischerella* sp. isolated from Ajigol wetland of Iran. South African Journal of Botany, 137, 423-433.

Okmen, G., Bozanta, E., Ugur, A., Ceyhan, N., 2011. Zinc effect on chlorophyll a, total carbohydrate, total protein contents and biomass of cyanobacterial species. Journal of applied biological sciences, 5(2), 67-73.

Olson, B. J., Markwell, J., 2007. Assays for determination of protein concentration. Current protocols in pharmacology, 38(1), A-3A.

Ozturk, S., Aslim, B., 2010. Modification of exopolysaccharide composition and production by three cyanobacterial isolates under salt stress. Environmental Science and Pollution Research, 17(3), 595-602.

Parikh, A., Madamwar, D., 2006. Partial characterization of extracellular polysaccharides from cyanobacteria. Bioresource technology, 97(15), 1822-1827.

Pereira, S., Micheletti, E., Zille, A., Santos, A., Moradas-Ferreira, P., Tamagnini, P., De Philippis, R., 2011. Using extracellular polymeric substances (EPS)-producing cyanobacteria for the bioremediation of heavy metals: do cations compete for the EPS functional groups and also accumulate inside the cell? Microbiology, 157(2), 451-458.

Pereira, S., Zille, A., Micheletti, E., Moradas-Ferreira, P., De Philippis, R., Tamagnini, P., 2009. Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS microbiology reviews, 33(5), 917-941.

Pereira, S. B., Mota, R., Santos, C. L., De Philippis, R., Tamagnini, P., 2013. Assembly and export of extracellular polymeric substances (EPS) in cyanobacteria: a phylogenomic approach. Advances in Botanical Research, 65, 235-279.

Pereira, S. B., Mota, R., Vieira, C. P., Vieira, J., Tamagnini, P., 2015. Phylum-wide analysis of genes/proteins related to the last steps of assembly and export of extracellular polymeric substances (EPS) in cyanobacteria. Scientific reports, 5(1), 14835.

Rossi, F., De Philippis, R., 2015. Role of cyanobacterial exopolysaccharides in phototrophic biofilms and in complex microbial mats. Life, 5(2), 1218-1238.

Shukla, M. K., Tripathi, R. D., Sharma, N., Dwivedi, S., Mishra, S., Singh, R., ... Rai, U. N., 2009. Responses of cyanobacterium *Anabaena doliolum* during nickel stress. Journal of Environmental Biology, 30(5), 871.

Surosz, W., Palinska, K. A., 2004. Effects of heavy-metal stress on cyanobacterium *Anabaena flos-aquae*. Archives of environmental contamination and toxicology, 48(1), 40-48.

Xie, X. H., Li, E. L., Kang Tang, Z., 2011. Mediator toxicity and dual effect of glucose on the lifespan for current generation by Cyanobacterium *Synechocystis* PCC 6714 based photoelectrochemical cells. Journal of Chemical Technology & Biotechnology, 86(1), 109-114.

Yu, H., 2012. Effect of mixed carbon substrate on exopolysaccharide production of cyanobacterium *Nostoc flagelliforme* in mixotrophic cultures. Journal of applied phycology, 24(4), 669-673.

Yu, H., Jia, S., Dai, Y., 2010. Accumulation of exopolysaccharides in liquid suspension culture of *Nostoc flagelliforme* cells. Applied Biochemistry and Biotechnology, 160(2), 552-560.

Zhang, G., Zhang, W., Sun, L., Sadiq, F. A., Yang, Y., Gao, J., Sang, Y., 2019. Preparation screening, production optimization and characterization of exopolysaccharides produced by Lactobacillus sanfranciscensis Ls-1001 isolated from Chinese traditional sourdough. International Journal of Biological Macromolecules, 139, 1295-1303.

ABS

Review

Continuous Bioprocessing for Recombinant Protein Production in *Bacillus subtilis*: Opportunities and Challenges

Andrej Ivanovski [1,*], Tomaž Bratkovič [2]

Abstract

The Gram-positive bacterium *Bacillus subtilis* has long been used for industrial protein production owing to its well-characterised genetic background, capacity for high-density fermentation, and effective protein secretion. With the growing global demand for recombinant proteins across various sectors, there is an increasing need for more efficient and sustainable protein production methods. This review explores the application of continuous bioprocessing for recombinant protein production using *B. subtilis*, critically evaluating current technologies and discussing their potential for integration into streamlined, integrated biomanufacturing framework. We primarily focus on transgenic *B. subtilis*, however the solutions for continuous fermentation and protein isolation/purification are the same for both endogenous and heterologous (i.e., recombinant) proteins.

Keywords

Continuous Bioprocessing; *Bacillus subtilis*; Upstream Processing; Downstream Processing; Recombinant Protein Production

1 University of Ljubljana, Biotechnical faculty, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

2 University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia

*** Corresponding author:**
E-mail address: ai70758@student.uni-lj.si

Citation: Ivanovski, A., Bratkovič, T., (2025). Continuous Bioprocessing for Recombinant Protein Production in *Bacillus subtilis*: Opportunities and Challenges. Acta Biologica Slovenica 68 (4)

Received: 13.05.2025 / **Accepted:** 14.07.2025 / **Published:** 21.07.2025

https://doi.org/10.14720/abs.68.4.22623

Kontinuirno bioprocesništvo za proizvodnjo rekombinantnih proteinov v *Bacillus subtilis*: priložnosti in izzivi

Izvleček

Po Gramu pozitivno bakterijo *Bacillus subtilis* že dolgo uporabljamo v industrijski proizvodnji proteinov. K temu prispevajo njena dobra raziskana genetska zasnova, možnost doseganja visoke biomase med fermentacijo in učinkovito izločanje proteinov. Zaradi naraščajočega svetovnega povpraševanja po rekombinantnih proteinih v različnih sektorjih, se povečuje tudi potreba po učinkovitejših in trajnostnih metodah proizvodnje proteinov. V članku predstavljamo potencial kontinuirnega bioprocesništva pri proizvodnji rekombinantnih proteinov z uporabo *B. subtilis*. Ponujamo pregled obstoječih tehnologij in njihovega potenciala za vključitev v integrirani koncept učinkovitejše bioproizvodnje rekombinantnih proteinov. Čeprav se prispevek primarno posveča transgenim bakterijam *B. subtilis*, so rešitve kontinuirne fermentacije in izolacije/čiščenja proteinov enake za endogene in heterologne (tj. rekombinantne) proteine.

Ključne besede

Kontinuirno bioprocesiranje; *Bacillus subtilis*; pripravljalni procesi; zaključni postopki; proizvodnja rekombinantnih proteinov

Introduction

The demand for recombinant proteins is rapidly increasing, driven by their expanding applications in healthcare, agricultural and material sector. While the expanding biopharmaceutical market remains a key driver, the need for recombinant enzymes, bio-pesticides, bio-stimulants and bio-based materials is also steadily rising, encouraging the development of efficient and scalable production strategies (Evens and Pharm, 2022; Kergaravat et al., 2025; Marrone, 2024). To meet this growing demand, the bioindustry is actively exploring strategies for process intensification. Continuous processing has already demonstrated its ability to boost productivity and reduce turnaround times across various industries. Accordingly, the bioindustry is shifting towards continuous manufacturing to improve productivity and ensure consistent product quality, while also reducing environmental impact (Jungbauer et al., 2024; Peebo and Neubauer, 2018). Importantly, the motivation for adopting continuous biomanufacturing is not solely economic. Regulatory bodies have recognized several advantages of continuous processing that contribute to improved product quality. Maintaining a steady-state operation is crucial for ensuring consistent quality, while the elimination of interruptions between process steps enhances both the reliability and safety of manufacturing (Godawat et al., 2015; Subramanian, 2017; Walther et al., 2015). In the bioindustry, the cultivation of microorganisms is a critical step in protein production (Yang et al., 2021). In this context, *Bacillus* species represent an attractive platform due to their high secretion capacity and ability to produce a range of recombinant proteins at high titers, supporting the needs of the expanding market. This paper focuses on *Bacillus subtilis*, a microorganism long employed for producing diverse proteins, enzymes, and various chemicals (Lopez et al., 2009). The aim is to review approaches in upstream and downstream processing for recombinant protein production using *B. subtilis* and to explore the potential of continuous biomanufacturing as a solution for process intensification.

Bacillus subtilis as a Host for Recombinant Protein Production

Escherichia coli remains the most widely used prokaryotic host for recombinant protein production. However, *B. subtilis* is widely used in industrial homologous protein production and its popularity for recombinant protein expression is increasing (Errington and van der Aa, 2020). Classified as Generally Recognised as Safe (GRAS), *B. subtilis* offers several advantages: a well-characterised genetic

framework, suitability for high-density fermentation, lack of lipopolysaccharides, and an inherent ability to secrete proteins (Yang et al., 2020, 2021). Numerous strategies, including selection of parental strains and strain engineering, have been used to enhance recombinant protein production in *B. subtilis*. (Zhang et al., 2020a). The initial step is to select a parental strain that shows promising characteristics in terms of its genetic background. This is followed by strain engineering, a process that is essential for meeting the demands of modern recombinant protein production (Zhang et al., 2018). One of the major challenges is that heterologous proteins are generally more susceptible to proteolytic degradation than native proteins. This vulnerability arises primarily from slower folding kinetics and an increased tendency for misfolding after membrane translocation (Li et al., 2004). Two main strategies have been proposed to mitigate these issues. The first focuses on improving the secretory protein-folding environment, while the second aims to reduce extracellular proteolytic activity (Zhang et al., 2020a). Regarding the first approach, *B. subtilis* naturally produces intracellular chaperones that assist in the folding of nascent polypeptides and prevent the formation of intracellular protein aggregates – processes that have been identified as potential bottlenecks in recombinant protein production. Advances in genome editing technologies have enabled the generation of multiple *B. subtilis* variants in which secretory pathways and signal peptides have been optimized, and key intracellular chaperones have been characterized and overexpressed (Zhang et al., 2020b). The second approach targets extracellular proteolysis. *B. subtilis* secretes seven extracellular proteases, each capable of degrading proteins at different cleavage sites, often contributing to nutrient recycling. By precisely modulating protease expression levels and activities, researchers have significantly improved the yield of extracellular recombinant proteins (Zhang et al., 2018).

Upstream Processing

Upstream processing represents a critical phase in each biomanufacturing operation and has specific characteristic when using *B. subtilis* as a host. Unlike *E. coli*, *B. subtilis* naturally secrets many proteins into the medium, avoiding the need for cell lysis (Chen et al., 2024). Generally, three primary modes of upstream processing are distinguished: batch, fed-batch, and continuous (Subramanian, 2017) with various sub-variants, such as repetitive fed-batch processes developed to bridge the gap between traditional fed-batch and fully continuous upstream processing through semi-continuous strategies (Kopp et al., 2020). All these approaches are applicable to *B. subtilis*. However, their implementation must consider organism-specific challenges, including oxygen transfer rates due to the aerobic nature of *B. subtilis*, as well as the control of sporulation and protease activity during cultivation (Chen et al., 2024). Despite differences in operation, all three modes share a common initiation process. Each begins with a working cell bank (WCB) aliquots revival carried out in shake flasks, followed by seed expansion in a bioreactor and, ultimately, inoculation of the production bioreactor. The major distinctions between batch, fed-batch, and continuous processes emerge during the production phase itself (Subramanian, 2017). A brief comparison of these three operational modes is provided in the following section.

Batch mode

In batch processing, the bioreactor is filled with growth medium up to the final working volume, inoculated, and processed until completion. The process is monitored online using integrated sensors, and regular sampling is performed for in-process analysis (Subramanian, 2017). For *B. subtilis*, batch production typically spans 1-2 days (Ji et al., 2015). When the bioprocess is complete, the culture is transferred to downstream processing. Manufacturing is carried out through successive batch operations, with the product accumulated at the end of each cycle.

Fed-batch mode

In fed-batch processing, the bioreactor is initially filled with growth medium to approximately 65-90% of the final working volume, followed by inoculation. Feeding with nutrient supplements is then initiated based on predefined criteria and continues until the final working volume is reached. As with batch mode, the process is monitored online through integrated sensors, and samples are collected for in-process analysis, including product yield, cell density and biochemical profiling (Subramanian, 2017). For *B. subtilis*, fed-batch production typically lasts between one and four days, depending on the feeding strategy employed (Klausmann et al., 2021). After the bioprocess concludes, the culture is transferred to the downstream processing.

Like batch mode, production in fed-batch mode is achieved through successive, repeated cycles.

Continuous mode

In continuous processing, the bioreactor is initially filled to 40-50% of the final working volume and inoculated. Once the target cell density is reached, a continuous dilution process is initiated, involving the steady feeding of fresh medium while simultaneously harvesting culture at the same flow rate (Subramanian, 2017). The system is maintained at a steady state, ensuring a constant cell density and a constant working volume throughout the operation. For *B. subtilis*, continuous bioprocesses typically run for five to ten days (Kittler et al., 2025). Culture containing the desired product is continuously harvested and sent directly to downstream processing, running in parallel with upstream operations.

Continuous Upstream Processing

Historically, conventional fed-batch bioprocesses have been the industrial standard for *B. subtilis*, primarily due to their operational robustness and high space-time product yields (Zhang et al., 2020b). However, technological advances now support continuous processing, and model-based studies suggest that continuous cultivation of microorganisms can achieve similarly high, and sometimes superior production rates. The primary advantage of a continuous bioprocessing lies in the ability to maintain cells in a steady state, enabling sustainable and consistent protein production (Kittler et al., 2025; Subramanian, 2017). For example, continuous cultures of *B. subtilis* have demonstrated the capacity to produce recombinant proteins at stable, high levels over extended periods. One study reported that continuous cultivation-maintained chloramphenicol acetyltransferase production at relatively high level, with stable protein expression observed at a dilution rate of 0.2 h^{-1} (Vierheller et al., 1995). In contrast, batch and fed-batch processes often experience fluctuations in nutrient concentrations and perturbation of cellular metabolic states, leading to variability in both protein yield and quality. While fed-batch processes allow for controlled nutrient addition, they still face challenges such as accumulation of inhibitory by-products and undesirable metabolism shifts. Despite optimization, fed-batch processes for *B. subtilis* continues to encounter issues related to oxygen limitation and by-product accumulation (Öztürk et al., 2016).

Nonetheless, continuous bioprocessing is not without challenges. Prolonged cultivation times increase the risk of contamination, and maintaining genetic stability over extended periods is critical. Loss of plasmids, spontaneous mutations, and genetic drift can lead to significant reductions in productivity (Csörgo et al., 2012; Croughan et al., 2015). Thus, the development of a robust expression system is imperative for successful continuous protein production. Recent studies have further nuanced this discussion. For instance, a study with *Bacillus lichenioformis* reported that an optimized fed-batch process outperformed a continuous process in terms of productivity (Kittler et al., 2025). Therefore, while continuous upstream processing can enhance sustainability and process consistency, it typically demands more sophisticated control systems and may result in lower per-cycle productivity compared to highly optimized fed-batch operations.

Downstream Processing

Downstream processing (DSP) aims to eliminate impurities and product variants while preserving the integrity of the final product (Jungbauer et al., 2024). When using *B. subtilis* for recombinant protein production, DSP benefits from the organism's natural capacity to secrete proteins into the extracellular environment, which avoids the need for cell lysis in many cases and simplifies the clarification step (Chen et al., 2024). However, *B. subtilis*-specific challenges include the removal of secreted host-cell proteins, notably extracellular proteases that can degrade the product. Therefore, the implementation of protease-deficient strains could be critical to preserve product integrity (Zhang et al., 2018). A streamlined biomanufacturing workflow relies on the integration of various technologies, including cell lysis, filtration, chromatography, refolding, precipitation, and extraction (Jungbauer, 2013; Jungbauer et al., 2024). The following section provides a brief general overview of key the technologies used in the context of continuous downstream protein processing while assessing their relevance for *B. subtilis*-based recombinant protein production.

Cell lysis

When the product is expressed intracellularly, the first step of DSP is typically cell lysis, which ruptures the cell membrane to release the intracellular components. High-pressure

homogenisation is the most widely used industrial method due to its high efficiency and robustness. In this method, a piston pump generates high pressures, leading to slit cavitation around the valve area. The sudden release of the pressure causes effective cell breakage. Modern high-pressure homogenisers are capable of operating in continuous mode, enabling integration into continuous biomanufacturing workflows (Subramanian, 2017). In the production of recombinant proteins with *B. subtilis*, the product is most often found extracellularly. Therefore, cell lysis is generally bypassed in *B. subtilis*-based processes unless targeting specific intracellular product (Yang et al., 2021).

Centrifugation

Centrifugation separates solids from liquids and is a crucial step in DSP. In *B. subtilis*-based recombinant protein production, this operation primarily used for clarification of the fermentation broth by separating the biomass from the extracellularly secreted target proteins (Patel et al., 2019). The most common centrifuge designs are the tubular, chamber and disk-stack centrifuge. Among these, the disk-stack centrifuge is particularly suitable for pseudo-continuous operation, allowing the transient removal of liquid and solid phases during processing (Jungbauer, 2013).

Filtration

Filtration plays a vital in DSP, supporting tasks such as cell-liquid separation, protein concentration, and buffer exchange. Filtration processes are classified into microfiltration, ultrafiltration and nanofiltration, distinguished by their pore sizes. Microfiltration, with the largest pore size, is used for cell and cell debris separation, whereas ultrafiltration and nanofiltration are commonly employed for protein concentration and buffer exchange. For *Bacillus* cultures, the high tendency for foaming and the presence of DNA fragments can increase the viscosity of the broth, requiring adapted filtration strategies or precipitation approaches to ensure cell debris and DNA removal, respectively, during continuous processing (Prabakaran and Hoti, 2008). Continuous filtration is an established technique in biomanufacturing and can be achieved by connecting multiple filtration units in series, where the permeate from one unit feeds into the next enabling uninterrupted flow. Engineering strategies for implementing continuous filtration have been comprehensively reviewed (Jungbauer, 2013).

Chromatography

Chromatography is often an indispensable step in DSP. Conventional packed-column chromatography operates in batch mode, with sequential loading, washing, and elution steps. However, significant research efforts have focused on developing continuous chromatography methods to improve productivity, purity, cost efficiency, and equipment footprint (Subramanian, 2017; Vogel et al., 2002). Depending on the desired purity of the final product, multiple chromatographic stages (i.e. purification train) may be implemented, utilising various physical and chemical differences between proteins to achieve a higher level of purity (Liu et al., 2010). The simplest form of continuous chromatography involves operating multiple columns in parallel, where columns alternate between loading, washing, elution, regeneration, and re-equilibration phases. This rapid cycling approach is already widely applied in the bioindustry. Additionally, alternative technologies such as rotating chromatography devices or counter-current chromatography have been developed to minimise equipment needs and streamline continuous operations (Jungbauer, 2013). When applying these strategies to *B. subtilis*, careful optimisation of chromatographic conditions becomes essential due to its high secretion of endogenous proteases and other host proteins, which can co-elute with the target recombinant proteins, requiring tailored purification strategies to achieve the desired purity levels (Shih et al., 2013).

Extraction

Liquid–liquid extraction (LLE) has emerged as a promising alternative to chromatography-based purification and has been extensively studied over recent decades (Subramanian, 2017). LLE relies on the differential partitioning of target products between the two immiscible aqueous phases. For example, phase-separating mixtures of polyethylene glycol (PEG) or dextran with appropriate salts can be used to achieve selective protein separation. Several continuous LLE methods have been developed and successfully applied for protein separation (Kee et al., 2021; Muniasamy et al., 2022). Since *B. subtilis* translocate recombinant proteins in the extracellular space, LLE can be applied directly to fermentation broth, enabling in situ or integrated extraction approaches (Kee et al., 2021).

Integration of Upstream and Downstream Processing

Current trends in the bioindustry are increasingly shifting toward integrated continuous bioprocessing. Efforts are focused on developing solutions at every stage of production to realise the fully integrated continuous bioprocessing concept (Jungbauer et al., 2024), as illustrated in Figure 1. However, literature regarding the continuous production of recombinant proteins using *B. subtilis* remains limited. Nevertheless, several studies have explored integrative approaches with *B. subtilis* for enzyme production, providing useful insights into potential strategies for continuous recombinant protein manufacturing. The following paragraph presents various applications of integrative approaches for protein production using *B. subtilis*. Kee et all (2021) implemented an in situ extractive approach for the production and recovery of *B. subtilis* xylanase. In their study, crude fermentation broth was directly fed to a biphasic (alcohol/salt) flotation unit during the bioprocess. The authors demonstrated that gas bubbles facilitated the partitioning process, with xylanase being retained in the upper alcohol phase. Muniasamy et all (2022) combined *B. subtilis* fermentation with in situ liquid-liquid extraction of a secreted fibrinolytic protease (FLP). In this process, shrimp waste hydrolysate served as a sustainable, low-cost substrate, while the FLP enzyme was concurrently extracted in a micellar two-phase system. Additionally, gel chromatography was used to quantify the amount of recovered enzyme, followed by final purification through anion exchange chromatography to achieve maximum purity and enzyme activity. Ng et all (2018) reported the direct extraction of xylanase enzyme from fermentation broth with alcohol/salt aqueous systems. Two earlier studies also highlight that interest in continuous biomanufacturing has existed for several decades. Stredanský et all (1993) demonstrated the simultaneous production and purification of *B. subtilis* α-amylase. Their bioreactor contained an aqueous two-phase system composed of PEG and dextran. During fermentation, culture medium was transferred to an external settler; following phase separation, the dextran-rich bottom phase, containing cells, was returned to the bioreactor, while the PEG-rich upper phase was pumped to an affinity column where the amylase was bound. The PEG flowthrough from the column was then recycled back into the bioreactor. Cooper et all (1981) developed a large-scale continuous bioprocess for the production of surfactin, a cyclic lipopeptide surfactant. In their system, surfactin accumulated in the foamed broth within the bioreactor, and the foam was collected in an external separator for product extraction via acid precipitation.

Challenges and Future prospects

In the bioindustry, continuous processing is the dominant approach for several applications, including wastewater treatment, composting, and specific bioenergy processes such as biogas and bioethanol production (Brethauer and Wyman, 2010; Matassa et al., 2016; Subramanian, 2017).

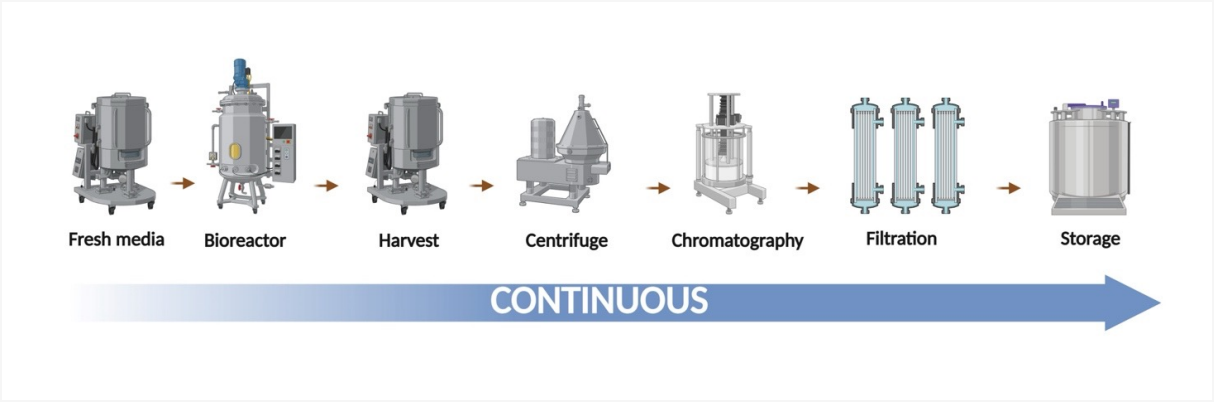


Figure 1. Fully integrated continuously manufacturing platform concept. Created in BioRender.

Slika 1. Koncept popolnoma integrirane platforme za neprekinjeno proizvodnjo. Ustvarjeno v BioRender.

Nevertheless, most industrial bioprocesses still operate in batch or fed-batch modes, with fed-batch currently being the predominant production strategy. Despite the potential advantages of continuous processing, several organism-specific challenges must be addressed for recombinant protein production in *B. subtilis* including control of sporulation and protease activity during prolonged cultivations, maintenance of genetic stability, and adaptation of oxygen transfer systems due to the aerobic nature of the microorganism (Chen et al., 2024). Strategies such as the use of sporulation-deficient strains, targeted protease knockouts, and optimized feeding strategies could help mitigate these limitations while supporting steady-state productivity (K. Zhang et al., 2018, 2020a). Furthermore, the integration of synthetic biology tools for strain stabilization, combined with advanced process monitoring under Quality-by-Design framework, may facilitate the consistent quality and yield of recombinant proteins during continuous production. In DSP, continuous strategies such as counter-current chromatography, continuous filtration, and integrated LLE systems are emerging to complement continuous upstream operations. These approaches can reduce buffer consumption, equipment footprint and overall processing times while increasing productivity and cost efficiency. However, implementing continuous DSP for *B. subtilis* remains challenging due to the complexity of fermentation broth and the secretion of host proteins that may co-elute with target products, requiring careful optimisation, as well as real-time monitoring and control. While challenges remain, ongoing advances in synthetic biology and process optimisation hold significant promise for facilitating the transition of the bioindustry towards continuous biomanufacturing.

Author Contribution

Conceptualization, A.I. and T.B.; investigation, A.I.; writing—original draft preparation, A.I.; writing—review and editing, A.I. and T.B.; supervision, T.B. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Conflicts of Interest

The authors declare no conflict of interest.

References

Brethauer, S., Wyman, C.E., 2010. Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. Bioresource Technology, 101(13), 4862–4874. https://doi.org/10.1016/J.BIORTECH.2009.11.009

Chen, Y., Li, M., Yan, M., Chen, Y., Saeed, M., Ni, Z., Fang, Z., Chen, H., 2024. *Bacillus subtilis*: current and future modification strategies as a protein secreting factory. World Journal of Microbiology and Biotechnology, 40(6). https://doi.org/10.1007/S11274-024-03997-X,

Cooper, D. G., Macdonald, C. R., Duff, S. J. B., Kosaric, N., 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Applied and Environmental Microbiology, 42(3), 408–412. https://doi.org/10.1128/AEM.42.3.408-412.1981;PAGE:STRING:ARTICLE/CHAPTER

Croughan, M. S., Konstantinov, K. B., Cooney, C., 2015. The future of industrial bioprocessing: Batch or continuous? Biotechnology and Bioengineering, 112(4), 648–651. https://doi.org/10.1002/BIT.25529

Csörgo, B., Fehér, T., Tímár, E., Blattner, F. R., Pósfai, G., 2012. Low-mutation-rate, reduced-genome *Escherichia coli*: An improved host for faithful maintenance of engineered genetic constructs. Microbial Cell Factories, 11(1), 1–13. https://doi.org/10.1186/1475-2859-11-11/FIGURES/7

Errington, J., van der Aa, L.T., 2020. Microbe profile: *Bacillus subtilis*: Model organism for cellular development, and industrial workhorse. Microbiology (United Kingdom), 166(5), 425–427. https://doi.org/10.1099/MIC.0.000922/CITE/REFWORKS

Evens, R.P., Pharm, B.S., 2022. Biotechnology – A Continual Revolution in Product Development and Healthcare – 1st 20 Years versus 2nd 20 Years and Beyond. Medical Research Archives, 10(4). https://doi.org/10.18103/MRA.V10I4.2719

Godawat, R., Konstantinov, K., Rohani, M., Warikoo, V., 2015. End-to-end integrated fully continuous production of recombinant monoclonal antibodies. Journal of Biotechnology, 213, 13–19. https://doi.org/10.1016/J.JBIOTEC.2015.06.393

Ji, S., Li, W., Xin, H., Wang, S., Cao, B., 2015. Improved Production of Sublancin 168 Biosynthesized by *Bacillus subtilis* 168 Using Chemometric Methodology and Statistical Experimental Designs. BioMed Research International, 2015(1), 687915. https://doi.org/10.1155/2015/687915

Jungbauer, A., 2013. Continuous downstream processing of biopharmaceuticals. Trends in Biotechnology, 31(8), 479–492. https://doi.org/10.1016/J.TIBTECH.2013.05.011

Jungbauer, A., Satzer, P., Duerauer, A., Azevedo, A., Aires-Barros, R., Nilsson, B., Farid, S., Goldrick, S., Ottens, M., Sponchioni, M., Marcelo Fernandez Lahore, H., 2024. Continuous downstream processing. Separation and Purification Technology, 338, 126439. https://doi.org/10.1016/J.SEPPUR.2024.126439

Kee, P.E., Cheah, L.S., Wan, P.K., Show, P.L., Lan, J.C.W., Chow, Y.H., Ng, H.S., 2021. Primary capture of *Bacillus subtilis* xylanase from crude feedstock using alcohol/salt liquid biphasic flotation. Biochemical Engineering Journal, 165, 107835. https://doi.org/10.1016/J.BEJ.2020.107835

Kergaravat, B., Plener, L., Castan, M., Grizard, D., Chabrière, E., Daude, D., 2025. Enzymes, Proteins, and Peptides as Promising Biosolutions for Crop Production. Journal of Agricultural and Food Chemistry, 73(19), 11546–11555. https://doi.org/10.1021/ACS.JAFC.5C01107/ASSET/IMAGES/LARGE/JF5C01107_0001.JPEG

Kittler, S., Müller, F., Elshazly, M., Wandrey, G.B., Klein, T., Daub, A., Spadiut, O., Kopp, J., 2025. Transferability of bioprocessing modes for recombinant protease production: from fed-batch to continuous cultivation with *Bacillus licheniformis*. BMC Biotechnology, 25(1), 1–13. https://doi.org/10.1186/S12896-025-00947-9/TABLES/4

Klausmann, P., Hennemann, K., Hoffmann, M., Treinen, C., Aschern, M., Lilge, L., Morabbi Heravi, K., Henkel, M., Hausmann, R., 2021. *Bacillus subtilis* High Cell Density Fermentation Using a Sporulation-Deficient Strain for the Production of Surfactin. Applied Microbiology and Biotechnology, 105(10), 4141–4151. https://doi.org/10.1007/S00253-021-11330-X/TABLES/1

Kopp, J., Kittler, S., Slouka, C., Herwig, C., Spadiut, O., Wurm, D.J., 2020. Repetitive Fed-Batch: A Promising Process Mode for Biomanufacturing With *E. coli*. Frontiers in Bioengineering and Biotechnology, 8, 573607. https://doi.org/10.3389/FBIOE.2020.573607/BIBTEX

Li, W., Zhou, X., Lu, P., 2004.Bottlenecks in the expression and secretion of heterologous proteins in *Bacillus subtilis*. Research in Microbiology, 155(8), 605–610. https://doi.org/10.1016/J.RESMIC.2004.05.002

Liu, H.F., Ma, J., Winter, C., Bayer, R., 2010. Recovery and purification process development for monoclonal antibody production. MAbs, 2(5), 480–499. https://doi.org/10.4161/MABS.2.5.12645;WGROUP:STRING:PUBLICATION

Lopez, D., Vlamakis, H., Kolter, R., 2009. Generation of multiple cell types in *Bacillus subtilis*. FEMS Microbiology Reviews, 33(1), 152–163. https://doi.org/10.1111/J.1574-6976.2008.00148.X,

Marrone, P.G., 2024. Status of the biopesticide market and prospects for new bioherbicides. Pest Management Science, 80(1), 81–86. https://doi.org/10.1002/PS.7403;PAGEGROUP:STRING:PUBLICATION

Matassa, S., Boon, N., Pikaar, I., Verstraete, W., 2016. Microbial protein: future sustainable food supply route with low environmental footprint. Microbial Biotechnology, 9(5), 568–575. https://doi.org/10.1111/1751-7915.12369;REQUESTEDJOURNAL:JOURNAL:17517915;WGROUP:STRING:PUBLICATION

Muniasamy, R., Balamurugan, B.S., Rajamahendran, D., Rathnasamy, S., 2022. Switchable deep eutectic solvent driven micellar extractive fermentation of ultrapure fibrin digesting enzyme from *Bacillus subtilis*. Scientific Reports, 12(1), 1–14. https://doi.org/10.1038/S41598-022-04788-W;SUBJMETA=1647,2188,2196,631;KWRD=ANALYTICAL+BIOCHEMISTRY,LIQUID

Ng, H.S., Chai, C.X.Y., Chow, Y.H., Loh, W.L.C., Yim, H. S., Tan, J.S., Lan, J.C.W., 2018. Direct recovery of *Bacillus subtilis* xylanase from fermentation broth with an alcohol/salt aqueous biphasic system. Journal of Bioscience and Bioengineering, 125(5), 585–589. https://doi.org/10.1016/J.JBIOSC.2017.12.010

Öztürk, S., Çalik, P., Özdamar, T. H., 2016. Fed-Batch Biomolecule Production by *Bacillus subtilis*: A State of the Art Review. Trends in Biotechnology, 34(4), 329–345. https://doi.org/10.1016/J.TIBTECH.2015.12.008

Patel, A. R., Mokashe, N. U., Chaudhari, D. S., Jadhav, A. G., Patil, U. K., 2019. Production optimisation and characterisation of extracellular protease secreted by newly isolated *Bacillus subtilis* AU-2 strain obtained from *Tribolium castaneum* gut. Biocatalysis and Agricultural Biotechnology, 19, 101122. https://doi.org/10.1016/J.BCAB.2019.101122

Peebo, K., Neubauer, P., 2018. Application of Continuous Culture Methods to Recombinant Protein Production in Microorganisms. Microorganisms, 6(3), 56. https://doi.org/10.3390/MICROORGANISMS6030056

Prabakaran, G., Hoti, S.L., 2008. Application of different downstream processing methods and their comparison for the large-scale preparation of *Bacillus thuringiensis* var. *israelensis* after fermentation for mosquito control. Biologicals, 36(6), 412–415. https://doi.org/10.1016/J.BIOLOGICALS.2008.06.001

Shih, K.S., Lin, C.C., Hung, H.F., Yang, Y.C., Wang, C.A., Jeng, K.C., Fu, H.W., 2013. One-Step Chromatographic Purification of *Helicobacter pylori* Neutrophil-Activating Protein Expressed in *Bacillus subtilis*. PLOS ONE, 8(4), e60786. https://doi.org/10.1371/JOURNAL.PONE.0060786

Stredanský, M., Kremnický, L., Sturdik, E., Fecková, A., 1993. Simultaneous production and purification of *Bacillus subtilis* α-amylase. Applied Biochemistry and Biotechnology, 38(3), 269–276. https://doi.org/10.1007/BF02916406

Subramanian, G., 2017. Continuous Biomanufacturing - Innovative Technologies and Methods. Continuous Biomanufacturing - Innovative Technologies and Methods. https://doi.org/10.1002/9783527699902

Vierheller, C., Goel, A., Peterson, M., Domach, M.M., Ataai, M.M., 1995. Sustained and constitutive high levels of protein production in continuous cultures of *Bacillus subtilis*. Biotechnology and Bioengineering, 47(5), 520–524. https://doi.org/10.1002/BIT.260470503;WGROUP:STRING:PUBLICATION

Vogel, J. H., Nguyen, H., Pritschet, M., Van Wegen, R., Konstantinov, K., 2002. Continuous annular chromatography: General characterization and application for the isolation of recombinant protein drugs. Biotechnology and Bioengineering, 80(5), 559–568. https://doi.org/10.1002/BIT.10411

Walther, J., Godawat, R., Hwang, C., Abe, Y., Sinclair, A., Konstantinov, K., 2015. The business impact of an integrated continuous biomanufacturing platform for recombinant protein production. Journal of Biotechnology, 213, 3–12. https://doi.org/10.1016/J.JBIOTEC.2015.05.010

Yang, H., Ma, Y., Zhao, Y., Shen, W., Chen, X., 2020. Systematic engineering of transport and transcription to boost alkaline α-amylase production in *Bacillus subtilis*. Applied Microbiology and Biotechnology, 104(7), 2973–2985. https://doi.org/10.1007/S00253-020-10435-Z/FIGURES/8

Yang, H., Qu, J., Zou, W., Shen, W., Chen, X., 2021. An overview and future prospects of recombinant protein production in *Bacillus subtilis*. Applied Microbiology and Biotechnology, 105(18), 6607–6626. https://doi.org/10.1007/S00253-021-11533-2

Zhang, K., Su, L., Wu, J., 2018. Enhanced extracellular pullulanase production in Bacillus subtilis using protease-deficient strains and optimal feeding. Applied Microbiology and Biotechnology, 102(12), 5089–5103. https://doi.org/10.1007/S00253-018-8965-X/FIGURES/6

Zhang, K., Su, L., Wu, J., 2020a. Recent Advances in Recombinant Protein Production by Bacillus subtilis. Annual Review of Food Science and Technology, 11, 295–318. https://doi.org/10.1146/ANNUREV-FOOD-032519-051750/CITE/REFWORKS

Zhang, Y., Nie, Y., Zhou, X., Bi, J., Xu, Y., 2020b. Enhancement of pullulanase production from recombinant *Bacillus subtilis* by optimization of feeding strategy and fermentation conditions. AMB Express, 10(1), 1–9. https://doi.org/10.1186/S13568-020-0948-5/FIGURES/6

News

FEMS MICRO Milan 2025 – 11. kongres evropskih mikrobiologov

Janez Mulec[1,*], Jerneja Ambrožič Avguštin[1]

14. – 17. julij 2025, Milano, Italija

FEMS-ov (Federation of European Microbiological Societies) kongres mikrobiologov, ki je bil letos v Milanu v Italiji, je privabil kar 1953 udeležencev iz 95 držav. Z več kot 100 udeleženci iz posamezne države so prišli predstavniki Nemčije, Italije, Južne Koreje, Španije in Združenega kraljestva. Tekom dogodka je bilo v treh dneh na ogled več kot 1400 posterjev. FEMS je tokrat finančno podprl udeležbo kar 300 raziskovalcem na začetku znanstvene kariere, kar se je odrazilo tudi v večjem številu mlajših predavateljev, vključno z doktorskimi študenti in podoktorskimi raziskovalci. Za plenarna predavanja je bil predviden čas eno uro, ostala predavanja so trajala 20, 10 ali 5 minut, zato so se morali predavatelji dobro potruditi, da so svoje znanstvene izsledke predstavili zgoščeno. Tokratni kongres je bil posvečen štirim tematskim sklopom. Prvi sklop, poimenovan »Eko inovacije (Eco Innovations)«, je bil posvečen raziskavam o zapletenem/ prepletenem medsebojnem delovanju med mikrobi in njihovim okoljem, tudi v povezavi s podnebnimi spremembami, urbanizacijo, ekosistemi in onesnaževanjem. Drugi sklop z naslovom »Obzorja zdravja (Health Horizons)« je bil namenjen predstavitvi raziskav o vlogi mikrobov in mikrobiomov, povezanih s človekovim zdravjem, vključno s predstavitvijo novih diagnostičnih in terapevtskih metod ter boja proti mikrobom, odpornih proti protimikrobnim učinkovinam in novo porajajočim patogenov. Prelomne mikrobne tehnologije, od sintetične biologije do trajnostnih rešitev, ki omogočajo preoblikovanje industrije in obravnavajo globalne probleme in izzive, so bile predstavljene v sklopu »Biotehnologija (Biotechnology)«. Zadnji, četrti sklop, z zanimivim naslovom »Vključevanje in rast (Engagement & Growth)«, pa je bil posvečen razvoju ključnih veščin za profesionalnega mikrobiologa, vključno s predstavitvijo možnih kariernih poti, financiranja znanstvenega komuniciranja ter podjetništva. Udeleženci kongresa so imeli možnost izbire prisostvovanja ozko specializiranim znanstvenim sekcijam posameznih sklopov, istočasno jih je potekalo do osem, med celotnim kongresom jih je bilo skupaj na voljo kar 82.

1 Karst Research Institute ZRC SAZU, Titov trg 2, SI-6230 Postojna, Slovenia

2 University in Ljubljana, Biotechnical Faculty, Dept. of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

*** Corresponding author:**
E-mail address: janez.mulec@zrc-sazu.si

Citation: Mulec, J., Ambrožič Avguštin, J., (2025). FEMS MICRO Milan 2025 – 11. kongres evropskih mikrobiologov. Acta Biologica Slovenica 68 (4)

https://doi.org/10.14720/abs.68.4.23502

Slika 1. Iz Slovenije je bilo na kongresu v Milanu s prispevki prijavljenih 35 udeležencev. Aktivno so na kongresu svoje raziskave predstavili na sliki od leve proti desni: Nurainina Binti Ayob (Univerza v Ljubljani), Maša Vodovnik (Univerza v Ljubljani), Marko Verce (Univerza v Ljubljani), Ines Mandić Mulec (Univerza v Ljubljani), Janez Mulec (Znanstvenoraziskovalni center SAZU), Jerneja Ambrožič Avguštin (Univerza v Ljubljani), Marjanca Starčič Erjavec (Univerza v Mariboru), Tadeja Vajdič (Univerza v Mariboru), Urša Miklavčič (Nacionalni laboratorij za zdravje, okolje in hrano), Sabina Mlakar (Nacionalni laboratorij za zdravje, okolje in hrano, Univerza v Mariboru).

Na kongresu so bila predstavljena številna nova dognanja. Na kratko lahko izpostavimo samo nekatera. Lwoffova nagrajenka za dosežke v mikrobiologiji Carmen Buchrieser je potegnila vzporednico mehanizma vstopa in razmnoževanja bakterije *Legionella pneumophila* v gostiteljski celici, ki je okoljska ameba, ali alveolarni makrofag. Pri tem igrajo ključno vlogo številni proteini legionele, ki so podobni evkariontskim, kar kaže na koevolucijo in horizontalne prenose genov.

Če si je kdo predstavljal, da so težave z odpornostjo proti protimikrobnim učinkovinam (AMR – Antimicrobial Resistance) problem samo bogatega sveta in bolnišničnega okolja, temu ni tako. Evolucijsko so se geni za AMR razvili z rekombinacijami in mutacijami in »raznesli« v različne bakterije in ekosisteme s horizontalnimi prenosi. Čeprav so bolnišnice še vedno ene od najpomembnejših vročih točk za razvoj in širjenje AMR v domača okolja in naravo, v tem pogledu ne zaostajajo čistilne naprave, ki omogočajo številne prenose tudi na neklinične seve in povečajo breme teh genov v naravnih ekosistemih. V tem pogledu se raziskuje tudi možen prenos tovrstnih genov na sadje in zelenjavo ter pitno vodo zaradi uporabe vode iz čistilnih naprav v kmetijstvu.

Da klasična mikrobiologija »ne izumira« in bo vedno aktualna, dokazuje zavedanje o fenotipski heterogenosti mikrobnih (čistih) kultur in s tem povezano tveganje pri interpretaciji, zlasti kliničnih rezultatov raziskav. Prav tako zanimivo je iskanje novih gojišč in pristopov, s katerimi bi lahko spoznali »mikrobno temno snov (microbial dark matter)«. Izraz, izposojen pri astrofizikih, je bil pogosto uporabljen v različnih sekcijah kongresa in opisuje mikrobe, katerih genetske sledi (sekvenciranje DNA, RNA) poznamo, vendar o njih in njihovi funkciji vemo malo ali nič, ker jih ne moremo gojiti in posledično raziskovati s klasičnimi metodami. Prav tako ne poznamo funkcije veliko genov, tudi kultivabilnih bakterij, vendar je za njihovo karakterizacijo eden, ali več izmed štirih pristopov »omik« (genomika, transkriptomika, proteomika, metabolomika) v veliko pomoč. Takšen celostni pristop je zlasti pomemben za boljše razumevanje mikrobne ekologije, saj ni dovolj vedeti zgolj kateri mikrob zaseda neko nišo, ampak zlasti kaj in koliko tam nečesa počne. Mikrobne interakcije so ključne za razumevanje mikrobne dinamike in fizioloških prilagoditev, kar so npr. izpostavili na primeru interakcije med probiotičnim in sporulirajočim sevom *Bacillus subtilis* ter patogenom *Salmonella typhimurium*, ali pa v primeru prevzemanja/ mikrobni kraji sideroforov med *Pseudomonas putida*, *Escherichia coli* in *Corynebacterium glutamicum*.

Atmosfera ne predstavlja samo način prenosa mikrobov, ampak tudi njihov habitat in vir energije, svetlobne, pa tudi kemijske v obliki plinov v sledovih, kot so vodik, ogljikov monoksid in metan, ki so sicer vedno prisotni. Atmosferski mikrobi kot rezultat svojih encimatskih reakcij proizvedejo vodo (1 milijon vodnih molekul v minuti v posamezni celici), ki je posebej pomembna za življenje v sušnih področjih, za nekatere mikrobe, ekstremne habitate. Na ekstremne

razmere za nekatere mikroorganizme, kot je npr. visoka slanost, ne naletimo zgolj v naravnih hiperslanih okoljih, ampak tudi v antropogenih, kot so zanimivo, lahko tudi klobase.

Odmevno je bilo uvodno plenarno predavanje z naslovom »Dinamika talnih mikroorganizmov v vesolju«. Predvideva se, da bi lahko talni mikroorganizmi igrali ključno vlogo pri osvajanju zunajzemeljskih okolij, npr. Marsa v smislu ustvarjanja primerne prsti za rast rastlin in užitnih pridelkov. Vendar se je treba zavedati, da nezemeljske razmere, npr. gravitacija, dostopnost vode, pomembno vplivajo na fiziološki odziv mikroorganizmov. V okviru astro(mikro)biologije ostaja še veliko odprtih vprašanj.

Veliko obeta koncept oziroma metoda za ocenjevanje geografske porazdelitve in raznolikosti mikrobiomov (npr. Microbiome Geographic Population Structure – mGPS) za mikrobne združbe, ki naseljujejo okolja kot so človeško telo, tla, voda ali zrak. Koncept temelji na predpostavki, da so mikrobiomi geografsko strukturirani. Potencialni primeri uporabe je forenzična mikrobiologija, npr. za določitev lokacije izvora bioloških delcev, ki lahko ključno vplivajo na zdravje in varnost ljudi. Da se svet in varnostne razmere spreminjajo, je dejstvo. Varnost, vključno z biološko varnostjo, postaja zato vedno bolj cenjena dobrina, kar smo občutili udeleženci kongresa tudi na lastni koži. Naj omenimo, da je bilo treba pred vstopom v predavalnice opraviti kar tri varnostne preglede (izkaz udeležbe pred vstopom na območje kongresnega centra, zaznavanje kovinskih predmetov pred vstopom v kongresno stavbo ter izkaz udeležbe pred vstopom na prizorišče kongresnih aktivnosti).

Na kongresu je bil viden trend drobljenja mikrobiologije po posameznih zelo specializiranih področjih, vendar je še vedno ključno ohranjati celosten pregled, medicinska ali okoljska mikrobiologija, mikrobov ne zanima. Vse je »ena mikrobiologija«.

FEMS je postal pomembna platforma za ustvarjanje povezav in izmenjavo informacij. Med drugim FEMS izdaja tudi več revij: FEMS Microbiology Letters, FEMS Microbiology Reviews, FEMS Microbiology Ecology, FEMS Pathogens and Disease, FEMS Yeast Research, FEMS Microbes, microLife. Kot spodbudo (mladim) udeležencem in vabilo k objavi rezultatov je vsaka od navedenih revij na koncu kongresa v Milanu podelila nagrado za poster oziroma ustno predstavitev s svojega področja.

Kot je bilo napovedano na zaključnem plenarnem zasedanju, bo naslednji, 12. kongres FEMS od 5. do 8. julija 2027 v Ljubljani, ki ga bo gostilo SMD – Slovensko mikrobiološko društvo. To je zelo pomenljivo, saj se skoraj četrt stoletja po prvem kongresu FEMS, le-ta ponovno vrača v mesto, kjer se je vse začelo – Ljubljana je namreč leta 2003 gostovala prvi kongres FEMS. Vabimo k udeležbi na kongresu, ki ne omogoča samo predstavitve našega dela širši mikrobiološki skupnosti »v živo«, ampak omogoča mladim raziskovalcem na začetku njihove karierne poti prve korake v svet znanstvenih srečanj, mreženja ter strokovne rasti.

Slika 2. Matjaž Ocepek z Veterinarske fakultete Univerze v Ljubljani, slovenski delegat pri FEMS-u, je na zaključni prireditvi sprejel zlati mikroskop, popotnico naslednjemu prireditelju kongresa, ki bo v Ljubljani od 5. do 8. julija 2027. Na sliki od leve proti desni: Tadeja Vajdič, Jerneja Ambrožič Avguštin, Marjanca Starčič Erjavec, Matjaž Ocepek, Ines Mandić Mulec, Marko Verce.

ABS

Acta Biologica Slovenica 2025 Vol. 68 | Št. 4