C O M M U N I C A T I O N S

FACULTY OF SCIENCES
UNIVERSITY OF ANKARA

DE LA FACULTE DES SCIENCES
DE L'UNIVERSITE D'ANKARA

Series C: Biology

VOLUME: 32 **Number: 1** **YEAR: 2023**

Faculty of Sciences, Ankara University
06100 Beşevler, Ankara-Türkiye
ISSN 1303-5991 e-ISSN 2618-6470

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S e r i e s C : B i o l o g y

Volume: 32 **Number: 1** **Year: 2023**

This Journal is published two issues in a year by the Faculty of Sciences, University of Ankara. Articles and any other material published in this journal represent the opinions of the author(s) and should not be construed to reflect the opinions of the Editor(s) and the Publisher(s).

Correspondence Address:
COMMUNICATIONS EDITORIAL OFFICE
Ankara University, Faculty of Sciences,
06100 Tandoğan, ANKARA – TÜRKİYE
Tel: (90) 312-2126720 Fax: (90) 312-2235000
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http://communications.science.ankara.edu.tr/index.php?series=A1

Print:
Ankara University Press
İncitaş Sokak No:10 06510 Beşevler
ANKARA – TÜRKİYE
Tel: (90) 312-2136655

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http://communications.science.ankara.edu.tr

Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 32, Number 1, Pages 1-13 (2023)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1135560

Research Article; Received: June 24, 2022; Accepted: December 27, 2022

COPPER(II) BIOREMOVAL BY THERMOPHILE *CYANOBACTERIUM APONINUM*

SEDA ŞEN[1], NUR KOÇBERBER KILIÇ[1], GÖNÜL DÖNMEZ[1]

[1]Department of Chemistry, Faculty of Science, Ankara University, Ankara, TÜRKİYE

ABSTRACT. In the current study, bioremediation of Cu(II) by thermophile *Cyanobacterium aponinum* has been studied in BG11 media under different conditions. The optimum pH was 9 due to the maximum Cu(II) bioremoval efficiency as 71% in the medium with12.8 mg/L Cu(II). According to the results obtained from the trials, the highest bioremoval was 76.6% in the medium including 9.7 mg/L Cu(II) for incubation period of 10 days. When the effect of increasing temperature (25-45 °C) and biomass [20% and 40% (v/v)] concentrations on bioremediation by *C. aponinum* was investigated, the highest heavy metal removal was found 75.8% at 45 °C, 12.8 mg/L Cu(II), and 20% (v/v) biomass concentration. It was 76.3% in the medium with 13.8 mg/L pollutant, 40% (v/v) biomass concentration. The q_m (maximum specific Cu(II) removal) was found as 6.1 mg/g at 45 °C in BG11 with 40% (v/v) biomass and 13.8 mg/L Cu(II). It has been concluded that Cu(II) bioremediation by thermophile *C. aponinum* was firstly investigated at various environmental conditions in this study. The results indicated that the tested cyanobacterium had a great potential to remove heavy metals from the aquatic environments, containing Cu(II).

1. INTRODUCTION

Water is an indispensable resource for life on earth. Access to clean water is extremely important for all living forms. Recently, water quality has decreased in consequence of industrial activities, rapid population growth, unplanned urbanization, and overuse of natural resources. The spread of activities such as agriculture, industry, urbanization, and population growth cause many pollutants that to be toxic to the environment [1].

Heavy metal pollution is a critical problem owing to their harmful properties to people, animals, and plants [2]. On the other hand, these metals are non-degradable and having possibility of accumulation in organisms. The most common heavy metals presenting in industrial wastewaters might be copper, nickel, lead, uranium, chromium, arsenic, zinc, silver, cadmium, and iron [3]. Wastewaters containing heavy metals, which is given to the nature without treatment, join the ecosystem and could be accumulated in various organs of organisms and create toxicity. Among these heavy metals, presence of copper in excess causes serious problems. Therefore, copper-contaminated wastewaters must be remediated before discharging them to the environment [4].

Keywords. Cyanobacterium aponinum, heavy metals, thermophile, bioremediation, wastewaters
sedasensa@gmail.com- 0000-0002-1187-2811
nur.kocberber@gmail.com- Corresponding author; 0000-0003-2668-3789
gdonmez@ankara.edu.tr- 0000-0001-7972-5570

There is a need for inexpensive, sustainable, and environmentally friendly methods for the bioremediation of metal-contaminated waters. Biological methods are preferred instead of chemical methods in heavy metal removal. Studies have shown that microorganisms with bioremediation capacity were very effective in removing metals from the environment [5]. Bioremediation is defined as the conversion of harmful and toxic substances by microorganisms into less toxic or non-toxic forms by microorganisms [6]. Prokaryotic and eukaryotic [7] microorganisms are used in the biological removal of heavy metals. Cyanobacteria are the largest photosynthetic prokaryotes and are capable of living in a variety of extreme habitats, from fresh and marine waters to terrestrial environments [8]. Cyanobacterial strains having a significant capability of heavy metal sorption were used in bioremediation processes [9]. Cyanobacteria have many features that make them preferred for the selective bioremoval and reduction of metals. These might be high removal capacity via absorption, tolerance to heavy metals by surviving in metal containing areas and having large surface area [10]. In recent years, extremophile microorganisms have attracted considerable interest. Among them, thermophile ones are preferred in many biotechnological applications.

Thermophile cyanobacteria have developed resistance pathways to adapt against several pollutants. These organisms have physiological and enzymatic properties to grow in undesirable conditions. These features make them as promising candidates for use in environmental biotechnological applications [11,12].

According to the previous studies, bioremediation of heavy metals done with cyanobacteria were mostly performed with mesophiles. De Philippis et al. [13] used *Cyanospira capsulata* and *Nostoc PCC7936* cyanobacteria producing exopolysaccharide for the bioremoval of heavy metals like nickel(II), copper(II), and zinc(II). In the same study, two cultures of cyanobacteria were shown to remove heavy metals, and it was stated that *C. capsulata* was more effective in bioremoval of heavy metals than other cyanobacteria, and was especially could tolerate Cu(II). In another study, the bioremoval of some metals [Zn(II), Cd(II), Cu(II), Pb(II)] by the secreted substances of *Cylindrospermopsis raciborskii* was investigated and it was found that the molecules secreted by the cyanobacteria showed the highest affinity for Cu(II) [14]. Heidari et al. [15] showed that when *Oscillatoria* sp. and *Leptolyngbya* sp. cyanobacteria were used as a consortium; consortia could remove chromium with the highest bioremoval capacity. In the same study, copper ions were removed with the lowest efficiency. In the study of Balaji et al., biological treatment of cadmium, chromium and lead heavy metals from leather industry wastewaters was carried out by Spirulina species [16]. In another study, Hazarika et al., showed bioremediation of heavy metals as Cu(II), Pb(II), Cd(II), and Zn(II) by *Noctoc muscorum* [17].

According to our knowledge is there is no study investigating Cu(II) bioremediation by thermophile *C. aponinum*. Therefore, the current study will be the first paper for the literature. The purpose of the study is to research Cu(II) bioremediation performance of thermophile *C. aponinum.* Effect of different environmental conditions such as pH, increasing metal concentrations, temperatures, and biomasses were studied on Cu(II) bioremediation capacity of *C. aponinum*.

2. MATERIALS AND METHODS

2.1. Cyanobacteria and culture conditions

C. aponinum was supplied from cyanobacteria collection of Biology Department, Faculty of Science, Ankara University [18]. Cyanobacterium was grown in containing 100 mL of BG11 media (pH 7) with using 250 mL Erlenmeyer [19] at 25 °C at a constant 2400 lx illumination in a climate cabinet [BINDER; KBW 400 (E5.1); 15–13640] for incubation period of 10 days.

2.2. Metal solution

Copper(II) stock solution was made by diluting $CuSO_4.5H_2O$ (Merck) to 1 g/L. The desired volumes of Cu(II) solutions were prepared from the stock Cu(II) solution.

2.3. pH Effect on Cu(II) bioremoval

pH media including nearly 10 mg/L Cu(II) was adjusted to 6, 7, 8, and 9 by using 1M NaOH and 1M HCL. Cu(II) concentrations were 10.8 mg/L (pH 6), 10.1 mg/L (pH 7), 9.8 mg/L (pH 8), and 12.8 mg/L (pH 9). Cyanobacterial cultures (20 mL) were added to 100 mL of media. After incubation for 2 days, the residual Cu(II) in the media was measured. Further trials were carried out at the optimal pH that was determined from these studies.

2.4. Effect of increasing Cu(II) concentration on Cu(II) bioremoval

In these trials, in the media at pH 9, the initial metal concentrations were 9.7, 14.2, and 22 mg/L. A 20% (v/v) biomass of cyanobacteria was inoculated into the prepared media. The remaining Cu(II) concentration was found after incubation for 10 day.

2.5. Effect of increasing temperatures and biomasses on Cu(II) bioremoval

For these experiments, different biomass concentrations [20% (v/v) and 40% (v/v)] of the tested cyanobacteria were used. Erlenmeyer flasks including approximately 15 mg/L Cu(II) at pH 9 were set at 25-45 °C.

In these experiments carried out with 20% (v/v) *C. aponinum* biomass, the concentration of Cu(II) was 15 mg/L at 25 °C, 14.6 mg/L at 35 °C, 12.8 mg/L at 45 °C. Copper(II) concentrations in media with 40% (v/v) biomass were 13.1 mg/L at 25 °C, 12.4 mg/L at 35 °C, and 13.8 mg/L at 45 °C. After incubation (10 days), the remaining Cu(II) concentrations were found.

2.6 Analytical methods

Three millilitres of samples were taken daily and centrifuged for 10 minutes at 6.000 rpm (Hettich®; EBA12; Germany) to remove the biomass. Microbial growth was followed by measuring the dry weight. The concentration of Cu(II) in the supernatant was found spectrophotometrically (Shimadzu UV 2001) at 460 nm by using sodium diethyl di-thiocarbamate as the complexing agent [20]. The BG11 medium without cell was used as the blank.

Copper(II) bioremoval yield was studied as a function of the pH, metal concentration, temperatures, and biomasses. The percentage of Cu(II) removal yield was found with Equation (1):

$$Y\% = \left(\frac{Co - Cf}{Co} \right) \times 100$$

Copper(II) bioremoval capacity can be measured based on the mass balance principle with Equation (2):

$$qm = [(Co — Cf)] / Xm$$

The maximal specific Cu(II) bioremoval (q_m) shows the maximal amount of heavy metal (mg) per unit dry weight of the cyanobacterial cells (g), the maximal dried cyanobacterial cell mass is *Xm* (g/L), and *Co* is the initial and *Cf* is the final concentration (mg/L) of Cu(II).

2.7. Statistical analysis

The results were subjected to analysis of the remarkable differences was performed by using variance method (ANOVA) and then compared by using standard deviations (±S.E.) The trials were done with two repetitions.

1. RESULTS AND DISCUSSION

3.1. Copper(II) bioremoval at different pH

The trials were performed in medium having with nearly 10 mg/L of Cu(II) at various pHs levels of 6, 7, 8, and 9 to determine appropriate pH level for the

maximum bioremoval of the pollutant (Table 1). Our results showed that, Cu(II) bioremoval by living *C. aponinum* biomass also increased depending on increase in pH. In the BG11 media including 12.8 mg/L Cu(II), the *C. aponinum* bioremoved heavy metal with the highest yield of 71% at pH 9. Due to the data obtained from these experiments, further trials were done in BG11 media at pH 9.As a result of statistical analysis, a significant difference was found in terms of bioremediation efficiency by using different pH.

Zinicovscaia et al. [21] studied the bioremoval of various metals like Zn, Cr, Cu, Fe, and Ni by *Spirulina platensis* in natural wastewaters. In that study, it was underlined that Cu(II) is in hydroxide form at pH 9.5, and therefore, metal bioremoval can only be done on the cell surface via microprecipitation. Sen et al. [22] showed that a consortium of *Limnococcus limneticus* and *Leptolyngbya subtilis* cyanobacteria removed 15 mg/L Cr(VI) with the highest efficiency at pH 9. In our study, *C. aponinum* had also the highest metal bioremoval at pH 9 as supported by the previous studies.

TABLE 1. Cu(II) bioremoval by *C. aponinum* at various pH (C_o: initial Cu(II) concentration, Y%: Cu(II) bioremediation yield; Biomass concentration: 20% (v/v); pH: 6–9; Temperature: 25 °C; Light intensity: 2400 lx; Incubation: 2 days; $p = 0.000$, F for different pH and yields: 98.3).

pH	Cu(II)	
	C_o (mg/L)	**Y%**
6	10.8	47.0 ± 1.0
7	10.1	57.0
8	9.8	66.0 ± 1.0
9	12.8	71.0

3.2. Copper(II) bioremoval at different pollutant concentrations

The trials were performed in BG11 media including nearly 10, 15, and 20 mg/L pollutants to investigate Cu(II) bioremoval by *C. aponinum*.

C. aponinum removed the applied 9.7 mg/L Cu(II) with a bioremoval yield as 61.6% after incubation for 3 days (Figure 1). In the same media, bioremediation of Cu(II) reached the maximum efficiency as 76.6% at the end of 10 days. In media including 14.2 mg/L Cu(II), the tested cyanobacterium removed 61.5% of the pollutant after incubation for 3 days; bioremoval yield was 71.5% after incubation for 10 days.

When the Cu(II) concentration was 22 mg/L, *C. aponinum* was successfully removed the applied pollutant with a yield as 71.0% at the end of the incubation period.

Panta et al. [23] used *A. variabilis* GITAM RGP to remove Cu(II) and other heavy metals from different wastewaters; they found 87.5% Cu(II) bioremoval efficiency at a very low metal concentration as 1.93 mg/L Cu(II). Fawzy [24] investigated the bioremoval of Cu(II) and cadmium(II) by *Merismopedia tenuissima;* the bioremoval of Cu(II) was 52% in the medium containing 1.2 mg/L Cu(II). In the same study, it was determined that the bioremoval efficiency decreases with the rise of metal concentration. In the present study, there was a slight decline in bioremoval efficiency with a rise in heavy metal concentration.

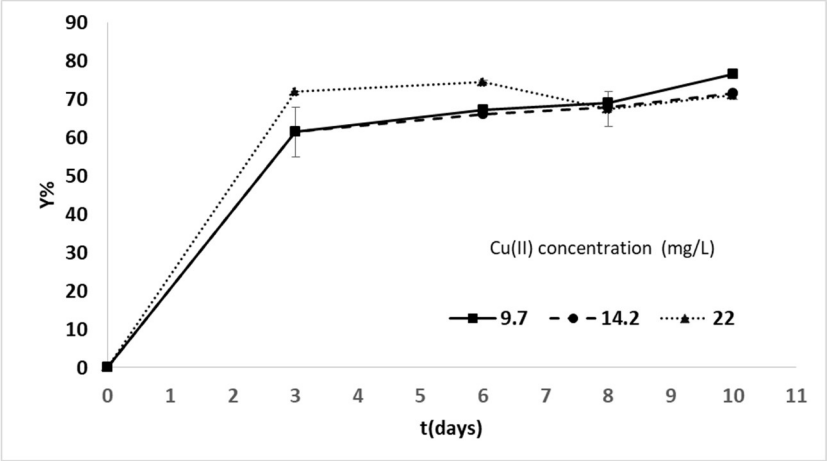

FIGURE 1. Cu(II) bioremoval by *C. aponinum* at increasing Cu(II) concentrations (Biomass concentration: 20% (v/v); Light intensity: 2400lx; pH 9; Temperature: 25 °C; Incubation: 10 days; $p < 0.05$, F for increasing Cu(II) concentrations and days: 0.229).

Cyanobacterial growth was not inhibited in the medium with increasing Cu(II) concentrations and was found at similar rates. However, as the metal concentration increases, the number of functional groups to which the metal is attached is similar. According to a previous study, there is a certain saturation capacity in removing metals from the environment and metal bioremoval capacity becomes saturated after a certain concentration of the pollutant [25]. In our study, as the metal concentration is increased, the number of functional groups to which the metal will be attached was similar in all tested media. Therefore, Cu(II) bioremoval slightly decreased in the media with increasing heavy metal. In addition, according to statistical data, when the experiments with increasing copper concentrations were evaluated, there was no significant difference between the concentrations in terms of removal efficiency.

In the current study, we found higher efficiency of pollutant removal at higher initial Cu(II) concentrations than the mentioned studies, and heavy metal bioremediation was achieved above 71% in the media with all the Cu(II) concentrations tested.

3.3. Copper(II) bioremoval at different temperatures and biomass concentrations

In the media including 15 mg/L Cu(II) at 25 °C, the highest Cu(II) bioremoval was 70.5% in 20% (v/v) biomass-samples after 10 days incubation (Figure 2). With the increment in temperature, Cu(II) bioremoval rose to 72.6% in 20% (v/v) biomass and 14.6 mg/L Cu(II) after 10 days incubation. At 45 °C, there was a slight increment of the bioremediation yield, and the percentage of efficiency was 75.8% after incubation for 10 days.

The increment of biomass from 20% (v/v) to 40% (v/v), Cu(II) bioremoval yield increased from 70.5% to 71.5% in medium with 13.1 mg/L heavy metal at 25 °C after incubation for 10 days (Figure 3). In the media at 35 °C, the maximum Cu(II) bioremoval was 72.9 % in the BG11 media having 40% (v/v) biomass and 12.4 mg/L Cu(II) after incubation for 10 days. The bioremoval yield was found as 76.3% in the medium at 45 °C with 40% (v/v) biomass and 13.8 mg/L heavy metal concentration after incubation for 10 days.

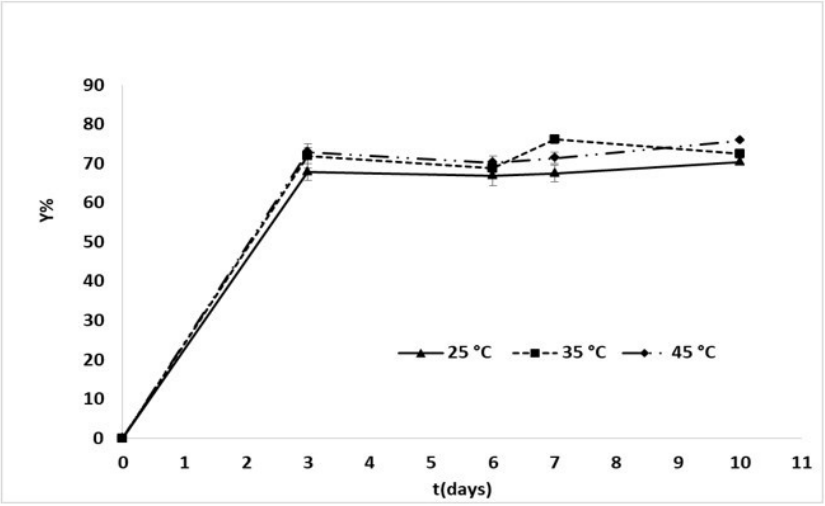

FIGURE 2. Cu(II) bioremoval by *C. aponinum* at increasing temperatures (C_o-25 °C:15 mg/L, C_o-35 °C: 14.6 mg/L, C_o-45 °C: 12.8 mg/L; Biomass concentration: 20% (v/v; Light intensity: 2400lx; pH 9; Temperatures: 25-45 °C; Incubation: 10 days; $p < 0.05$, F for increasing temperatures and days: 4.18).

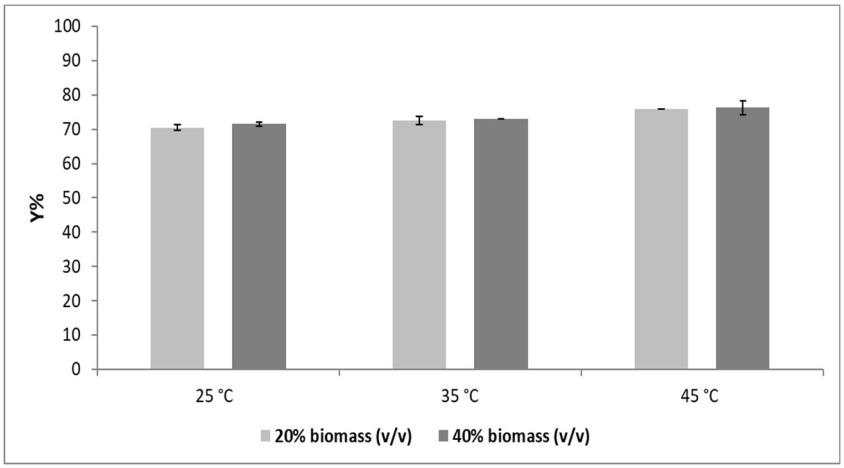

FIGURE 3. Comparison of Cu(II) bioremoval in BG11 with 20% (v/v) and 40% (v/v) biomass (C_o: nearly 15 mg/L; Light intensity: 2400lx; pH 9; Temperatures: 25-45 °C; Incubation: 10 days; $p < 0.05$, F for increasing biomass and yields: 0.102).

Like in the current study, Zinicovscaia et al. [26] emphasized that the bioremoval efficiency did not increase at the same rate by increasing the amount of cyanobacterial biomass from 10 g/L to 60 g/L. In that study, with using 6 times higher biomass, efficiency percentage only increased from 61% to 83%. Due to the statistical results, there was no meaningful difference for bioremoval efficiencies between 20% and 40% biomass at the mean 0.05 significance level.

In the medium containing approximately 15 mg/L Cu(II), the heavy metal bioremoval of cyanobacteria increased with the increase of the temperature from 25 to 45°C. Cyanobacterial growth was not affected negatively from the temperature increment and the growth rate of *C. aponinum* was similar at all the tested temperatures in the media with pollutant. According to the statistical data, a significant difference was found when bioremediation efficiencies at 25 °C compared with the trials performed at other temperatures.

The optimum growth temperature for *C. aponinum* has been previously shown to be between 35-40 °C in contaminant-free environments [27, 28]. In the current study, when *C. aponinum* was exposed to two stress conditions such as heavy metal and temperature, the cyanobacterium continued its growth in that media and performed effective heavy metal bioremoval. Cyanobacteria are known to synthesize small cysteine-ric proteins called metallothionein, which can bind toxic heavy metals [29]. In our study, *C. aponinum*, which was exposed to temperature stress in heavy metal-containing media, may have increased heavy metal bioremoval by synthesizing these types of proteins.

TABLE 2. The q_m values in the media with different biomass concentrations and temperatures (Biomass concentration: 20% (v/v), 40% (v/v); pH: 9; Light intensity: 2400 lx; Incubation: 10 days; in media with 20% (v/v) biomass, $p < 0.05$, F for at different temperatures and q_m: 14.9; in media with 40% (v/v) biomass, $p < 0.05$, F for at different temperatures and q_m: 14.9).

20% biomass (v/v)		
T (°C)	**C_o (mg/L)**	**q_m (mg/g)**
25	15.0	12.3 ± 0.4
35	14.6	13.5 ± 0.3
45	12.8	11.9 ± 0.01
40% biomass (v/v)		
T (°C)	**C_o (mg/L)**	**q_m (mg/g)**
25	13.1	5.3 ± 0.2
35	12.4	5.6 ± 0.6
45	13.8	6.1 ± 0.2

In the medium with 15 mg/L Cu(II), %20 (v/v) biomass at 25 °C, the q_m was 12.3 mg/g. In %20 (v/v) biomass-samples at 35 °C and 45 °C, the q_m values were 13.5 mg/g and 11.9 mg/g in the medium with 14.6 mg/L and 12.8 mg/L Cu(II), respectively. In BG11 medium with 40% (v/v) biomass and 13.1 mg/L Cu(II), q_m was 5.3 mg/g at 25 °C. Copper bioremoval per one gram of the *C. aponinum* biomass was 5.6 mg/g in the medium with12.4 mg/L Cu(II) and 40% (v/v) biomass at 35 °C. The q_m was 6.1 mg/g in samples with 13.8 mg/L Cu(II) and 40% (v/v) biomass at 45 °C.

When the q_m values were evaluated statistically in the experiments carried out at increasing temperatures and biomasses, a significant difference was found.

Copper(II) bioremovals per one gram of the cyanobacterial biomass increased in samples with increasing temperatures. In the BG11 with Cu(II), bioremoval yields increased with an increase in temperature, therefore, the maximum specific heavy metal bioremovals increased. The maximum specific Cu(II) bioremovals were found to be lower in samples with 40% biomass (v/v) compared to samples with 20% biomass (v/v). Since the biomass is doubled, a decrease in Cu(II) removal per 1 gram of biomass is an expected result. There was one exception in the 20% (v/v) biomass-samples at 45 °C that q_m decreased to 11.9 mg/g. This is because there was slightly more biomass at 45 °C than any other temperature tested. Thus, Cu(II) bioremoval per 1 gram of the biomass decreased. The maximum specific Cu(II) bioremovals were found to be lower in samples with 40% (v/v) biomass compared to samples with 20% (v/v) biomass.

Similar bioremoval percentages were found in the media with different biomass concentrations, therefore, q_m decreased.

At the end of these experiments, it was showed that an increment in the biomass concentration of *C. aponinum* and temperature had a favorable effect on the bioremediation of Cu(II). Statistical analysis showed that Cu(II) bioremediation by *C. aponinum* was mostly affected by pH and temperature.

4. CONCLUSIONS

In the current study, thermophile *C. aponinum* was used to bioremove Cu(II) on harsh conditions like alkaline media and high temperatures. The highest Cu(II) bioremoval by *C. aponinum* was found at pH 9. *C. aponinum* could grow at all the applied temperatures (25-45 °C) and it also had bioremoval capacity in samples with Cu(II). In BG11 medium with 13.8 mg/L Cu(II) and 45 °C, *C. aponinum* biomass [40% (v/v)] had the maximum bioremoval yield as 76.3%. The maximum specific Cu(II) bioremoval (q_m) was 13.5 mg/g in samples containing 14.6 mg/L Cu(II) and 20% (v/v) at 35 °C.

The results demonstrated that the thermophile *C. aponinum* tested in the present study had bioremediation capacity for the removal of Cu(II) from water. It was concluded that thermophile *C. aponinum* is a remarkable biologic sorbent with efficient heavy metal bioremediation capacity that might be applied in biological treatment of wastewaters.

Acknowledgement. The financial support was provided by the Ankara University Research Fund (Project No. 21L0430005).

Author Contribution Statements SŞ- data collection, management, and manuscript writing. NKK- project development, data analysis, manuscript editing, manuscript writing. GD- project development, data analysis. All authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest.

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http://communications.science.ankara.edu.tr

Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 32, Number 1, Pages 14-23 (2023)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1185771

Research Article; Received: October 22, 2022; Accepted: February 26, 2023

SEXUAL DIMORPHISM IN THE LACERTID LIZARD *Apathya cappadocica* (WERNER, 1902) (Reptilia: Lacertidae) FROM SOUTHEASTERN TÜRKİYE

KAMİL CANDAN[1,2], ELİF YILDIRIM CAYNAK[1,2], YUSUF KUMLUTAŞ[1,2], OSMAN SİNAN GÜNİSTER[1], ÇETİN ILGAZ[1,2]

[1]Dokuz Eylül University, Faculty of Science, Department of Biology, İzmir 35390, TÜRKİYE
[2]Dokuz Eylül University, Fauna and Flora Research and Application Center, İzmir 35390, TÜRKİYE

ABSTRACT. *Apathya cappadocica* is a medium-sized lizard species included in the family Lacertidae. The species is known from Türkiye, Northern Syria, Iraq, and Western Iran. Sexual dimorphism (SD), which is a phenomenon including phenotypic differences between males and females, has many effects on behaviour, shape and size characteristics. A total of 87 adult lizard specimens collected from south-eastern Anatolia were used in this study. The results of ANOVA showed that all morphometric features exhibited a pattern in which males have larger size than females. According to principal component analysis (PCA), the first three factors explain 81.553% of the total variance. Differences in head size between sexes have been well-documented in lizards and are associated with male-male aggression which results in mating success. This kind of pattern is supported by this study. Additionally, the results showed that males have more femoral pores, which is a signalling mechanism for sexual selection, than females. In conclusion, it can be accepted that the species in the genus *Apathya* has a similar pattern in accordance with the larger male theory.

1. INTRODUCTION

Apathya cappadocica (Werner, 1902), the Anatolian lizard, is a medium-sized lizard species included in the family Lacertidae. The species is known from Central, East, and South Anatolia, Northern Syria, Iraq, and Western Iran [1-3]. Although systematic, phylogenetic and ecological studies about the species group have been carried out, evolutionary evaluations including different aspects, such as sexual dimorphism are scarce [1,2,4-6]. Sexual dimorphism (SD) is a common phenomenon based on phenotypic differences between males and females of a species [7-9]. It consists of three main categories: (i) sexual size dimorphism (SSD), (ii) SD in body shape and (iii) SD in ornamentation [10-12]. Sexual size dimorphism (SSD), which was reported in lacertid lizards, is a widespread biological event in nature [5,13-16]. In most lizards, males have larger morphological features providing physical advantages than compared to

Keywords. Apathya cappadocica, lizard, sexual dimorphism, morphology
kamil.candan@deu.edu.tr- Corresponding author; 0000-0002-6934-3971
yildirim.elif@deu.edu.tr- 0000-0001-9614-5754
yusuf.kumlutas@deu.edu.tr- 0000-0003-1154-6757
sinan.gunister@outlook.com- 0000-0002-4483-8097
cetin.ilgaz@deu.edu.tr- 0000-0001-7862-9106

females [17-19]. In the current study, we investigated SSD in *A. cappadocica* considering morphological characteristics for the first time.

2. MATERIALS AND METHODS

A total of 87 adult (38 males and 49 females) lizard specimens, which were collected during herpetological field surveys from south-eastern Anatolia between 2001 and 2006, were obtained from the Zoology Lab of the Department of Biology in the Faculty of Science, Dokuz Eylül University. The localities of the collected specimens are given in Table 1.

TABLE 1. Localities of samples used in this study.

No	Locality	Sample Number and Sex
1	Damlacık Village, Viranşehir, Şanlıurfa, Türkiye	3 males, 5 females
2	Keklikoluk Village, 20 km N of Göksun, Kahramanmaraş, Türkiye	1 male, 2 females
3	Çermik, Diyarbakır, Türkiye	1 male, 6 females
4	between Diyarbakır and Siverek 48. km, Diyarbakır, Türkiye	2 males, 2 females
5	between Şambayat and Besni 4. km, Adıyaman, Türkiye	3 males, 1 female
6	Tek tek Mountain, Şanlıurfa, Türkiye	1 male, 4 females
7	Halfeti, Şanlıurfa, Türkiye	3 males, 3 females
8	between Bitlis and Tatvan 13. km, Bitlis, Türkiye	1 male, 3 females
9	between Birecik and Halfeti 16. km, Şanlıurfa, Türkiye	1 male, 2 females
10	between Şanlıurfa and Viranşehir 32. km, Şanlıurfa, Türkiye	1 male, 4 females
11	Gerger, Adıyaman, Türkiye	3 males, 5 females
12	between Besni and Gölbaşı 5 km, Adıyaman, Türkiye	2 males
13	between Şanlıurfa and Bozova 33. km, Şanlıurfa, Türkiye	1 male
14	Hasankeyf, Batman, Türkiye	9 males, 3 females
15	Acar village, Kilis, Türkiye	1 male
16	between Batman and Hasankeyf 13. km, Batman, Türkiye	5 males, 5 females
17	Küçükalanlı village, Şanlıurfa, Türkiye	2 females
18	Küplüce village, Kilis, Türkiye	1 female
19	Ballık village, Yavuzeli, Gaziantep, Türkiye	1 female

Metric measurements were taken using a digital calliper with a sensitivity of 0.01 mm, and pholidosis characteristics were counted under a stereo microscope. Mensural and meristic data were recorded following the system of [20] and [21].

Eleven morphometric and sixteen meristic characters were examined for all adult specimens. The following metric measurements were taken: snout-vent length (SVL), tail length (TL), width of pileus (PW), length of pileus (PL), width of head (HW), length of head (HL), length of forelimb (FLL), length of hindlimb (HLL), length of parietal (PAL), width of occipital (OCW), width of internasal (INW). The meristic (pholidosis) characteristics are as follows: number of postnasals (PON), number of sublabial scales on the left side (SBLL), number of sublabial scales on the right side (SBLR), number of supraciliary scales on the left side (SPCL), number of supraciliary scales on the right side (SPCR), number of supraciliary granules on the left side (SCGL), number of supraciliary granules on the right side (SCGR), number of palpebral scales (PPL), dorsalia (DS), gularia (GU), ventralia longitudinal (VL), preanale (PAN), number of femoral pores on the left side (FPL), number of femoral pores on the right side (FPR), number of subdigital lamellae on the left side (SDLL), number of subdigital lamellae on the right side (SDLR).

All statistical analyses were conducted using Statistical Package for the Social Sciences SPSS v24. Means, standard error of the mean, minimum and maximum values for each variable were calculated. To determine the characteristics that contribute to the discrimination of sexes, analysis of variance (ANOVA) were performed since all variables exhibited normal distribution. Considering the results of ANOVA, one morphometric and six meristic characteristics were discarded because they were uninformative. Finally, principal component analysis (PCA) was performed to detect the size of variation between sexes at the multivariate level using the remaining informative variables (ten for both morphometric and meristic). The significance level for all statistical tests was set at 0.05.

3. RESULTS

The results of ANOVA showed that all morphometric features exhibited a pattern in which males had larger size than females (Table 2). Among these characteristics, SVL alone did not vary depending on sex considering the significance level (p=0.441, Table 4), while the other features appear to have significant differences (all p values are less than 0.05, Table 2). Similarly, males had larger values for many metric characteristics than females, except for PON, VL and SBLL. PON and VL in females were significantly larger than males and provided discrimination when considering sex-dependent variety (p=0.008 and F=7.344 for PON, p=0.000 and F=13.603 for VL, Tables 3 and 4).

TABLE 2. Results of the one-way ANOVA test for metric characteristics for both sexes. SEM: Standard. Error Mean, D of d: Direction of difference, Min: Minimum value; Max: Maximum value. All measurements are shown in millimetres.

SEX	♂ (n=38)				♀ (n=49)				D. of d.	F-value	P-value
	MEAN	SEM	MIN	MAX	MEAN	SEM	MIN	MAX			
TL	130.82	3.604	86.00	176.00	107.88	2.119	70.00	155.00	M>F	33.205	0.000
PW	7.48	0.18582	5.62	10.00	6.6645	0.09152	5.28	7.92	M>F	17.728	0.000
PL	17.2005	0.41791	13.16	22.22	14.9555	0.20583	11.88	17.70	M>F	26.562	0.000
HW	9.9347	0.27415	7.18	13.00	8.5371	0.14245	6.70	10.84	M>F	23.164	0.000
HL	18.8458	0.43243	14.54	24.02	16.44	0.20822	13.40	19.36	M>F	28.856	0.000
FLL	24.5763	0.42873	20.34	29.00	22.4078	0.27267	18.54	26.34	M>F	19.743	0.000
HLL	39.3395	0.82325	31.30	47.58	35.5155	0.41803	28.62	41.56	M>F	19.504	0.000
PAL	5.7784	0.16429	4.04	7.54	4.8682	0.08187	3.58	6.14	M>F	28.063	0.000
OCW	2.0374	0.07384	1.22	3.08	1.7306	0.04393	1.02	2.64	M>F	14.026	0.000
INW	2.9295	0.07757	2.14	4.04	2.7024	0.04728	1.92	3.36	M>F	6.835	0.011

TABLE 3. Results of one-way ANOVA test for meristic characteristics for both sexes. SEM: Standard. Error Mean, D of d: Direction of difference, Min: Minimum value; Max: Maximum value. All measurements are shown in millimetres.

SEX	♂ (n=38)				♀ (n=49)				D. of d.	F-value	P-value
	MEAN	SEM	MIN	MAX	MEAN	SEM	MIN	MAX			
PON	2.39	0.104	1	3	2.73	0.076	2	4	F>M	7.344	0.008
SPCL	6.61	0.167	5	9	6.22	0.089	5	8	M>F	4.578	0.035
PPL	5.84	0.179	4	8	5.33	0.128	4	9	M>F	5.769	0.018
GU	27.74	0.339	24	34	26.45	0.235	23	30	M>F	10.351	0.002
VL	25.61	0.201	24	28	26.45	0.127	25	28	F>M	13.603	0.000
PAN	5.63	0.157	4	8	5.08	0.108	4	7	M>F	8.801	0.004
FPL	22.24	0.228	18	25	20.61	0.204	17	23	M>F	28.161	0.000
FPR	22.37	0.240	18	26	20.69	0.217	17	23	M>F	26.673	0.000
SDLL	24.66	0.283	22	29	23.27	0.212	20	28	M>F	16.176	0.000
SDLR	24.79	0.318	22	30	23.27	0.214	21	28	M>F	16.914	0.000

TABLE 4. Results of one-way ANOVA test showed no significant in metric (**bold**) and meristic (no bold) characteristics for both sexes. SEM: Standard. Error Mean, D of d: Direction of difference, Min: Minimum; Max: Maximum.

SEX	♂ (n=38)				♀ (n=49)				D. of d.	F-value	P-value
	MEAN	SEM	MIN	MAX	MEAN	SEM	MIN	MAX			
SVL	68.8526	1.52251	53.60	85.86	67.4988	0.99041	51.72	82.36	M>F	0.599	0.441
SBLL	6.34	0.087	5	7	6.37	0.081	5	8	F>M	0.045	0.833
SBLR	6.53	0.098	6	8	6.47	0.078	6	8	M>F	0.213	0.646
SPCR	6.63	0.166	5	9	6.33	0.107	5	8	M>F	2.580	0.112
SCGL	13.84	0.363	7	19	13.39	0.247	11	19	M>F	1.143	0.288
SCGR	13.82	0.365	9	18	13.24	0.300	6	18	M>F	1.487	0.226
DS	62.58	0.485	58	69	61.92	0.416	56	68	M>F	1.074	0.303

PCA was performed to compare sexes at a multivariate level using all morphological variables which were standardized to obtain an unbiased statistical analysis (Figure 1). Considering the results of PCA, PON was not correlated with other characteristics, and SPCL, PPL and PAN which had less than 0.50 values of communalities and were discarded. According to PCA, the first three factors explained 81.553% of the total variance (Table 5). The most crucial contributors to the first component (PC1), which explained 54.043%, were determined to be PL, PW, HL, HW, FLL, HLL and PAL, while SDLL, SDLR, FPL, FPR and GU were the main contributors to the second component (PC2) which explained 20.684%. Also, 6.868% of variance was explained by PC3 that included VL, FPL and FPR, which play the most important roles (Table 5).

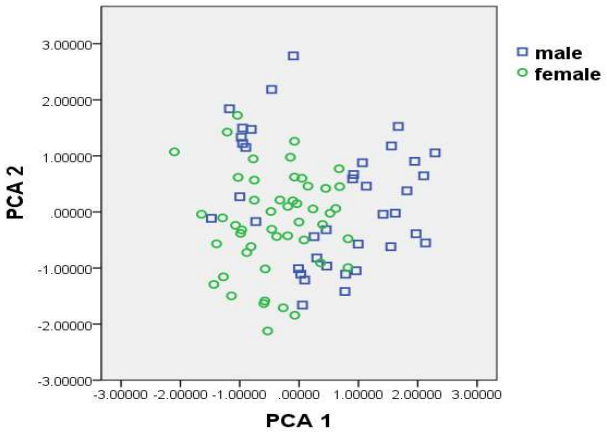

FIGURE 1. Ordination of individual males and females of *Apathya cappadocica* on the first two principal components.

TABLE 5. Loadings from principal component analysis of metric and meristic characteristics. Variables in bold represent strong loadings.

Variables	PC1	PC2	PC3
TL	**.718**	.296	-.055
PW	**.948**	-.036	.121
PL	**.986**	.039	.078
HW	**.972**	.023	.067
HL	**.985**	.042	.056
FLL	**.932**	.056	.030
HLL	**.969**	-.017	-.022
PAL	**.940**	.030	.050
OCW	**.753**	.098	.023
INW	**.888**	-.100	.142
GU	-.099	**.717**	.385
VL	**-.461**	.276	**.590**
FPL	.097	**.733**	**-.523**
FPR	.100	**.804**	**-.440**
SDLL	-.180	**.843**	.184
SDLR	-.156	**.841**	.196
Eigenvalues	8.647	3.309	1.092
% of Variance	54.043	20.684	6.826
Cumulative %	54.043	74.727	81.553

4. DISCUSSION

To compare SD in reptiles, scientists believe that body size should be used as a potential determinant and indicator of reproductive output [5,15,22,23]. Rensch's rule, which is a common perspective for SSD, stated there were two major issues: (i) females are prone to be larger than males in small species, whereas males are larger than females in large species and (ii) SSD increases with size when males are the larger sex and decreases with size when females are the larger sex [5,14,22,24-28]. In most lizards, males represent the larger body size, which is related to physical advantages that are critical for territorial defence and mating success [17-19]. Combat success between males is generally positively correlated with larger body size [15,29].

Considering morphometric characteristics which are closely related to body size, our results showed that males have larger size than females (Tables 2 and 3). Although a statistically significant difference could not be obtained, males were relatively larger than females in terms of SVL. Head size, another important size characteristic, showed that males were significantly larger than females (Table 2). Differences in head size between sexes were well-documented in lizards and

associated with male-male aggression which results in mating success [30-33]. Males with larger heads may produce greater bite force and they can use this to provide a mating advantage by repelling other males [13,34,35]. This kind of pattern is also supported by our study considering HW, HL, PW and PL are larger in males than females (Table 2). Considering another lizard species in the same genus, *A. yassujica*, the species has a similar pattern based on the larger male theory [5].

As another important point of discrimination between sexes, femoral pores are also useful characteristics in lacertid lizards [15, 23]. Femoral pores are clear and large in males because of holocrine secretion which is a signalling mechanism for sexual selection during the reproductive period [36-38]. Our results showed that males (mean for left/right side: 22.24/22.37) have significantly more femoral pores than females (mean for left/right side: 20.61/20.69) (Table 3). Similar results were found for *A. yassujica* [5]. Based on these conditions, species in the genus *Apathya* exhibit the expected pattern for lacertids.

Author Contribution Statements KC- conceptualization, analysis, writing, review and editing EYC- validation, writing ÇI and YK- resources OSG- analysis.

Declaration of Competing Interests The authors declare no conflict of interest.

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http://communications.science.ankara.edu.tr

Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 32, Number 1, Pages 24-35 (2023)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1184290

Research Article; Received: October 4, 2022; Accepted: January 26, 2023

AN INVESTIGATION OF PSEUDOMONAS MARINCOLA TO DETERMINE PB(II) BIOSENSOR POTENTIAL

CİHAN BOSTANCI[1], HASAN NAZIR[2], GÖNÜL DÖNMEZ[1]

[1]Department of Biology, Faculty of Science, Ankara University, Ankara, TÜRKİYE
[2]Department of Chemistry, Faculty of Science, Ankara University, Ankara, TÜRKİYE

ABSTRACT. Biosensors are strong alternatives to conventional analytical techniques such as HPLC and spectroscopic techniques for water quality and heavy metal detection. Heavy metal contaminated waters can monitor by microbial biosensors efficiently. For this purpose, newly isolated *Pseudomonas sp.* is used to develop a highly sensitive low-cost microbial biosensor for water quality monitoring. The objective of the study is the invention of new high sensitive low-cost microbial biosensors to determine heavy metals in aqueous solutions and optimise the working conditions *Pseudomonas marincola* cells were embedded onto the Screen Printed Electrode (SPE) carbon surface and dried for 30 minutes at laminar flow cabinet. Developed microbial sensors were immersed into the Pb(II) solution for electrochemical analysis. After the exposure time, cyclic voltammetry (CV) and differential pulse voltammetry (DPV) analyses were carried out. The study shows that the sensor was found in a linear range between $1x10^{-8}$ M and $8x10^{-8}$ M, with the lowest detection limit 10^{-9} M. The optimum pre-concentration time and scan rate were measured as 10 minutes and 10 mV/s, respectively. The results support that the new isolated *Pseudomonas sp*. has significant potential to determine the trace amount of lead in aqueous solutions.

1. INTRODUCTION

Pseudomonas marincola; Aerobic, gram-negative, unpigmented, motile, rod-shaped cells, 1.8-2.5 µm long and 0.4-0.6 µm in diameter. Oxidase and catalase positive. Colonies are smooth, unpigmented, off-white, transparent, and measure 3-4 mm in diameter. Strains produce brownish diffusible pigments on peptone-enriched media. Growth is observed in 0-8% (w / v) NaCl and at 5-37 °C (optimal at 25-28 °C); weak growth occurs at 4 °C and no growth above 38 ° C is observed. The pH range is 5.5-9.5 (optimal pH 6.5-8.5). Slowly produces H_2S. *Pseudomonas*-like bacteria are reviewed as usual members of microbial communities in aquatic environments. Few species belonging to the genus *Pseudomonas* have been retrieved in seawater or marine sediments. We recently introduced a new *Pseudomonas* member from Burdur Lake, Burdur, Türkiye [1].

Heavy metal pollution significantly harms soils, water, atmosphere and human health [2]. According to the World Health Organisation (WHO), Pb poisoning is

Keywords. *Pseudomanas marincola*, biosensor, lead, heavy metal
cihan.bostanci@ankara.edu.tr- Corresponding author; 0000-0001-7846-9181
nazir@science.ankara.edu.tr- 0000-0002-8423-751X
gdonmez@ankara.edu.tr- 0000-0001-7972-5570

among the most paediatric health problems worldwide. Pb, like other heavy metals, leads to respiratory and gastric dysfunctions in the human body [3-4]. Lead ion (Pb^{+2}), one of the toxic heavy metal ions, can cause severe damage to the brain and central nervous system [5]. The World Health Organization (WHO) requires that the Pb(II) content in the drinking water should be no more than 10 $\mu g \cdot L^{-1}$. However, due to the un-degradability and extensive use of lead in paints, batteries and ceramics industry, Pb(II) has become a major environmental contaminant in water, soils and air. Therefore, considerable efforts have been devoted to the ultrasensitive and quantitative detection of Pb(II). Up to date, the conventional methods, such as atomic absorption/emission spectroscopy [6], inductively coupled plasma atomic emission spectroscopy [7], and surface plasma resonance [8] are reliable and accurate. However, the requirement of expensive instruments, professional operation and elaborated sample pre-treatment limit the application of these methods for the on-site and real-time determination. In contrast, the electrochemical method could compensate for these disadvantages with high sensitivity and sufficient accuracy, which has attracted considerable attention and progress.

Biosensors have an essential role in detecting environmental contamination, including heavy metals and hydrocarbons, human health, sample quality and many other applications [9-10]. Using biosensors for quantitative analysis as an alternative to conventional methods has many advantages, such as being fast, compact, cheap and allowing to work on-site applications [11].

In this study, *Pseudomonas marincola* was isolated from Burdur Lake, Burdur, by using Nutrient Broth, and then Pb(II) interest of the bacterium was determined by electrochemical ways. The bacterium is used to prepare a modified carbon paste electrode (MCPE) by using whole living cells. Pb interest, current changes of surface and calibration curve were studied by using MCPE. Although *Pseudomonas sp*. was studied in many biotechnological applications, including heavy metal adsorption and biosensors, this is the first report of *P. marincola* about Pb(II) interest.

2. MATERIALS AND METHODS

2.1. Chemicals

All heavy metals in nitrates form, such as Pb(II), Cu(II), Ni(II), Zn(II) and Co(II), were purchased from (Merck, Germany). Heavy metal solutions were prepared freshly from metal nitrates before experiments and then deoxygenated by passing pure nitrogen gas through each heavy metal solution. The buffer solution was prepared from Tris–HCl's analytical grade (Sigma, Germany). Graphite powder and paraffin oil (Merck, Germany) were used to prepare both modified and non-

modified carbon paste electrodes (CPE). The pH of the supporting electrolyte solution was set using 0.1 M NaOH or 0.1 M HCl (Merck, Germany).

2.2. Isolation of microorganisms and culture conditions

Aqueous samples were collected from Burdur Lake, Türkiye, then 10% inoculated into tubes containing Nutrient Broth (NB) at 30 °C and pH 6.2 for 3 days. After incubation, colonies were collected and purified on Nutrient Agar (NA) plates. The pure cultures were kept at 4 °C and were transferred to a fresh medium every 3 months. Fresh -24h incubated on NA at 30 °C, the bacterium used for experiments.

2.3. PCR and Sequencing

Whole cells from exponentially growing cultures of the isolates were used for genomic DNA isolation and amplification of internal transcribed spacer (ITS) regions of 18s rRNA by conventional PCR. Genomic DNA of bacteria strains were isolated using Qiagen DNeasy Blood and Tissue kit. ITS regions were then PCR amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3') primers. PCR was carried out with using 50 µL 1x Taq Buffer (Thermo Fisher Scientific, USA), 1,5mM MgCl, 0,2 mM dNTP, 0,3 pmol/uL primers (27F and 907R) and 1.24 U Taq polymerase (Thermo Fisher Scientific, USA) for each primer. DNA sequencing was performed at ABI 3100 Genetic Analyzer device with DNA sequence analysing BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) in an external laboratory (Refgen, Ankara, Türkiye). Strains were identified according to the assessment of the results of DNA, 18S rDNA gene sequence and PCR analyses performed.

2.4. Preparation of Designed Microbial Biosensor

The designed carbon paste electrode as a working electrode was formed using teflon tube. Electrical conductivity was established by copper wire (5 mm diameter). The designed carbon paste electrode was filled with carbon paste. Carbon paste was prepared as 10 mg of living whole-cell bacteria, 90 mg of graphite powder, and paraffin oil. The non-modified carbon paste was prepared only with graphite powder and paraffin oil. Once the designed carbon paste electrode body was filled with freshly prepared carbon paste mixture, the electrode surface was smoothed on paper to obtain more homogenous results and prevent undesired noises.

Screen Printed Electrode (DS150) is purchased from Metrohm (Switzerland). SPE was prepared freshly before using each experiment set. The bacteria culture was diluted with 0.01 M Tris-HCl buffer solution and $8x10^7$ ($5.33x10^9$ CFU/ml) bacteria cells were embedded onto the electrode surface via drying at 30 °C under laminar flow.

2.5. Electrochemical Methods and Tools

All electrochemical measurements were accomplished at room temperature with a CH Instruments 660B model potentiostat (CH Instrument, USA). The instrument was supplied with a common three-electrode cell, including buffer solution 0.01 M Tris–HCl, deoxygenated by pure nitrogen through it before the measurements. CPEs modified with *P. marincola* were used as working electrodes. The platinum wire and Ag/AgCl electrode (saturated KCl) were used as counter and reference electrodes, respectively. Electrochemical experiments were done once accumulation was finished [12].

In the accumulation step, the working electrode was immersed in 10 ml of prepared heavy metal solution at a definite concentration for a selected time at an open circuit. Voltammetric analyses were performed immediately once the accumulation was completed. Cyclic voltammetry (CV) was carried out for bare and modified working electrodes. The CV working conditions were set from -1 to +1 V (from negative to positive direction) with a 0.01 V/s scan rate.

After obtaining the optimum working conditions for Pb(II), other model heavy metal solutions were prepared with 0.01 M Tris-HCl solutions to determine their interference effect with Pb(II). For this purpose, 10 ml of heavy metal solutions ($1x10^{-8}$ M for each heavy metal) containing a particular concentration of Ni(II), Co(II), Zn(II), and Cu(II) were used.

2.6. Surface Measurements

Modified Screen-Printed Electrodes (8x107 bacteria cells and $1x10^{-5}$ M Pb) were coated with Au-Pd. The surface morphology of the microbial biosensor was examined by Environmental Scanning Electron Microscopy (FEI Quanta 200 FEG ESEM Thermo Fisher, USA) at UNAM Bilkent University, Ankara.

Modified electrode surface changes at lower Pb concentration ($1x10^{-8}$ M) were analysed at Atomic Force Microscopy (Park System XE-100 PSIA, South Korea) with the non-contact mode at UNAM Bilkent University, Ankara.

XPS analysis (Thermo Scientific K-Alpha, USA) was performed with modified electrodes (treated $1x10^{-8}$ M) at UNAM Bilkent University, Ankara.

3. RESULTS AND DISCUSSION

3.1. Electrochemical behaviour of *Pseudomonas marincola*

The electrochemical behaviour of the bacterium was shown in Figure 1. Cyclic voltammograms of both modified and bare CPE were obtained in 0.01 M Tris-HCl for $1x10^{-5}$ M Pb(II). As seen in Figure 1, the reducing peak, which was characteristic for Pb(II), around -0.6 V was observed at the modified electrode. The cathodic peak around -0.4 to -0.6 V also address to Pb(II) by other researchers [12-13]. The cathodic peak corresponds to a reduction of surface-bonded Pb(II); thus, the peak area was decreased after each cycle was completed. This result is the first time showing *P. marincola* interacted with Pb ions.

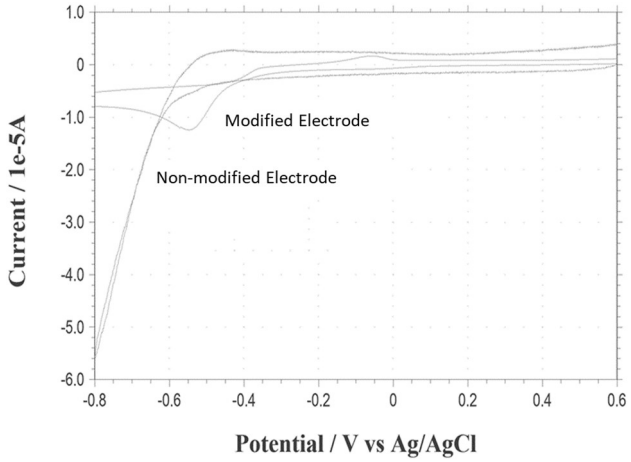

FIGURE 1. Cyclic Voltommograms of *P. marincola* ($1x10^{-5}$ M Pb(II) in 0.01 M Tris-HCl pH:7)

3.2. The effect of pre-concentration time

Pre-concentration time has significant importance in determining the bacteria response time. For this reason, modified CPE was placed in an electrochemical cell containing 10 mL $1x10^{-5}$ M Pb(II) solution at Open Circuit Potential (OCP) for 1000 seconds then-current changes were recorded in Figure 2. As seen in Figure 2, the current was increased until 10 minutes and then stabilised with minor increases-decreases. Until 10 minutes, Pb(II) ions were bound by bacteria located on the surface. After 10 minutes, the surface was saturated by ions then minor ion changes were started from surface to solution without reduction. Consequently, pre-concentration time was selected as 600 seconds and was applied for further experiments.

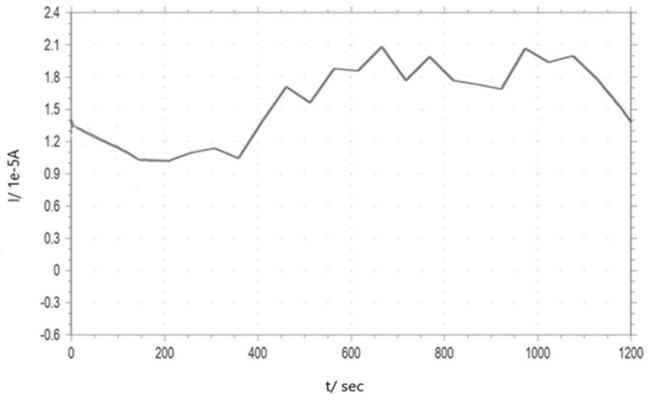

FIGURE 2. Current/ Pre-concentration time of *P. marincola*.

3.3. Detection of working limits of the designed sensor

Bacteria cells were diluted with 0.01M Tris-HCl (pH: 7) buffer solution in a sterile Eppendorf tube, and then 15 µl of the diluted solution was dropped onto the SPE surface in the sterile laminar flow cabinet. SPE was dried for 30 min at 30 °C in the laminar flow cabinet. Ligament or any mediator was not used for fixing the bacteria cells. Therefore bacteria cells were fixed on the bare SPE surface directly. All SPE were prepared freshly before the experiments. The minimum limits for Pb solutions were tested with modified SPE. Figure 3 shows the calibration range for modified SPE between $1x10^{-8}$ and $8x10^{-8}$ M Pb. The Limit of Detection (LOD) was obtained as $1x10^{-9}$ M using the formula $3\sigma/s$ [14-15].

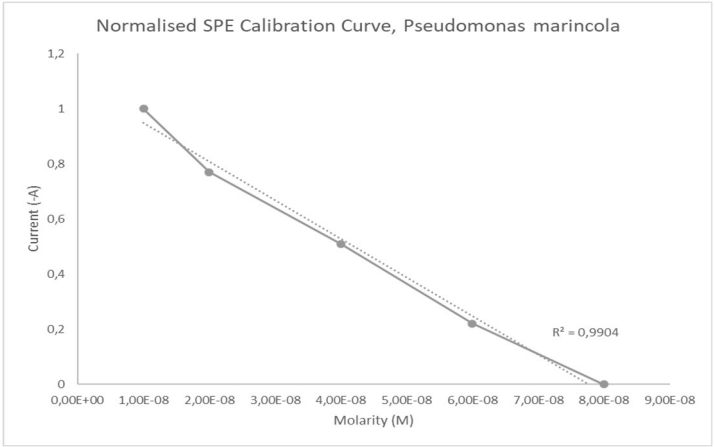

FIGURE 3. Fitting curve between $1x10^{-8}$ and $8x10^{-8}$ M for modified screen-printed electrode

3.4 Surface analysis of modified screen-printed electrode

SEM images were used to show the embedded bacteria cells and components on the electrode surface. As seen in the SEM images, bacteria cells were perfectly fixed onto the surface without using any ligament such as glutaraldehyde (Figure 4).

AFM was used for the detailed topographical analysis of modified SPE treated $1x10^{-8}$ M Pb. The topographical image shows the bacteria cells fixed in the pits on the SPE surface. Once the modified electrode was treated with $1x10^{-8}$ M Pb solution, Pb ions were accumulated on the electrode surface. While Pb ions were accumulating onto the surface, surface thickness was not changed significantly. On the other hand, the surface smoothness and homogeneity were collapsed by Pb ions, as seen in Figure 5.

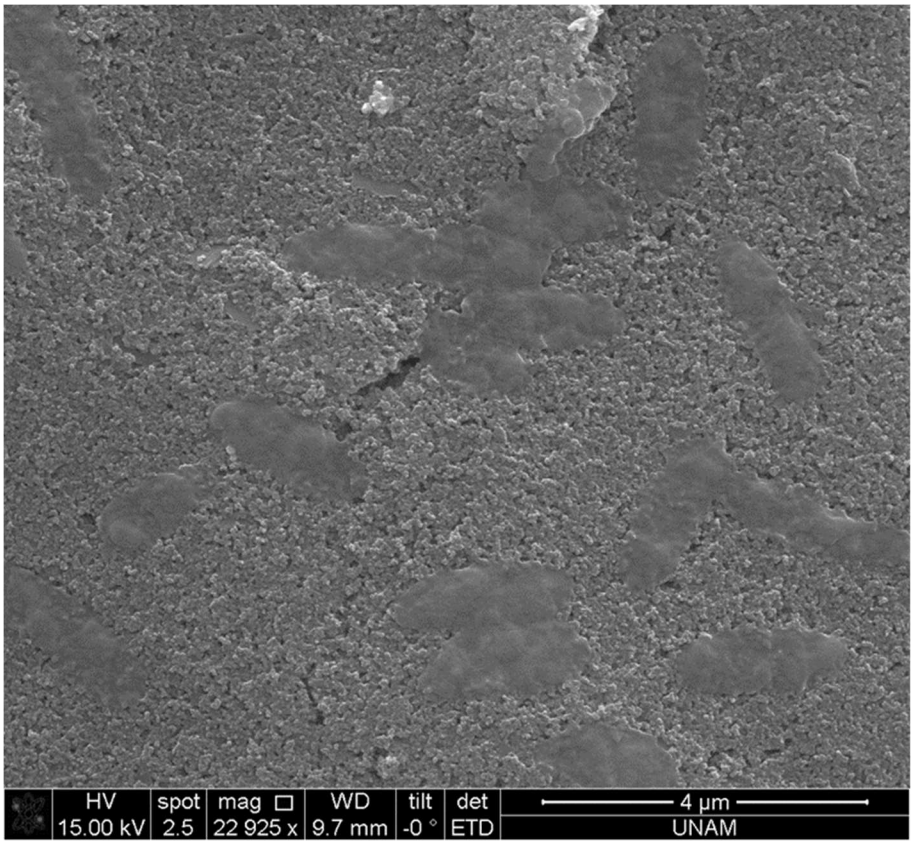

FIGURE 4. The bacteria cells onto the modified screen-printed electrode (treated $1x10^{-5}$ M)

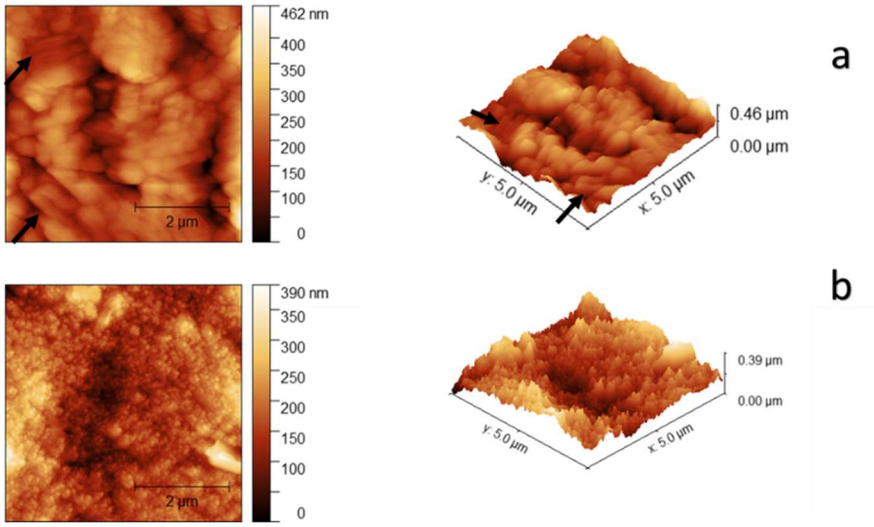

FIGURE 5. Modified electrode surface without Pb treat (a), modified electrode surface after Pb treat (b)

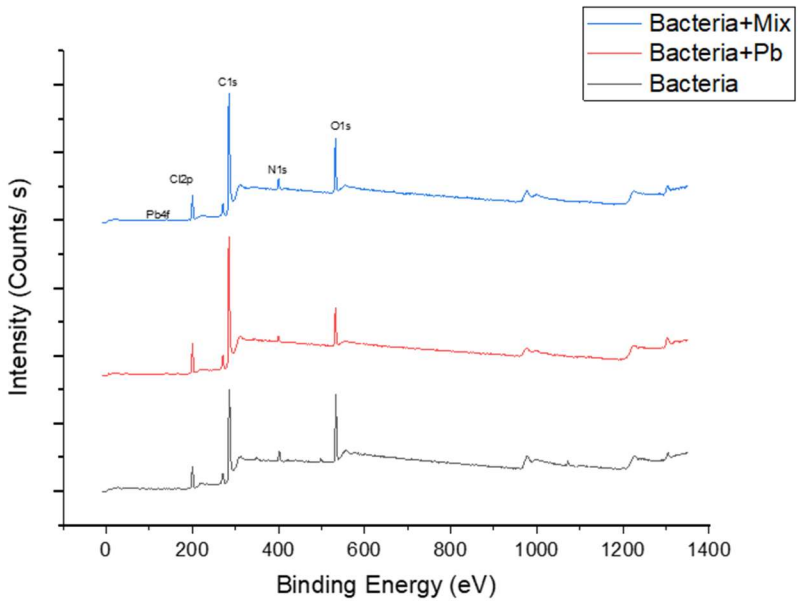

FIGURE 6. XPS scan for the non-treated electrode (bacteria), treated with $1x10^{-8}$ M Pb (bacteria+Pb) and treated with $1x10^{-8}$ M mixed heavy metal solution (bacteria+Mix)

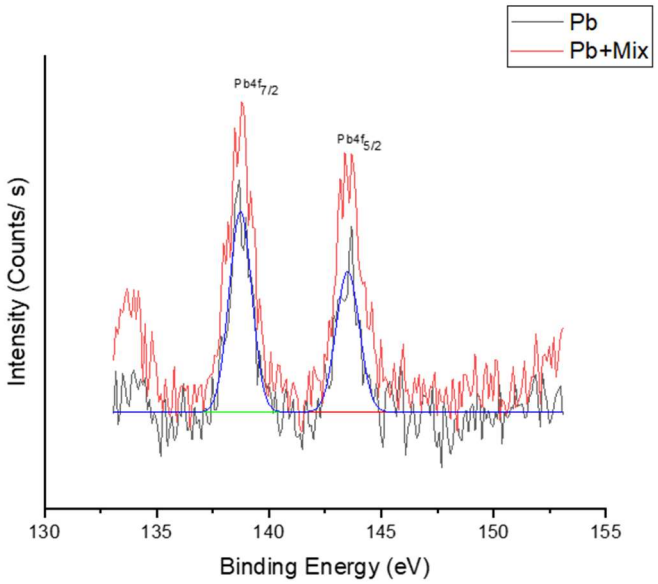

FIGURE 7. High resolution of Pb with treated $1x10^{-8}$ M Pb and $1x10^{-8}$ M mixed heavy metal solution

The whole-cell microbial biosensors have been recognised as a novel, cost-efficient replacement for conventional sensors. Another advantage of whole-cell microbial biosensors is the fast and specific detection of heavy metals. Thus whole-cell microbial biosensors can use for in-situ water monitoring [22]. On the other hand, the detection limit is a common issue for whole-cell microbial biosensors. The average LOD is 0.1 µM for whole-cell microbial biosensors; however, the operating range of the designed sensor with *P. marincola* is well beyond this limit [23]. Moreover, the developed sensor is significantly specific for Pb(II) even in 0.01 µM mixed heavy metal solution. As seen in Figure 8, the lead accumulation is not changed in mixed heavy metal solution, and the almost exact amount of lead is accumulated on the electrode surface.

4. CONCLUSIONS

In this study, newly isolated *P. marincola* from Burdur Lake, Burdur, was cultured and used as biomaterial to detect Pb(II) in aqueous solutions with a $1x10^{-9}$ M limit of detection. This study is the first report of using living whole-cell *P. marincola* as biomaterial to detect Pb(II) by electrochemical ways. Compared with other electrochemical determinations, the method presented here has a lower detection limit and a wide working range. *P. marincola*, which has biotechnological importance, may uptake Pb(II) ions on the surface in 10 minutes. Moreover, the designed low-cost biosensor does not require any

mediator for electrochemical response or fixator to embed on the SPE surface. Living cells can fix on the surface under laminar flow conditions without extra effort. The designed biosensor can be prepared and detect Pb in under 30 min from the initial step to the final stage. *P. marincola* is a promising Pb agent for potential biosensor, bioremediation and biosorption studies.

Acknowledgement. C. Bostancı was supported by funding from the Scientific and Technological Research Council of Türkiye (TUBITAK-BIDEB 2211-C and 2214A), Council of Higher Education (YOK-100/2000), American Society for Microbiology and Ankara University (Project Number: 19L0430010).

Author Contribution Statement CB- data collection, management and manuscript writing. HN- data analysis GD- project development, manuscript writing and manuscript editing. All authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest.

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Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 32, Number 1, Pages 36-53 (2023)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1254635

Research Article; Received: February 23, 2023; Accepted: April 6, 2023

AN EVALUATION OF THE MACRO ANATOMY OF THE HAZEL DORMOUSE'S (*MUSCARDINUS AVELLANARIUS* LINNAEUS, 1758) (RODENTIA: MAMMALIA) SKELETAL SYSTEM IN TÜRKİYE

ESRA ENGIN KILIÇ[1], HATİCE MUTLU EYİSON[1], ERKUT KIVANÇ[1]

[1]Department of Biology, Faculty of Science, Ankara University, Ankara, TÜRKİYE

ABSTRACT. *Muscardinus avellanarius,* one of the thirty species of the Gliridae family, is found in areas covered with deciduous forests, with a range stretching from Bursa (Uludağ) to Trabzon in Türkiye. Hazel Dormouse, classified as an endangered species and characterized by their deep hibernation patterns, possess significant value as bioindicators of environmental change. In this study, a macro-anatomical evaluation of the skeletal structure of forty-six specimens collected from the Eastern and Western Black Sea Regions between 1979 and 1983 were conducted. The evaluation revealed that the whole skeleton of *Muscardinus avellanarius* consisted of 225 bones. The morphometric variances and variations between the Eastern Black Sea and Western Black Sea specimens were detected by defining the skull and baculum bones that hold taxonomic value.

1. INTRODUCTION

The hazel dormouse (*Muscardinus avellanarius* L., 1758), a member of the Gliridae family, is a hibernating rodent that lives in Europe and Northern Anatolia [1, 2]. The hazel dormouse is a protected species by strict regulations through its inclusion in Annex IV of the Habitats Directive (Council Directive 92/43/EEC on the Conservation of natural habitats and of wild fauna and flora) and Annex II of the Bern Convention in Europe [3-5]. The main threats to the hazel dormouse population are habitat loss, habitat fragmentation and unsuitable forest management practices [6-8]. Special care and consideration are given to the protection of the hazel dormouse in certain Western European countries such as the United Kingdom, Germany, Denmark, the Netherlands, and Belgium [8]. Studies reported that the hazel dormouse population in the United Kingdom declined by 72%. The drop in the population has been attributed to climate change and forest habitat structure and quality [9,10]. *M. avellanarius* is an arboreal species living in woodland and scrub habitats and is often associated with wooded areas [11-13]. This glirid is known as an important bioindicator of environmental change due to its sensitivity to both climate and other environmental factors [14].

Keywords. Muscardinus avellanarius, macro anatomy, skeletal system, baculum, Türkiye
enginesra@hotmail.com- 0000-0002-2062-3139
hmutlu@science.ankara.edu.tr- Corresponding author; 0000-0002-4637-5268
kivanc@science.ankara.edu.tr- 0000-0001-6829-2335

The first record of *M. avellanarius* from Turkey was given by Nehring in 1903 from Istanbul-Alemdağ [15]. Miller (1908) [16] introduced a new record for *Muscardinus* from Trabzon-Coşandere named *M. trapezius,* but Ellerman (1948) [17] later described them as *M. avellanarius trapezius.* Kıvanç (1983) described *M. avellanarius abanticus* from Soğuksu (Bolu/Abant) [18]. Following their taxonomical descriptions based on morphologic differences [19,20], molecular studies also revealed the presence of these two subspecies [21,22].

Numerous studies have been conducted on the biology [23-26], ecology [24-30], population dynamics [24,31], behavior [32-35], dentitional variations [19,20] and karyotype [21,22] of *M. avellanarius*. Recent studies mostly focus on conservation biology and investigating the adverse effects of habitat fragmentation on the species [10,36,37].

Anatomical studies related to the hazel dormice are limited in number; however, there are studies available on the cranial and vertebral column of the *M. avellanarius* [38,39].

Despite the extensive research conducted on the taxonomy, biology, ecology, population dynamics, behaviour, dentitional variations, and karyotype of *M. avellanarius*, the skeletal structure and the bones that hold taxonomic value have yet to be fully revealed. To address the gaps in this subject, it is crucial to conduct further research to complete the existing deficiencies and disseminate the findings to the scientific community. Moreover, a comprehensive examination of *M. avellanarius'* skeleton including a complete definition and a total number of all bones has yet to be reported in any existing literature. This study aimed to identify the skeletal structure and bones with a taxonomic value of *M. avellanarius*, as well as to determine the differences between *M. avellanarius trapezius* (Eastern) and *M. avellanarius abanticus* (Western) specimens.

2. MATERIALS AND METHODS

In this study, skull and skeleton samples of 46 hazel dormouse collected from Northern Turkey (Black Sea Region) between 1979-1983 were evaluated. Samples boiled in a 15% ammonia solution at 70 ºC in a water bath for maceration, later cleaned and left to dry. Afterwards, the shapes of the bones were illustrated under magnification. Photographs were taken and bones were evaluated morphologically and morphometrically. Comparative analysis of the skeletal measurements was made between the specimens from the Western Black Sea region and Northern Black Sea Region to investigate the differences between subspecies of hazel dormice. Findings compared with the previous studies in the literature. A total of 12-character sizes (Figure 1) and Os glandis (baculum)

(Figure 2) measurements were taken with a digital caliper with an accuracy of up to 0-150 mm.

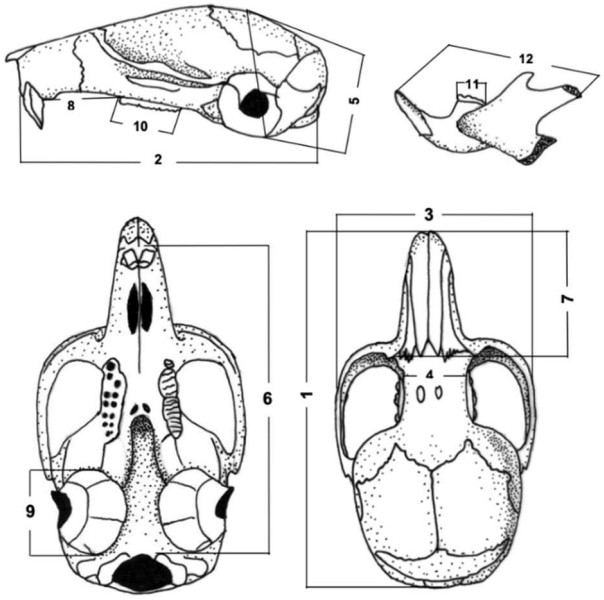

FIGURE 1. Internal character measurements are taken from the skull, teeth, and lower jaw. **1.** Occipito-nasal length, **2.** Condylo-basal length, **3.** Zygomatic width, **4.** Interorbital width, **5.** Brain capsule width, **6.** Basal length, **7.** Nasal length, **8.** Diastema length, **9.** Tympanic bulla length, **10.** Crown length of the upper molar, **11.** Crown length of the lower molar, **12.** Length of the mandible.

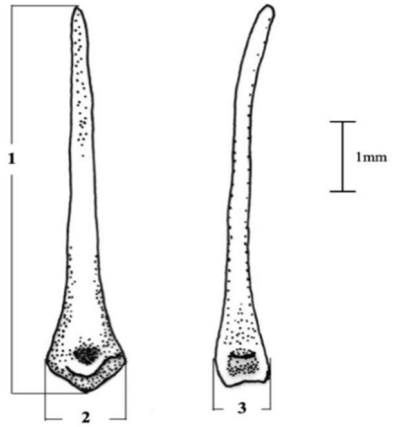

FIGURE 2. Definition of Os glandis (baculum) measurements. **1.** Total length and baculum length (BL), **2**. Proximal width from ventral and baculum base width (BaW) **3**. Proximal width from lateral and baculum base thickness (BaH)

3. RESULTS

The missing skeleton parts of the samples under examination in this study were completed from available samples. The study revealed that the entire skeleton consists of 225 bones, as shown in Figure 3. The skeletal system of *M. avellanarius* was examined in two parts, axial and appendicular.

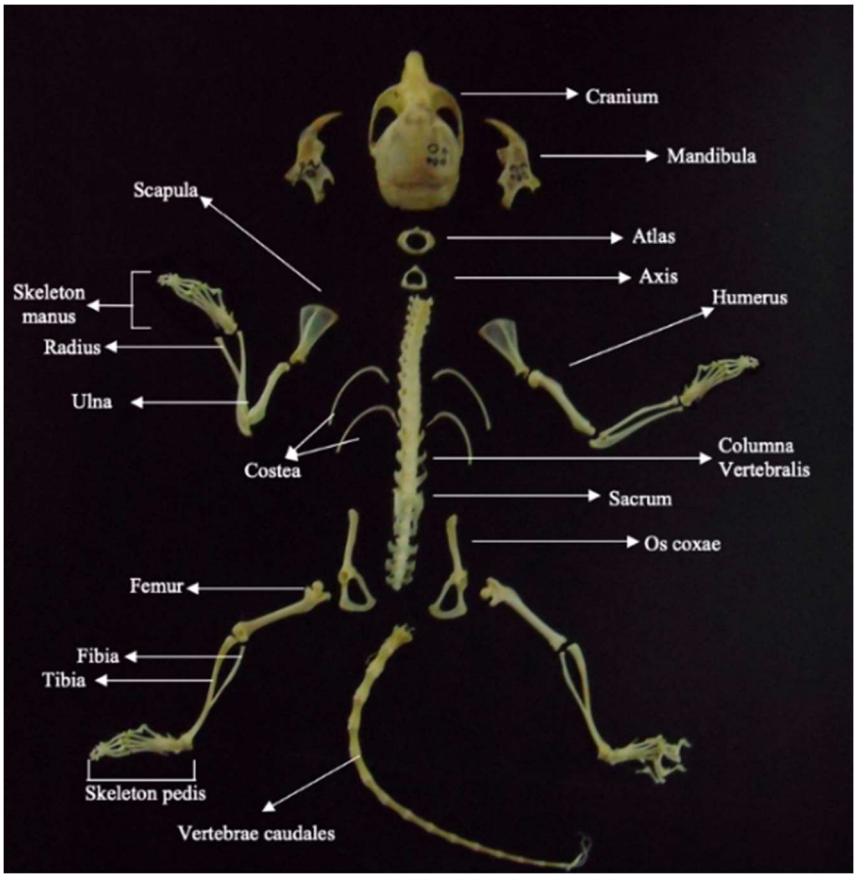

FIGURE 3. Skeleton of *Muscardinus avellanarius.*

Skeleton Axiale: The axial skeleton is comprised of three parts skull bones, the spinal column, and the rib cage. The bone characteristics of the axial skeleton of *M. avellanarius* are presented in Table 1, including the quantity of the bones and their names.

TABLE 1. The bone characteristics of the skeleton axiale of the *Muscardinus avellanarius*.

Division of the skeleton	Regions of Skeleton Axiale		Name of the bone	Quantity	Local bone count/total bone count
Skeleton Axiale	Ossa Cranii	Neurocranium	Os Occipitale	1	11
			Os Interparietale	1	
			Os Sphenoidale	2	
			Os Pterygoideum	1	
			Os Temporale	1	
			Os Parietale	2	
			Os Frontale	1	
			Os Ethmoidale	1	
			Os Vomer	1	
		Viscerocranium (Ossa Faciei)	Os Nasale	2	13
			Os Lacrimale	2	
			Os Maxillare	2	
			Os Incisivum	2	
			Os Palatinum	1	
			Os Zygomaticum	2	
			Mandibula	2	
	Columna Vertebralis		Vertebrae cervicales	7	54
			Vertebrae thoracicae	13	
			Vertebrae lumbales	6	
			Os Sacrum	3	
			Vertebrae Caudales	25	
	Skeleton Thoracis		Costae	26	27
			Sternum	1	
			Total	105	105

The cranial region, called Ossa cranii, consists of 24 bones. The anatomical structures, photographs, and drawings of *Muscardinus avellanarius* cranium are given in Figure 4. Evaluations showed that this species' cranium is thin and fragile. The nasal section is tapered, and its average length is 8.2 mm in western specimens and 7.9 mm in eastern specimens. The zygomatic arches widely outward with an average width of 13.7 mm in western specimens and 12.5 mm in eastern specimens. The pterygoid processes extend to the tympanic bulla and are in contact with them, which are snail-like shaped. The average bulla length is 6 mm in western samples and 5.4 mm in eastern samples. The average bulla length is 6 mm in western specimens and 5.4 mm in eastern specimens. Cranium measurements of the Western and Eastern Black Sea samples are illustrated in Table 2 and Table 3, respectively.

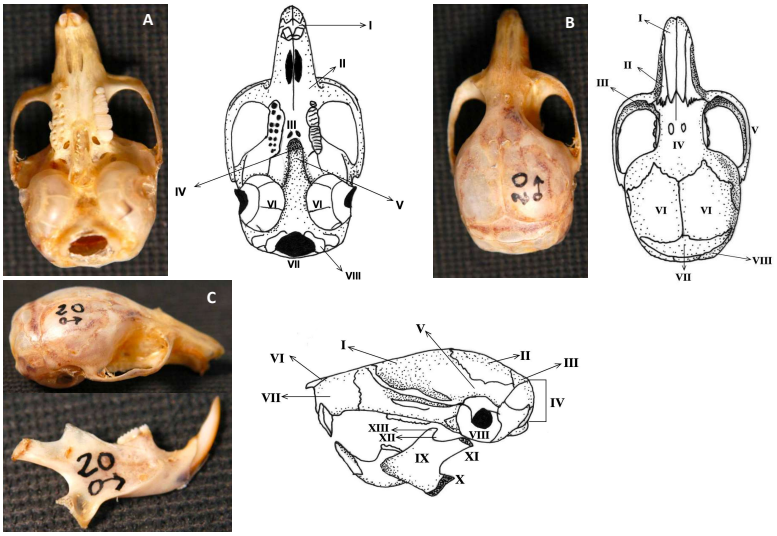

FIGURE 4. Ventral (**A**), dorsal **(B**) and lateral **(C)** view of the skull and lateral view of the mandible **(C)** of *M. avellanarius*; **A:** I) Os incisivum, II) Os maxillare, III) Os palatinum, IV) Presphenoidale, V) Basisphenoidale, VI) Bulla tympanica, VII) Foramen magnum, VIII) Condylus occipitalis. **B**: I) Os nasale, II) Os incisivum, III) Os maxIllare, IV) Os frontale, V) Os zygomaticum, VI) Os parietale, VII) Os interparietale, VIII) Os occipitale. **C:** I) os frontale, II) os parietale, III) Os interparietale, IV) Os occipitale, V) Os temporale, VI) Os nasale, VII) Os incisivum, VIII) bulla tympanica, IX) mandibula, X) processus angularis, XI) processus condyloideus, XII) incisura mandibulae, XIII) processus coronoideus.

TABLE 2. Internal measurements of the skull of *M. avellanarius* from Western Black Sea Specimens

Characters	Number	Min. – Max. (mm)	Mean (mm)	Standard deviation (mm)
Occipito-nasal length	20	23.2-25.7	24.0	3.8
Condylo-basal length	21	20.8-24.0	22.4	0.7
Zygomatic width	20	12.8-14.7	13.7	0.5
Interorbital width	23	3.3-3.7	3.5	0.1
Braincase width / cranial width	22	7.2-7.7	7.3	0.1
Basal length	21	19.0-22.4	20.9	0.7
Nasal length	22	7.6-9.2	8.2	0.4
Tympanic bulla length	17	5.6-6.4	6.0	0.2
Diaestema length	23	5.7-6.7	6.0	0.3
Mandibulae length	23	14.0-15.8	14.7	0.5
Upper molar length from crown	23	4.5-5.0	4.7	0.1
Lower molar length from crown	23	4.3-4.7	4.5	0.1

TABLE 3. Internal measurements of the skull of *M. avellanarius* from Eastern Black Sea Specimens

Characters	Number	Min. – Max. (mm)	Mean (mm)	Standard deviation (mm)
Occipito-nasal length	11	21.6-24.1	23.0	0.9
Condylo-basal length	11	19.7-22.4	21.3	0.8
Zygomatic width	10	11.3-13.6	12.5	0.8
Interorbital width	11	3.3-3.7	3.4	0.1
Braincase width / cranial width	11	6.7-7.4	7.0	0.2
Basal length	11	18.2-20.7	19.7	0.9
Nasal length	11	7.3-8.8	7.9	0.4
Tympanic bulla length	11	4.9-6.0	5.4	0.3
Diaestema length	11	5.3-6.4	5.9	0.4
Mandibulae length	23	13.4-14.7	14.0	0.4
Upper molar length from crown	23	4.0-4.6	4.3	0.2
Lower molar length from crown	23	4.0-4.4	4.1	0.1

Dental anatomy: In studies regarding taxonomy, dental formula and dental structures have special importance. In this regard, particular attention was paid to the dental structure of *M. avellanarius* when evaluating the skull bones. The total number of teeth in *M. avellanarius* is 20, with a dental formula of I(incisor) / C(canine) / P(premolar) / M(molar) of 1013 / 1013 on the upper and lower jaws. Additionally, the Eastern and Western specimens showed variations in the root structure of their upper and lower molars. Besides, the observed variations in the upper and lower molar lengths between Eastern and Western specimens were significant enough to allow for accurate taxonomic differentiation between the two subspecies.

P^1 (Upper Premolar) is the smallest tooth and has a single root. However, three specimens with two rooted P^1 were found among the Western Black Sea samples, while all the Eastern Black Sea samples are single-rooted. For M^1, out of the 44 specimens, 40 have four roots and two specimens from each of the Eastern and Western Black Sea regions have a small additional fifth root. The M^2 (second upper molar) has 4 roots in all specimens from both the Eastern and Western Black Sea regions. The M^3 (Third upper molar) has 4 roots in all the Western Black Sea samples (n=26), whereas in the Eastern Black Sea samples, 10 have four roots, 3 have three roots, and 2 have two free and two transversely fused roots (Figure 5A).

P_1 (Lower premolar) is single-rooted in all specimens. M_1 (First lower premolar) has 3 roots in 21 of the Western Black Sea samples (n=27) and 11 of the Eastern Black Sea samples (n=16), four roots in one specimen from Western and 4 specimens from Eastern Black Sea samples, two free and two transversely fused roots (total of 4 roots) in 1 Western and 5 Eastern Black Sea samples. M_2 (second lower molar) has 4 roots in all 44 specimens from both the Western (n=27) and Eastern (n=17) Black Sea samples. M_3 (third lower molar) has 4 roots in all the Western Black Sea samples (n=27) and 13 Eastern Black Sea samples (n=17), however, 4 specimens from the Eastern Black Sea regions have 3 roots.

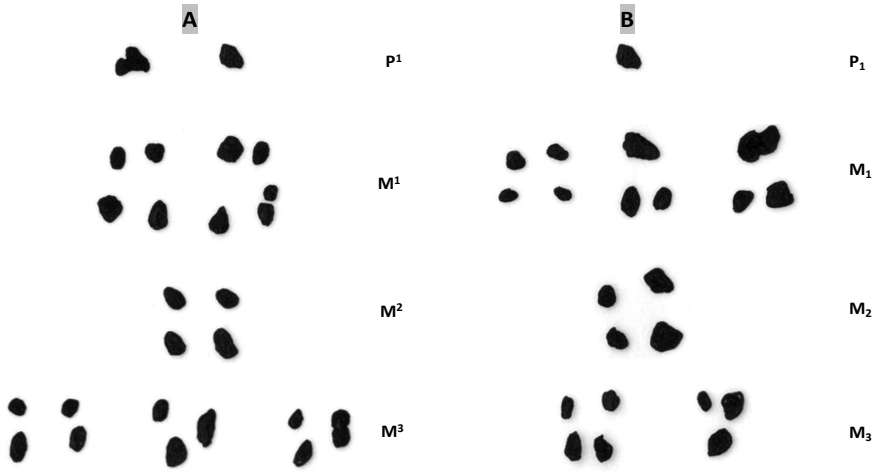

FIGURE 5. Variations on the roots of upper (A) and lower (B) molars

In *M. avellanarius*, the columna vertebralis is divided into five regions as in most vertebrates. These regions are vertebrae cervicales (Figure 6A), vertebrae thoracicae (Figure 6B), vertebrae lumbales (Figure 6C), vertebrae sacrales (Figure 6D, E) and vertebrae caudales (Figure 6G). Columna vertebrae consist of 54 vertebrae. The observations showed that the cervical vertebrae are formed by the union of 7 vertebrae, the thoracic vertebrae are formed by the union of 13 vertebrae, and the lumbar vertebrae are formed by the union of 6 vertebrae. Additionally, the os sacrum is formed by the union of 3 vertebrae, as illustrated in Figures 6D and E. Atlas and axis, the first two vertebrae of vertebrae cervicales display structural changes unique to *M. avellanarius.* The atlas is in the form of a ring. Arcus dorsalis is slightly curved medially. The tuberculum dorsal is underdeveloped, while the tuberculum ventral is more prominent (Figure 7A). The axis of *M. avellanarius* is characterized by the presence of short dens (Figure 7B).

Ala sacralis is reduced in the os sacrum (Figure 6F). The vertebrae caudales of *M. avellanarius* consist of 25 vertebrae, with the first three having a normal structure (Figure 6G). However, the corpus of the subsequent vertebrae is reduced, and the processus spinosus, arcus, and articular processes are absent (Figure 6H).

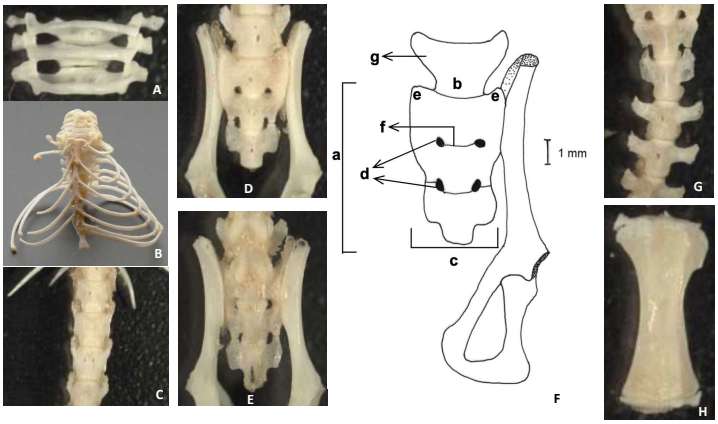

FIGURE 6. Vertebrae cervicales, **(B)** vertebrae thoracicae, **(C)** vertebrae lumbales, **(D)** ventral view of os sacrum, **(E)** dorsal view of os sacrum, **(F)** drawing of os sacrum and os coxae surface from ventral view; a) os sacrum, b) basis ossis sacri, c) apex ossis sacri, d) foramina sacralia ventralis, e) ala sacralis, g) sixth lumbar vertebrae, f) linea transverse. **(G)** first tail vertebrae, **(H)** general view of the tail vertebra of *Muscardinus avellanarius.*

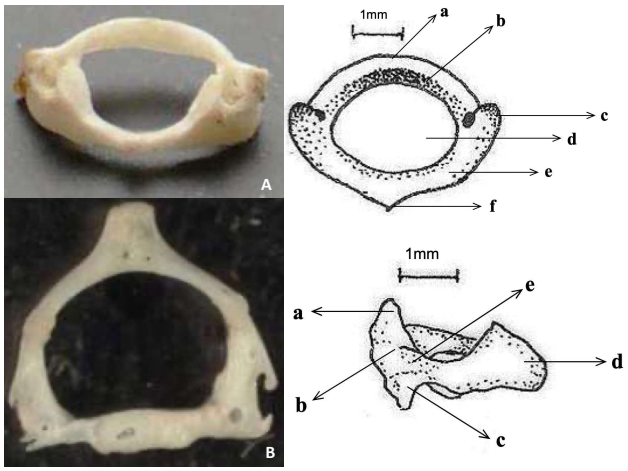

FIGURE 7. **(A)** atlas; a) tuberculum dorsale, b) arcus dorsalis, c) fovea articularis cranialis, d) foramen vertebrae, e) arcus ventralis, f) tuberculum ventrale, **(B)** axis; a) dens, b) facies articularis lateralis, c) processus costotransversarius, d) processus spinosus, e) foramen costotransversarium in *M. avellanarius*.

The sternum (breastbone) and costea (ribs) in the skeleton thoracis consist of a total of 27 bones (Figure 8-1). The sternum (B) is a solitary bone that consists of six sternebrae (b), whereas the corpus sterni (d) is formed by five sternebrae (as illustrated in Figure 8-3).

M. avellanarius possesses 13 pairs of costae, with five of them being attached to separate sternebrae in the sternum. The 6th to 10th costae attaches to the last sternebrae. It was determined that the 11th, 12th, and 13th costae do not attach to the sternum, and they form the costae fluctuantes (floating ribs).

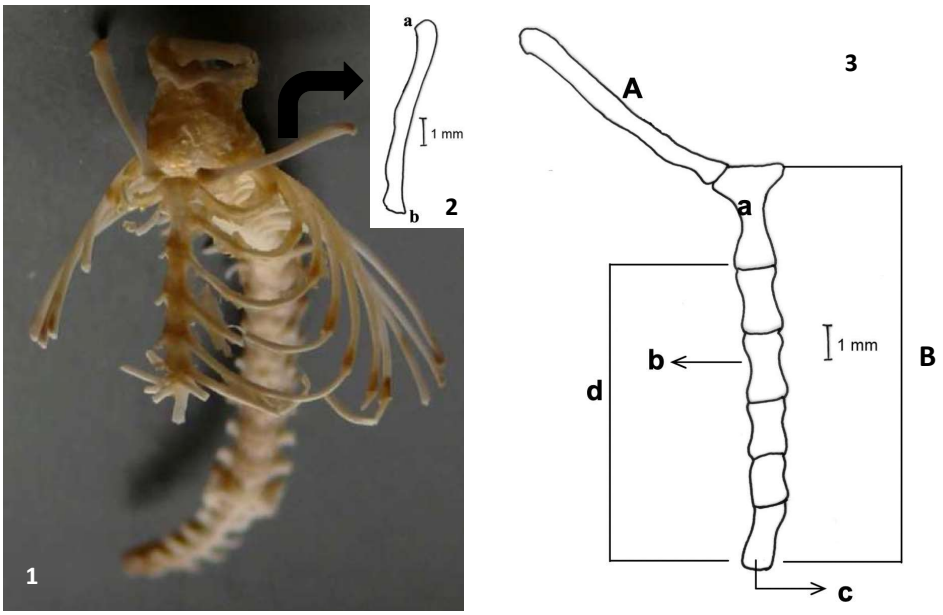

FIGURE 8. Thorax (**1**), clavicula (**2**); a) Extremites sternalis, b) Extremites acromialis, clavicula and sternum (**3**); A) Clavicula (collarbone), B) Sternum (breastbone), a) Manibrum sterni, b) Sternebrae, c) Processus xiphoideus, d) Corpus sterni of *M. avellanarius.*

Skeleton Appendiculare: Skeleton appendiculare of *M. avellanarius* consist of 120 bones (Table 4). Ossa membri thoracici (forelimb bones) have a total of 58 bones, comprising the cingulum membri thoracici, skeleton brachii, skeleton antebrachii and skeleton manus. The names and numbers of these bones are given in Table 4.

TABLE 4. Skeletal parts, names and numbers of bones in skeleton appendiculare in *M. avellanarius*

Division of the skeleton	Regions of appendiculare skeleton	Name of the bone		Number of bones	Regional number of bones
Skeleton Appendıculare	Ossa Membri Thoracici	Cingulum membri thoracici	Scapula	2	58
			Clavicula	2	
		Skeleton brachii (Humerus)		2	
		Skeleton Antebrachii	Radius	2	
			Ulna	2	
		Skeleton manus	Ossa capri	16	
			Ossa Metacarpilia	8	
			Ossa Digitorum Manus	24	
	Ossa Membri Pelvını	Os Coxae		2	62
		Skeleton femoris		2	
		Patella		2	
		Skeleton Cruris	Tibia	2	
			Fibula	2	
		Skeleton Pedis	Ossa Tarsi	14	
			Ossa Metatarsalia	10	
			Ossa Digitorum Pedis	28	
			Total	120	120

In *Muscardinus avellanarius*, the scapula exhibits a triangular morphology as depicted in Figure 9A-B. The fossa infraspinata is broader than the fossa supraspinata. From a lateral view, it was observed that the spina scapulae make up the processus hamatus, which protrudes like a hook at the acromion shoulder joint. Together with the scapula, a pair of clavicles are present in *M. avellanarius*, forming the cingulum membri thoracici (anterior limb girdle). The clavicle articulates with the scapula and sternum as shown in Figure 8-3.

The humerus bone is the thickest of the forelimb bones and articulates with the cingulum membrane thoracici (anterior limb girdle). It has been determined that the caput humeri of the humerus articulate with the cavitas glenoidalis of the scapula, as depicted in Figures 9C and 9D. In *M. avellanarius*, the skeleton antebrachia consists of two distinct long bones named the radius and the ulna. The radius is situated medially, as shown in Figure 4.36, while the ulna is situated laterally, as depicted in Figure 9E.

The forelimbs of *M. avellanarius* are adapted for climbing and have four toes. Due to the small size of the skeleton manus (forelimb skeleton) in this species, some of the bones were lost during the cleaning process, however, a total of 48 carpal, metacarpal and digitorum manus bones were identified.

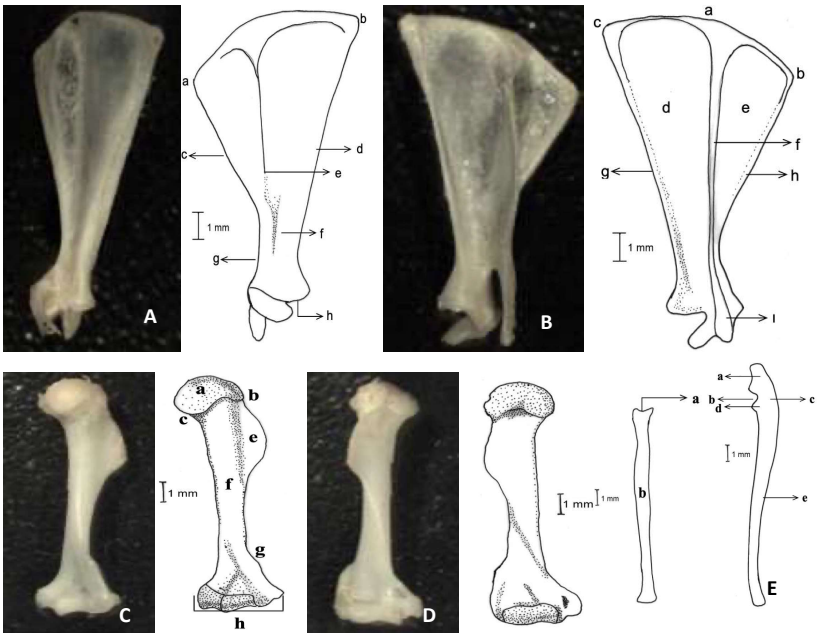

FIGURE 9. **(A)** View of right scapula from facies costalis; a) Angulus cranialis, b) Angulus caudalis, c) Margo cranialis, d) Margo caudalis, e) Fossa subscapularis, f) Angulus ventralis, g) Collum scapulae, h) Cavitas glenoidalis. **(B)** View of right scapula from facies lateralis; a) Margo dorsalis, b) Angulus cranialis, c) Angulus caudalis d) Fossa infraspinata, e) Fossa supraspinata, f) Spina scapulae, g) Margo caudalis h) Margo cranialis, i) Acromion. **(C)** Ventral view of the right humerus; a) Caput humeri, b) Tuberculum majus, c) Tuberculum minus d) Collum humeri, e) Tuberositas deltoidea, f) Corpus humeri, g) Sulcus m. brachialis h) Condylus humeri. **(D)** Dorsal view of the right humerus. **(E)** Radius; a) Fovea capites radii, b) Corpus radii; ulna a) Tuber olecrani, b) Processus anconeus, c) Olecranon, d) Incisura trochlearis, e) Corpus ulna of *Muscardinus avellanarius*.

Ossa membri pelvini: The specimens of *M. avellanarius* had os coxae formed by the fusion of three bones, as shown in Figure 10A. The ala ossis ilii was not large in the ilium, while the foramen obturatum was wide in the ischium. The corpus ossis ilii was long and the os pubis had a narrow structure.

The examination of the femur of *M. avellanarius* revealed that the caput ossis femoris and the trochanter major are not aligned, and there is no fovea capitis on the caput ossis femoris. The collum ossis femoris was observed to be thin, as shown in Figures 10B and C. The skeleton cruris corresponds to the skeleton

antebrachii on the front leg. In *M. avellanarius*, the hindlimb is composed of a fused tibia and fibula, as depicted in Figure 10D.

Due to the small size of the hindfoot skeleton in *M. avellanarius*, many of the bones were lost during the cleaning process, therefore not shown in the figures. However, tarsal, metatarsal and digitorum pedis bones were found to be 52 in total.

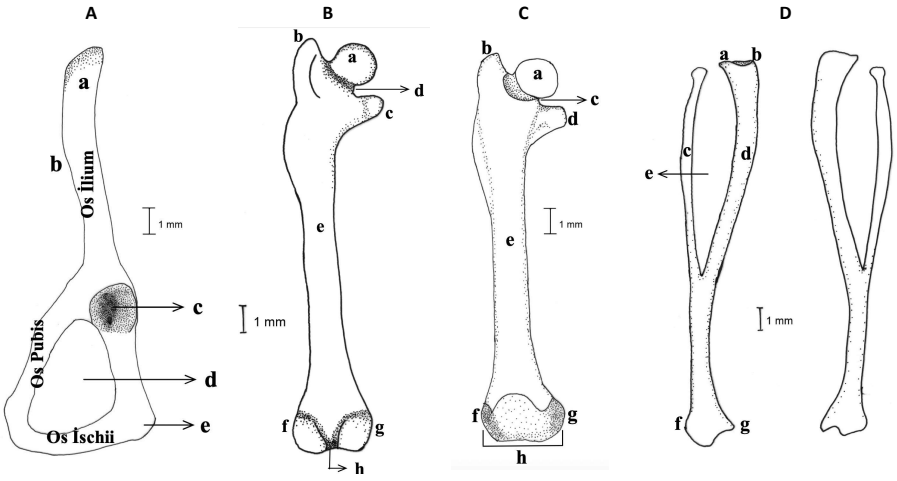

FIGURE 10. **(A)** Os coxae: a) Ala ossis ilii, b) corpus ossis ilii, c) Acetabulum, d) Foramen obturatum, e) Tuber ischiadicum. **(B)** Dorsal view of Os femoris: a) Caput ossis femoris, b) trochanter major, c) trochanter minor, d) Collum ossis femoris, e) corpus ossis femoris, f) condylus lateralis, g) condylus medialis, h) fossa intercondylaris. **(C)** Ventral view of Os femoris: a) Caput ossis femoris, b) trochanter major, c) collum ossis femoris, d) trochanter minor, e) corpus ossis femoris, f) condylus lateralis, g) condylus medialis, h) trochlea ossis femoris. **(D)** Skeleton cruris: a) Condylus lateralis, b) condylus medialis, c) fibula, d) corpus tibia, e) spatium interosseum cruris, f) malleolus lateralis, g) malleolus medialis.

Os glandis (Baculum): The os glandis of *M. avellanarius* is shown in Figure 11. The proximal part of the os glandis shaped like a spoon, and narrows distally, resembling an awl in appearance. The proximal ventral part of the os glandis has a slight depression, and the dorsal part appears to be raised. The measurements of the os glandis are listed in Table 5.

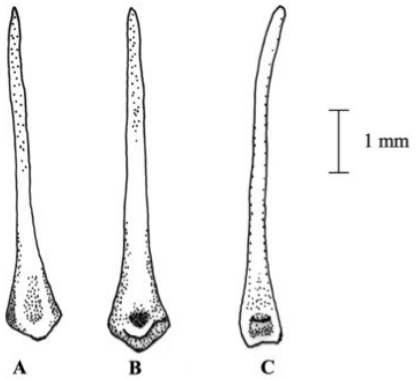

FIGURE 11. View of the os glandis (baculum) in *M. avellanarius* from **A)** dorsal, **B)** ventral, and **C)** lateral.

TABLE 5. Measurements of os glandis

Characters	Number	Min. – Max. (mm)	Mean (mm)	Standard deviation
Total length	5	5,72 - 6,05	5,94	0.16
Proximal width from the ventral	7	0.66 - 1.1	0.97	0.15
Proximal width from the lateral	7	0.44 - 1.21	0.75	0.23

4. DISCUSSION

Andreescu and Andreescu [38] recorded a total of 54-56 vertebrae in *M. avellanarius*, including 7 cervical vertebrae, 13 thoracic vertebrae, 6 lumbar vertebrae, 3 os sacrum and 25-27 caudal vertebrae. The number of vertebrae and regions of the vertebral column stated by Andreescu [38] were consistent with the findings of this study, and the number of vertebrae in the vertebrae caudales was found to be 25.

The British Museum's mammal collection catalogue [39] states that the vertebral column is comprised of 5 regions with 7 cervical vertebrae, 13 thoracic vertebrae, 6 lumbar vertebrae, 2 sacral bones, and 24 caudal vertebrae. However, this study found that while the vertebral counts in the various regions were consistent with the Turkish samples, the number of sacral bones was determined to be 3 and the number of caudal vertebrae was found to be 25.

The length of the upper and lower molar rows, as well as the variations in the premolar and molar roots, were found to be consistent with the findings reported by Kıvanç [18,19].

Hrabe [40] reported baculum length (BL) as approximately 6 mm and baculum base width (BaW) as 0,5 mm. Hrabe [40] also reported that the anterior of os glandis (baculum) is awl-shaped and the cross-section is circular. In their study of the phallus and the baculum of European edible dormice, Simson [41] reported that the baculum length of a single *Muscardinus* specimen from Italy was 4.84 mm, and the baculum base width was 0.86 mm. In this study, the overall length of the os glandis was found 5.94 mm on average, and the base part was 0.97 mm on average, which was in agreement with Hrabe [40] however was larger than those reported by Simson [41].

This study is a pioneer one that conducted a macro-anatomical analysis of the entire skeletal structure and determined the number of bones in the whole skeleton of *Muscardinus avellanarius* species living in Türkiye for the first time. In addition, bones that hold taxonomic value such as humerus, femur, radius-ulna, scapula, os coxae were defined and necessary information was provided to compare these bones with other species.

Author Contribution Statement EEK and EK experimental design and performance, EEK and HME-manuscript writing. HME-manuscript drawing. All authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest. This paper is a summary of a Master's thesis titled "Türkiye Fındıkfaresi'nin (*Muscardinus avellanarius* Linnaeus, 1758) (Rodentia: Mammalia) iskelet sisteminin makro anatomisi' submitted to Ankara University.

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Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 32, Number 1, Pages 54-58 (2023)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1202072

Research Article; Received: November 10, 2022; Accepted: April 12, 2023

NEW RECORD OF EXOTIC LAND SNAIL *RUMINA DECOLLATA* (LINNAEUS, 1758) IN IRAQ

RAGDA HASSANABD AL-QAISI[1], KHANSAA S. FARMAN[1]

[1] Department of Biology, College of Education for Pure Sciences, University of Diyala, IRAQ

ABSTRACT. A new record of the species *Rumina decollata* in Iraq when studying the terrestrial snail community in Diyala Governorate, as it was recorded in 5 sites with different densities for the period from October 2021-May 2022.

1. INTRODUCTION

Biodiversity itself is a relatively recent concept that was used for the first time in late 1980s [1] and, it has been defined in different ways, but simply it is the diversity of life, and soil biodiversity is generally defined as the diversity of living organisms in the soil and the ecological communities that are part of it, and this includes diversity within species, between species and ecosystems [2]. Biodiversity and the many ecosystem functions and services it supports are subject to large and often rapid changes throughout the world, as changes in species distribution and abundance affect all aspects of biodiversity [3]. The Gastropods is one of the most common and, widespread among the varieties of the phylum Mollusca, as it includes about 80% of its species, and it lives in aquatic and terrestrial environments, and this class includes approximately with about 60,000 to 80,000 living species [4] in addition to this large number of the diverse species, there are still undescribed and undiscovered species, because taxonomists consider the morphology of the exoskeleton only as special characteristics of these gastropods, and also the lack of taxonomic experts to study the richness in diversity and distribution of snails [5]. *Rumina decollata* called as the cut (bevelled shell) snail is a predatory European invasive terrestrial snail and belongs to the family Achatinidae, its origin from Mediterranean region in Europe and North Africa, it has been introduced all over the world in the early 1800s [6] *R. decollata* is known to prey on other mollusks, so used as a biological control agent for the brown garden snail *Cornu aspersum*. Although it is also considered a plant pest [7], It has been an agricultural pest in non-native areas, where it has been documented to cause damage in farms in Mexico [8], Cuba [9], and Brazil [10]. With regard to human and animal health, *R. decollata* can serve as an intermediate host for the number of rat nematodes *Angiostrongylus malaysiensis* and and *Angiostrongylus cantonensis* [11].

Keywords. *Rumina decollate*, land snail, Iraq's snail, Iraq's exotic species
raghda.hassan.abd@uodiyala.edu.iq- 0009-0004-6714-1681
khansaasf@uodiala.edu.iq- Corresponding author; 0000-0001-7550-7330

2. MATERIALS AND METHODS

The samples were collected monthly for the period from October 2021 to May 2022 from five restrict in Diyala governorate of Iraq: Baqubah, Al-khalis, Miqdadiyah, Khanaqin, and Mendeli, the samples were collected manually from among the plants and under the leaves of the accumulated plants or from above the small plants located within one square meter [12], and placed in plastic bottles with a capacity of 100 ml [13]. As for the soil samples, they were collected at a depth of (5-15) cm, according to what was stated in [14].

The samples of snails collected from the study sites were diagnosed using Dissecting Microscope type Heerbrugg Model Wild-M3B to species depending on the characteristics of the shell shape, colour, dimensions, shape and direction of its opening [6], the diagnosis was confirmed by the Natural History Museum /University of Baghdad. The physical and chemical factors were measured for each of the study sites by conducting standard soil tests.

1. RESULTS AND DISCUSSION

The shell of *Rumina decollata* is characterized by being calcareous, thick, spindle and elongated, of moderate size, with an average height of 15 individual reached to 26.5 mm and a diameter 10.2 mm (Figure 1). The shell colour of this species is medium brown, i.e., from light grey to dark brown. 5 slightly convex whorls with a surface sculptured with irregular growth lines, and the opening is oval with a simple peristome. The examined specimens showed the black body and the feet pale olive grey. It was found from the monthly averages of population density (Figure 2).

The highest value of *R. decollata* density was 142 ind/m² it recorded in Khanaqin restrict [15] which is located to the east of Iraq and has borders with Iran.

It was abundance in urban areas, this due to the availability of food in addition to the distance from predators [16], were confined to humid area, farmland, and in plant nurseries, this species introduced with imported vegetables ornamental plants.

The current study, showed that this species found in environmental conditions represented by a temperature that ranged between 7.9-37.1 °C, while the pH values were neutral and weak alkaline ranged between 7.0-8.7 and the salinity ranged between 0.1-8.73. In addition to them, the percentage of soil moisture ranged between 1.4-20.64%, while the organic matter ranged between 1.6-8.2%, and the calcium content ranged between 5.2-12.2 mg/kg, and the soil was classified as a loam soil.

The lowest density was recorded at 2 ind/m² in the Mendeli site, due to the increasing in salinity in the upper layer of the soil in those areas, which has an adverse effect on the density of land snails, where the electrical conductivity

recorded between 6.8-8.8 cm/ms and this is due to the dependence of those areas on well water for irrigation.

In conclusion, *Rumina decollata* was recorded as a new record to Iraq molluscan fauna, which is considered one of the invasive species that entered the country through the import of plants and fruits. Its importance is due to its predatory and harmful effects on mollusk populations, causing environmental imbalances.

FIGURE 1. *Rumina decollata*

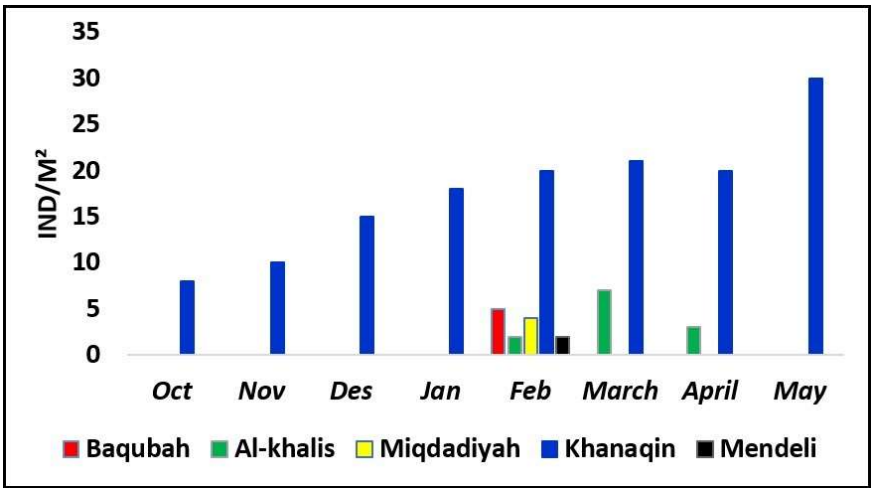

FIGURE 2. Density of *Rumina decollata* (ind/ $m^{2)}$ in study sites through the study months'

Acknowledgement. This work would not have been possible without the support of the College of Education for Pure Sciences at the University of Diyala and the assistance of researchers at the Natural History Museum / University of Baghdad.

Author Contribution Statements RH-specimen collection, Environmental data analysis. KH.F-specimen identification, data analysis, manuscript writing and editing

Declaration of Competing Interests The authors declare no conflict of interest.

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http://communications.science.ankara.edu.tr

Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 32, Number 1, Pages 59-69 (2023)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1225993

Research Article; Received: December 28, 2022; Accepted: March 27, 2023

THE EFFECT OF LEAD (PB) ON THE GROWTH RATES OF TWO AQUATIC MACROPHYTE SPECIES; *LIMNOBIUM LAEVIGATUM* (HUMB & BONPL. EX WILLD) HEINE AND *EGERIA DENSA* PLANCH. GROWN IN DIFFERENT EXPERIMENTAL MEDIA

FATİH DİKMEN[1], VAHİDE CANSU SEYMENOĞLU[2], MEHMET BORGA ERGÖNÜL[1]

[1]Department of Biology, Faculty of Science, Ankara University, Ankara, TÜRKİYE
[2]Department of Biology, Faculty of Science, Gazi University, Ankara, TÜRKİYE

ABSTRACT. Anthropogenic causes contribute to toxic pollutants in aquatic environments and heavy metal pollution. As a heavy metal, Lead (Pb), is one of the most common causes of pollution in water. Heavy metals must be removed from the aquatic environment because they adversely affect health and all living things in each environment. In this study we aimed to determine the effects of lead (Pb) exposure on the growth rates and biomass of two aquatic macrophyte species, *E. densa* and *L. laevigatum*. Plants grown in in two different experimental media. For this purpose, both plants were exposed to 3 different concentrations of lead (1 ppm, 5 ppm, 15 ppm). Samples were measured on the 1st, 4th and 7th days, and the first and last weights of the plants were compared. Bioexperiments were run in triplicate. Positive values were observed in the growth rates of both plants, except for the negative growth rates observed on the 1st day at 1 ppm and 5 ppm lead concentrations in the pond water environment. Both plants showed positive growth in 25% Hoagland medium at all concentrations and days, except for the 1 ppm lead concentration, being observed for *E. densa*. As a result of our study, lead exposure did not significantly alter the growth rates of *E. densa* and *L. laevigatum* in the experimental media used for short-term (up tp 7 days) durations.

1. INTRODUCTION

Water is an essential resource needed for life on earth. Therefore, access to safe water is vital for humans and other living organisms in the ecosystem. Water quality is adversely affected by population growth, industrialization, urbanization, and eventually pollution of water sources [1-2]. Potential major sources of water pollution are toxic compounds such as pesticides and heavy metals, sewage and household wastes, plastics, nanoparticles, industrial and agricultural wastes. [3]. Heavy metals are one of the most common pollutants discharged into natural environment [4].

Heavy metals have an atomic weight between 63.5 and 200.6 (Da) and a density greater than 5 g/cm^3. Heavy metals including arsenic (As), cadmium (Cd), lead

Keywords. Aquatic pollution, macrophytes, lead, heavy metals
fatidikmen@gmail.com- Corresponding author; 0000-0003-2544-4284
vahide.seymenoglu@aski.gov.tr- 0000-0001-6240-2644
ergonul@gmail.com- 0000-0002-0263-9129

(Pb), and mercury (Hg) are toxic even at lower concentrations. Lead (Pb) is a non-essential metal for living organisms and toxic to biota even at a very low dose. It is mainly found in the soil within a range of 15-40 ppm and not considered as a serious threat to living organisms as long as it does not exceed 150 ppm. However, if the lead concentration is >300 ppm in the environment, it poses a serious health concern [5-6-7].

In order to eliminate the potential hazards of heavy metals to the environment and living organisms, physical, chemical, physicochemical and biological techniques can be used to remove or minimize the toxic effects of heavy metals [8]. Since conventional methodologies including chemical precipitation, ion exchange, and electrochemical removal methods have many disadvantages, such as high energy requirements, incomplete removal and toxic waste generation after treatment, the phytoremediation technique have become a prominent technique, which is considered a cost-effective, environment-friendly and easy method [8-9].

Phytoremediation is a natural process being carried out by plants and those absorb pollutants such as pesticides, nanoparticles and heavy metals from the environment [10]. Several plant species including aquatic macrophytes can uptake and store heavy metals in high concentrations thus, they are used to remediate polluted areas or remove pollutants from polluted water bodies [9]. Phytoremediation capacity of each plant species depends on the tolerance of the plant to pollutant, the growth rate and efficiency, and the depth of the root systems [9]. Therefore, it is essential to investigate the optimum growth conditions, tolerance of plant species to specific pollutants and their removal capacities. Although, several macrophyte species are shown to be tolerant to pollutants, there is limited information on the growth performances *of L. laevigatum* and *E. densa* under heavy metal stress.

In this study we aimed to determine the effects of lead (Pb) exposure on the growth rates and biomass increment of two aquatic macrophyte species; *E. densa* and *L. laevigatum*. Plants were exposed to 3 different concentrations of lead (1 ppm, 5 ppm, 15 ppm) in pond water and 25% Hoagland solutions. Bioexperiments lasted for 7 days and plants were harvested on the 1st, 4th and 7th days and the weights of the plants were measured and compared among groups. Pond water was used to imitate a natural environment to reveal the effects of Pb in natural environment, thus providing a deeper understanding on the effects of lead on the growth performance of those aquatic macrophytes which is a critical factor for an effective removal process.

2. MATERIALS AND METHODS

2.1 Plant Material

Plants, *L. laevigatum* and *E. densa* were collected from a local shop and were identified [11] from fresh specimens. Plants were acclimatized to laboratory conditions for 4 weeks prior to use. The stock plant cultures were kept in 150 L aquaria supplied with 25% Hoagland solution [12]. Half of the water from the bottom of the stock culture tanks was removed and renewed every second day. No internal aquarium heaters or filters were used. The temperature was maintained at 19±3.2°C, under a 12:12 h light:dark cycle.

2.2 Bioexperiments

Experiments were carried out in 1 L glass beakers containing either 25% Hoagland solution or pond water. Pond water was used to imitate a natural waterbody and it was obtained from an open pond in Ankara University, Faculty of Science with 5L stainless steel containers. Pond water was transferred to the laboratory and allowed to reach ambient temperature and filtered through 0.2 mm stainless steel sieves remove large suspended particles.

Only green and healthy plants were used in bioexperiments. Plant fragments (2 fragments per beaker) of a similar size (approximately 10 cm long) were used for experiments carried out with *E. densa*. A different approach was followed for *L. laevigatum*; 5-8 individual plants in similar size with a total of 30-35 leaves were placed in each beaker. Plants were gently washed with distilled water twice to remove stock culture solution. Initial wet weight of the plants was weighed (Precisa-BJ 100M) carefully after gently touching with filter papers to remove excess water.

Each plant was exposed to 3 different lead concentrations (1ppm, 5ppm, and 15ppm) in each media in separate beakers. The tested lead concentrations were adopted from relevant studies [14-15]. Lead nitrate ($Pb(NO_3)_2$, (Merck) was used as a lead source. Bioexperiments were run in triplicate and were carried out in 25% Hoagland solution or pond water containing required amount of lead stock solutions. Beakers were covered with transparent stretch film to reduce evaporation. Experiments lasted 7 days and plants were harvested and weighed on 1^{st}, 4^{th} and 7^{th} days. Relative growth (%) of the plants were calculated using the formulae given below [13].

$RGR = (\ln W2 - \ln W1)/(t2 - t1)$ W=weight, t=time

All of the glass material used in the experiments were washed prior to use, with a mixture of 1:3 nitric acid (HNO_3 65% Merck) and hydrochloric acid (HCl 35% Merck) and rinsed 3 times with distilled water.

2.3. Statistical Analysis

The data presented as the mean ± standard deviation (SD) of the triplicates for each group. The data were tested for goodness of fit to a normal distribution prior to the analysis using Shapiro Wilk-W test. Since the normality was violated, non-parametric Kruskal-Wallis tests were performed to determine significant differences among the growth rates of each group. All of the statistical analyses were performed using SPSS 26.0.

1. RESULTS

3.1 Growth Rates in Pond Water

The weight measurement of the plants and growth rates (%) at 1st, 4th and 7th days for each plant exposed to three different lead concentrations in pond water are shown in Table 1.

TABLE 1. The relative growth rates of the plants exposed to Pb in pond water.

Medium	Plant	Day	1ppm			5ppm			15ppm		
			Intitial	Final	Percent %	Intitial	Final	Percent %	Intitial	Final	Percent %
Pond Water	*L. laevigatum*	1	1.63 ± 0.21	1.58 ±0.13	-3.21	1.17 ±0.22	1.15 ±0.26	-2.51	2.13 ±0.82	2.23 ±0.8	4.67
		4	1.60 ± 0.34	1.69 ± 0.30	5.21	1.35 ±0.27	1.41± 0.28	4.21	2.45 ±0.95	2.57 ±0.93	4.58
		7	1.50 ± 0.30	1.58 ±0.25	5.02	1.55 ±0.25	1.68 ±0.39	8.19	1.65 ±0.60	1.79 ±0.59	8.16
	E. densa	1	1.16 ± 0.26	1.10 ±0.22	-5.50	1.28 ±0.26	1.24 ±0.25	-2.73	1.31 ±0.23	1.33 ±0.27	1.30
		4	1.16 ± 0.39	1.28 ±0.40	9.17	1.15 ±0.34	1.16 ±0.36	0.46	1.28 ±0.29	1.36 ±0.34	5.36
		7	1.22 ± 0.37	1.33 ±0.43	9.36	1.32 ±0.29	1.41 ±0.40	6.14	1.31 ±0.22	1.34 ±0.29	2.27

The difference between the weight values of *L. laevigatum* at 1 ppm, 5ppm and 15ppm concentration was statistically significant ($P<0.05$), however, there was no statistical difference for *E. densa* according to the Kruskal Wallis test. Although there was an increase in weight values for both plants on the 1st, 4th and 7th days, the difference between these values was not statistically significant ($p>0.05$).

There was a decrease (-5.5%) in the final weight of *E. densa* when exposed to 1 ppm Pb in pond water. The maximum weight for *E. densa* was recorded on 4th day of the experiment when exposed to 5 and 15 ppm Pb. The growth rate on the 1st day was recorded as the minimum at the 1ppm concentration (-3.21%), while the highest rate was observed at the 15 ppm concentration (4.67%) for *L. laevigatum*. (Figure 1).

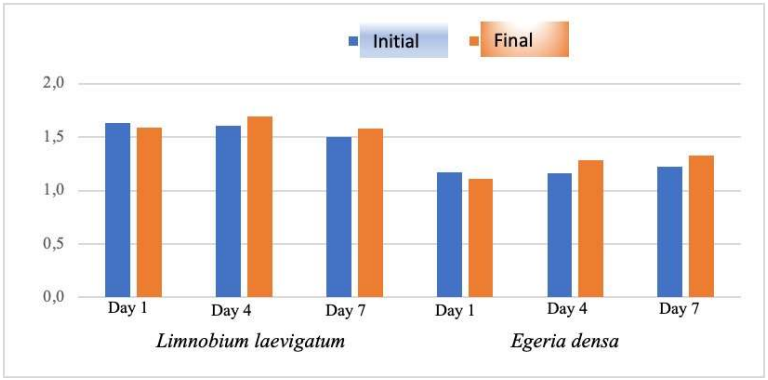

FIGURE 1. Initial and final weights of the plants exposed to 1 ppm Pb in pond water.

The final weights of both plants decreased when exposed to 5 ppm Pb at 1st day (%-2,-2,5), however showed a gradual increase after 4th day of the experiments reaching up to 6-8%. These values were similar to the plants exposed to 1 ppm Pb (Figure 2).

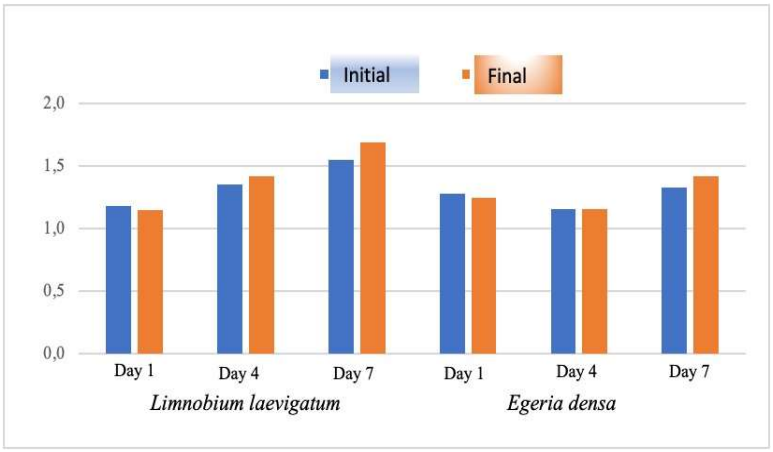

FIGURE 2. Initial and final weights of the plants exposed to 5 ppm Pb in pond water.

The final weights of both plants showed a slight increase when exposed to 15 ppm Pb in the pond water, this increase rate was observed similar on the 4th day (Figure 3).

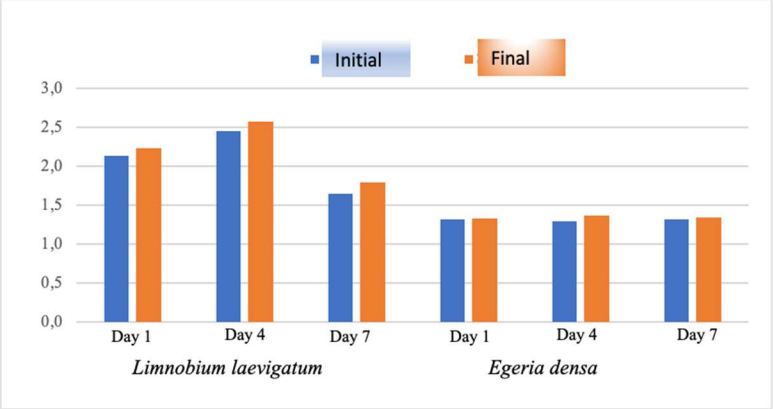

FIGURE 3. Initial and final weights of the plants exposed to 15 ppm Pb in pond water.

3.2. Growth Rates in 25% Hoagland Medium

The weight measurements and growth rates of plants exposed to 3 different concentrations of Pb in 25% Hoagland solution at 1st, 4th and 7th days are shown in Table 2.

TABLE 2. The relative growth rates of the plants exposed to Pb in 25% Hoagland solution.

Medium	Plant	Day	1ppm			5ppm			15ppm		
			Intitial	Final	Percent %	Initial	Final	Percent %	Intitial	Final	Percent %
25% Hoagland	*L. laevigatum*	1	4.31 ± 0.93	4.59 ±1.11	6.12	4.83 ±0.52	4.95 ±0.33	2.47	3.67 ±0.39	3.75 ±0.60	2.27
		4	4.48 ± 0.57	4.81 ±0.52	6.87	4.55 ±0.61	4.80 ±0.56	5.17	4.70 ±1.16	5.07± 1.13	7.16
		7	4.95 ± 0.94	5.33 ±1.25	8.40	4.74 ±0.98	4.79 ±1.04	0.88	6.78 ±1.67	6.95 ±1.5	2.45
	E. densa	1	0.91 ± 0.22	0.84 ±0.20	-8.06	1.06 ±0.27	1.16 ±0.19	8.27	0.83 ±0.14	0.89 ±0.18	6.34
		4	0.98 ± 0.33	0.98 ±0.36	0.19	0.85 ±0.21	0.91 ±0.20	11.64	0.87 ±0.11	0.93 ±0.12	9.91
		7	0.69 ± 0.15	0.73 ±0.15	5.94	0.64 ±0.08	0.71 ±0.23	7.64	0.83 ±0.12	0.89 ±0.17	5.95

The difference between the weight values of the *L. laevigatum* exposed to 3 different concentrations of Pb was not statistically significant ($P>0.05$) in the Hoagland solution. However, there was a significant difference in the weight values of *E. densa* at 1ppm and 5 ppm concentrations ($P<0.05$). When the data were compared among to experimental duration, we found a significant difference between the measured weights values for the both plants in the 25% Hoagland medium on all days ($P<0.05$).

The final weight values for *E. densa* were observed at the highest on the 4th day and a decrease was observed in the weight on the 7th day at 1 ppm Pb concentration (Figure 4).

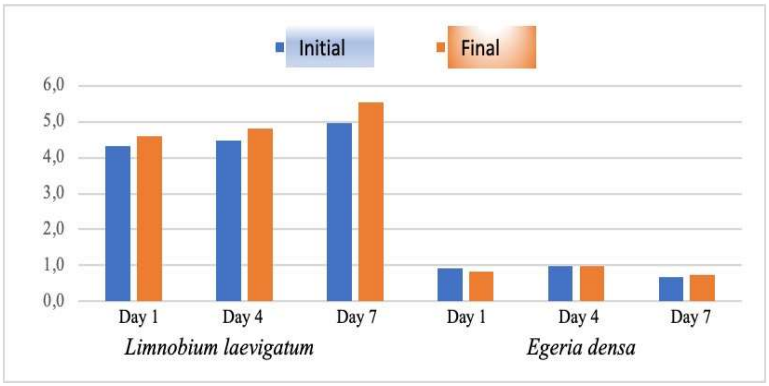

FIGURE 4. Initial and final weights of the plants exposed to 1 ppm Pb in 25% Hoagland solution.

The highest final weight for both plants were observed on the 4th day (5.17-11.64%) and showed a decrease on the 7th day at 5 ppm concentration (0.88-7.6%) (Figure 5).

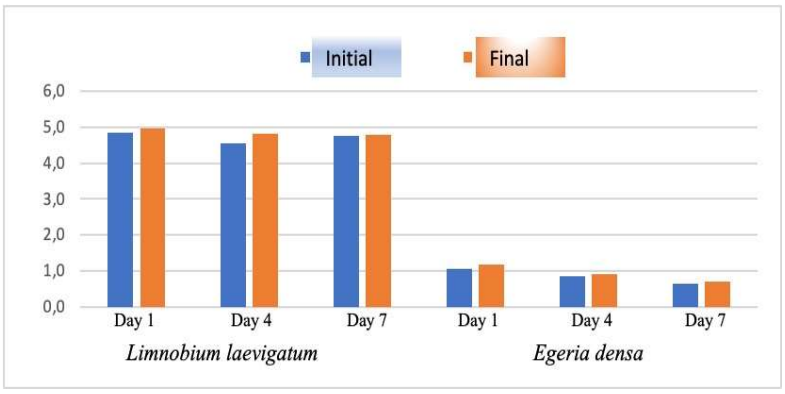

FIGURE 5. Initial and final weights of the plants exposed to 5 ppm Pb in 25% Hoagland solution

The highest final weight of the plants exposed to 15 ppm Pb was observed on the 4th day (7.16-12.91%) for *E. densa*, and it showed a decrease at the end of the 7th day (2.45-5.95%) (Figure 6).

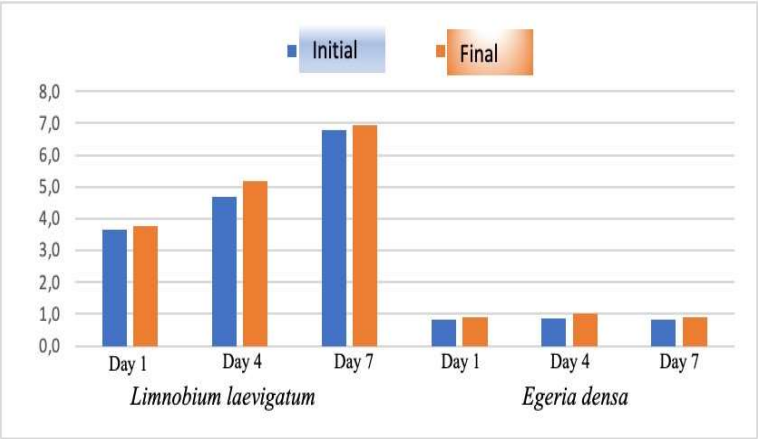

FIGURE 6. Initial and final weights of the plants exposed to 15 ppm Pb in 25% Hoagland solution

4. DISCUSSION

In this study we aimed to investigate the effects of Pb exposure on the growth rates of *L. laevigatum* and *E. densa* in two different experimental media. We found that, the stress caused by lead exposure did not suppress the growth rates of plants, in general. During the experiment, the plants generally showed no physical wear or deterioration. However, in some experiments we found growth rates were reduced followed by wilting and destruction of the leaves.

In general, the weight of *L. laevigatum* showed a gradual increase during the experiment, except for the 1st day, when it was exposed to 1 and 5 ppm Pb in pond water. The weight increment reached up to 9% in the following days of the experiment (Table 1). The final weight of the plants (*E. densa*) reached the highest level (11,64%) on the 4th day in 25% Hoagland solution and showed a gradual decrease at 7th days (Table 2). In a study where *Pistia stratiotes* L. was exposed to Pb for 7 days a decrease in the growth rates at the 7th day was observed [16]. In another study where *E. densa* was exposed to 3 different concentrations of vanadium, it was found that the average weight of the plant (*E. densa*) reached to its maximum level when exposed to highest V concentration (1.8 ppm) [17]. No significant changes were observed in the wet weight of the plant (*Scripus grossus* L. f.) in the first week when exposed to lead, but an increase of 25-50% was observed in the 2nd and 3rd weeks [18]. Interestingly [19], the growth rates for *P. stratiotes* decreased only at concentrations of 50 ppm and above when exposed to various Zn-Cd concentrations for 9 days.

In general, our results demonstrated that the plant growth was higher in Hoagland solutions indicating the necessity of macro and micronutrients to obtain a high

growth rate and therefore remediation capacity. In a similar study[20], it was shown that *Lemna* sp. L. and *P. stratiotes* L. showed the highest growth rates (14% and 12%) when grown in Hoagland solution. The decrease in the growth rates observed in the first day for both plants in both media might indicate the acclimation potential of plant in a very a short time (4 days or above).

When the responses of the two plants to different lead concentrations in the aquatic medium are examined (Tables 1 and 2), the growth rates of *E. densa* showed more variation than *L. laevigatum*. This change was more visible in *E. densa* in both directions (+,-). This might indicate that *E. densa* is less tolerant to Pb pollution when compared to *L. laevigatum*. A similar finding was also reported by Aran et. al., [14] stating that *L. laevigatum* was resistant to high lead and zinc concentrations. They also reported that the chlorophyll concentration of the plant showed only a slight decrease without exhibiting any morphological abnormalities in the leaves [15]. Higher growth rates were observed in *L. laevigatum* for %25 Hoagland media in 1 ppm Pb than *E. densa* during the 1st, 4th and 7th days.

This is the first study reporting the growth performance of *L. laevigatum* and *E. densa* exposed to lead (Pb). According to the results of this study, lead exposure did not significantly alter the growth rates of *E. densa* and *L. laevigatum* in the experimental media used for short term durations (up to 7 days). The results of this study may provide a better understanding on the responses of those two plant species under heavy metal stress.

Acknowledgement. I would like to special thank my friend Spec. Biologist Danial Nassouhi for his assistance during the experiment.

Author Contribution Statements. MBE-supervising, resources, conceptualization, writing–review & editing. FD-Conceptualization, Investigation, Data curation, Formal analysis, Writing. VCS-experimental analysis. All authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest

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Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 32, Number 1, Pages 70-86 (2023)
ISSN 1303-6025 E-ISSN 2651-3749
DOI: 10.53447/communc.1241869

Research Article; Received: January 24, 2023; Accepted: March 29, 2023

THE EVALUATION OF THE GROWTH PERFORMANCE OF A CYANOBACTERIAL ISOLATE *PHORMIDIUM LUCIDUM* (KÜTZİNG EX GOMONT 1892) GROWN UNDER DIFFERENT ENVIRONMENTAL CONDITIONS AND ITS USE AS A DIETARY SOURCE FOR *DAPHNIA MAGNA* (STRAUS 1820)

KÜBRA KARAKAŞ[1], KAMİL MERT ERYALÇIN[2], MEHMET BORGA ERGÖNÜL[1], TAHİR ATICI[3], SİBEL ATASAGUN[1],

[1]Department of Chemistry, Faculty of Science, Ankara University, Ankara, TÜRKİYE
[2]Department of Aquaculture and Fish Diseases, Faculty of Aquatic Sciences, Istanbul University, İstanbul, TÜRKİYE
[3]Department of Biology Education, Gazi Education Faculty, Gazi University, Ankara, TÜRKİYE

ABSTRACT. This study presents the effect of cyanobacterium isolated from Bolluk Lake (Konya, Türkiye) which is a saline lake on the growth performance of *Daphnia magna*. Isolated cyanobacteria species were identified as *Phormidium lucidum* according to its 16S rDNA sequences. The effects of different growth conditions including pH (7.18, 8.15, 9.17 and 10.26), light intensity (1200, 2400, 3600 and 4800 lux), temperature (10, 20, 25 and 30°C) and nitrogen concentrations (0.25, 0.5, 1.0 and 1.5 g/L) on *P. lucidum* was studied. Effects of each environmental factor on biochemical composition (total protein, total lipid and chlorophyll-a concentration) of *P. lucidum* were also studied. The optimum growth conditions were found as pH 7.18, ambient temperature 20°C, nitrogen 0.25 g/L and light intensity 3600 lux, after a 2-week incubation period. The effects of various mixtures of the cyanobacteria and *Chlorella vulgaris* which is a common feed for Daphniids were also evaluated for their effects on the growth rates of *D. magna*. The best growth rate for *D. magna* was obtained in the medium containing 100% *P. lucidum* at the end of the 13thday.

1. INTRODUCTION

Members of the cyanobacteria are called blue-green algae due to the pigments they contain. These prokaryotic organisms are known as the first photosynthetic organisms of the earth. They are mainly found in the oceans, thermal waters, freshwaters including lakes, rivers, streams, marshes, wetlands, permanent or temporary water bodies and saline ponds. They can also survive in very harsh environmental conditions such as temperatures up to 74°C, deserts, polar regions, rock interiors, terrestrial environments exposed to UV radiation [1-4].

Biomass increase in cyanobacterial cultures depends on various environmental factors such as pH, temperature, N and P content, salinity and light intensity [5-11]. Several studies indicated the changes in the concentration of metabolic

Keywords. Environmental conditions, *Phormidium lucidum*, biochemical composition, *Daphnia magna*, diet composition
erdmkubra@gmail.com- Corresponding author; 0000-0003-1376-9284
eryalcin@istanbul.edu.tr- 0000-0002-8336-957X
ergonul@gmail.com- 0000-0002-0263-9129
tahir@gazi.edu.tr- 0000-0002-3396-3407
satasagun@gmail.com- 0000-0001-8507-0961

products including total lipid, total protein and fatty acid produced when they are exposed to different environmental conditions such as pH, temperature, N and P amount, salinity and light intensity. It is also known that such parameters are species specific [11-13]. pH is considered one of the most important factors affecting the species diversity, growth and development of cyanobacteria [14-18]. Light intensity may lead to remarkable changes in the chemical composition, pigment content and photosynthetic activities of cyanobacteria [11,19-22]. Temperature may affect the growth and chemical composition of cyanobacteria through alterations in the metabolic rate [9]. Nitrogenous compounds are also known to effect cynabobacteria growth and chemical composition particularly lipid content [23-24].

Daphnia spp. is one of the most important and common food sources for freshwater fish both in aquaculture facilities and natural aquatic habitats. They are favored due to their rapid growth rate, high reproductive potential and short life cycles [25]. Daphniids tend to grow rapidly in environments where food items such as bacteria, yeast and microalgae are sufficient [26-27]. Several zooplanktonic species including Daphniids can not synthesize enough essential fatty acids and lipid. Although, several well known microalgal species are used widely in the culture of various zooplankton species including Daphniids [26-31], it is vital to determine lipid and fatty acid composition of new candidate microalgal and cyanobacterial species for Daphniid cultures. Extreme habitats such as alkaline lakes or saline lakes have a potential for isolation of new algal strains which could be used as an alternative dietary source for zooplankton cultures. Therefore, in this study we focused on a cyanobacteria species isolated from a saline lake, Bolluk Lake located in the Tuz Lake Basin which is home to several endemic taxa [32]. The strain was identified using 16S rDNA gene sequences and grown under several environmental conditions to determine optimum growth conditions. We compared chlorophyll-a, total protein and total lipid concentrations of *Phormidium lucidum* in order to assess its growth rates. Then, the growth rates of *Daphnia magna* fed with various mixtures of the isolate and a common green microalga, *Chlorella vulgaris* were tested to determine and compare the dietary potential of the isolate for *Daphnia magn*a culture. We choose *Chlorella vulgaris* as a control feed type since it is one of the most common feed types for Daphniids and thus enabling us to compare the nutritional value of the new isolate.

2. MATERIALS AND METHODS

2.1. Isolation of cyanobacteria *Phormidium lucidum*

Bolluk lake (38°32′25″N 32°56′34″E) is a salina lake located in Konya Closed Basin in Türkiye. This lake is chracterised by its high sodium sulphate content.

Water samples were taken with sterile dark colored glass bottles from 20 cm below the surface in duplicates. The samples were transferred to laboratory in ice boxes within 6 hours and were immediately inoculated in sterile flasks containing liquid BG-11 media. Samples were left to grow under a 2400 lux light intensity with white fluorescent bulbs, with a pH of 7.18 at ambient temperature (23 ± 2°C). Isolated algal cells were transferred to petri dishes and purified under aseptic conditions. Isolated algal culture under sterile conditions were added to 200 ml sterile flasks containing 100 ml of BG-11 medium for stress conditions experiment [33-34].

2.2. Phylogenetic analysis of isolated strain

Isolated cyanobacteria strain was identified according to its 16S rDNA gene sequences. EurX GeneMATRIX Bacterial & Yeast DNA isolation kit (Poland) was used for isolation of DNA from the isolated samples. In the PCR study (Kyratec thermocycler), gene regions targeted for species identification were amplified with 27F (5' AGAGTTTGATCMTGGCTCAG 3') – 1492R (5' TACGGYTACCTTGTTACGACTT) primers as universal primers. PCR steps were adjusted to 95°C for 5 min, 30 cycles of 95°C for 45 sec., 57°C for 45 sec. and 72°C for 60 sec. The amplification results obtained by PCR were carried out in 1.5% agarose gel prepared with 1x TAE buffer at 100 volts for 90 min and their image was taken in UV light using ethidium bromide dye.

2.3. Microalgae *Chlorella vulgaris* culture

Microalgae *Chlorella vulgaris* (CCAP-211/12) were provided by CCAP (Culture Collection of Algae and Protozoa, Scotland, UK). Freshwater microalgae *C. vulgaris* were cultured in 3N-BBM+V medium ($NaNO_3$+ $CaCl_2xH_2O$+ $MgSO_4x7H_2O$+ $K_2HPO_4x3H_2O$+ KH_2PO_4+ NaCl+ trace minerals+ Vitamin B1 and Vitamin B12) [35]. Cultured microalgae then harvested at stationary phase. In order to calculate growth numbers, microalgae were counted daily by Neubauer Hemocytometer. The microalgae culture was grown in liquid BG-11 medium at pH 7.18 for 14 days and freshly harvested *C. vulgaris* culture was used in the experiments.

2.4. Effects of experimental conditions (pH, light, nitrogen and temperature) on total protein, total lipid and chlorophyll-a concentrations in *P. lucidum*

In order to determine the optimum growth conditions for *P. lucidum* isolated from Bolluk Lake, we tested the growth performance of the cyanobacterium grown in BG-11 medium at different pH levels (7.18, 8.15, 9.17 and 10.26), temperature (10, 20, 25 and 30°C), light intensities (1200, 2400, 3600 and 4800 lux) and nitrogen concentrations (0.25, 0.5, 1.0 and 1.5 g/L ($NaNO_3$, Sigma

≥99.0%)). Experimental conditions tested were based on preliminary experiments and relevant studies [10-11,19,36]. Experiments were carried out in 200 ml flasks containing 100 ml of BG-11 at pH 7.18 for 14 days in triplicate. The results are given as the average of 3 repetitions.

2.5. Maintainace of *Daphnia magna* stock culture

Daphnia magna stock culture was maintained according to the procedure described by OECD guidline 211 (OECD, 2012) Stock cultures were kept in 100 lt aquaria under a photoperiod of 8 hours dark:16 hours light. One third of the water was changed every second day with dechlorinated tap water. The temperature for the stock cultures was kept constant at 20.2±1.3°C.

Daphnids used in the experiments were selected among individuals i. reproducing by parthenogenesis, and ii. were at least third generation and iii. age <24 hours. A total of 50 mature individuals were taken into the aquarium and the females were transferred to a new tank every week, and during this period the individuals ready to spawn were transferred to separate tanks and the newly hatched individuals were collected within 24 hours [37]. As a starting point, 10 individuals ready to lay eggs were used [38-40]. The experimental period lasted 21-days; a time in which *Daphnia magna* is reported to reach maxiumum output [26,41].

2.6. Growth rates of *Daphnia magna*

The nutritional value of *P. lucidium* were compared to *C. vulgaris* which is one of the most common feed types for Daphniid stock cultures. The mixtures tested were selected based on the approach reported by Lürling [42]. The mixtures including cynabocateria and *C. vulgaris* were mixed in a volume-to-volume ratio. To use cyanobacteria and microalgae cell density at approximate values, filamentous algae cells were transformed into smaller forms by morter. After obtaining the approximate cell density for both algae species, experimental feeding groups were created. Experimental feeding groups were 100% *Chlorella vulgaris* (100CV), 50% *Chlorella vulgaris* + 50% *Phormidium lucidum* (50CV+50PL), 25% *Chlorella vulgaris* + 75% *Phormidium lucidum* (25CV+75PL), 75% *Chlorella vulgaris* + 25% *Phormidium lucidum* (75CV+25PL) and 100% *Phormidium lucidum* (100PL) with 5 different food combinations (100 ml 10%, per 1 liter) have been evaluated. The use cell densities equally for all algae the filamentous cells were divided into small pieces in a mortar and the approximate cell number was obtained for both algae. The mixtures including cyanobacteria and microalgae were mixed in a volume-to-volume ratio.

The total number of daphniids were counted in each experimental food group every two days during the 21-day trial period to estimate the growth performance of *D. magna*. The formulae [43] given below was used for calculations (2.1).

$$r = (In\ Nt - In\ No)/t \quad (2.1)$$

No= Number of daphniids at the beginning
Nt= Number of daphniids at the end of each trial
t= Time (days) to reach the maximum number of individuals per unit volume (ml)
% r = Growth rate

2.7. Chlorophyll-a analysis

The chlorophyll-a concentration in the media for each cyanobacterium group was determined in aqueous 90% acetone (≥99.5%, Isolab) solution. The concentration of chlorophyll-a was found with optical absorption at 630, 645, 665 ve 750 nm, respectively [44]. Calculation is made and the amount of chlorophyll-a is calculated by writing the resulting value into the formula below (2.2).

Chlorophyll-a = (11.85 × OD665) – (1.54 × OD645) – (0.08 × OD630) × dilution rate (2.2)

2.8. Total protein (TP) and total lipid (TL) analysis

The protein concentration in the samples *P. lucidum* were measured spectrophotometrically [45] after homogenization within in a mortar. Total lipid concentration in each cyanobacterium group grown at different conditions was calculated using a modified method of Bligh and Dyer [46] with the following formulae (2.3).

Lipid% = [amount of lipid extracted (g)/ weight of microalgae sample (g)] × 100 (2.3)

2.9. Statistical analysis

The data are given as mean ± standard deviation (SD) of the 3 replicates for each group. The data were tested for goodness of fit to a normal distribution prior to the analyzes using Shapiro Wilk-W test. One Way ANOVA and Duncan multiple comparison tests were performed to analyze significant differences among groups. Results were considered significant where $P<0.05$. All statistical analyzes were performed using the statistical package program, SPPS (v23.0).

3. RESULTS

3.1. The physicochemical parameters of Bolluk Lake (Konya, Türkiye)

The physicochemical parameters of the lake water measured simultenaously a WTW portable multi meter device were as follows: temperature 28.9°C, pH 10.7, dissolved oxygen 5.37 mg/L and electrical conductivity 129 µs/cm.

3.2. Identification of isolated cyanobacteria

The 16S rDNA sequences of the isolated cyanobacterium indicated that the strain had a >99% similarity to *Phormidium lucidum* according to the NCBI Gen-Bank.

3.3. The effects of different growth conditions biochemical content in *P. lucidum*

3.3.1. pH

The total protein and lipid concentrations of the cyanobacterium *P. lucidum* grown at different pH levels (7.18, 8.15, 9.17 and 10.26) are given Figure 1a. The highest (26.7±0.02 mg/L) and lowest (13.4±0.07 mg/L) protein concentrations were observed in groups grown at 7.18 and 10.26, respectively. There was gradual decrease in the TP concentration with increasing pH levels. An opposite pattern was observed for TL concentration (Figure 1a); with the highest (11.5±0.07%) content observed at pH 10.26 and lowest (3.83±0.09%) when grown at pH 7.18. Chl-a concentrations of *P. lucidum* showed a decreasing pattern with increasing pH levels (Figure 1a). The highest and lowest chl-a concentrations were 1.88±0.41 µg/L and 0.41±0.26 µg/L when grown at pH levels of 7.18 and 10.26, respectively.

3.3.2. Light intensity

Biochemical responses of *P. lucidum* grown under various light intensities are summarized in Figure 1b. The highest TP (18.9±0.03 mg/L) and TL (7.96±0.39%) concentrations were observed in cyanobacterium grown under 3600 lux. Chl-a concentrations were found 0.661±0.034 µg/L, 0.537±0.013 µg/L, 0.459±0.016 µg/L and 0.343±0.032 µg/L when cultivated in media under 1200 lux, 2400 lux, 3600 lux and 4800 lux different light illumination, respectively.

3.3.3. Nitrogen concentrations

The effects of different nitrogen concentrations on the growth of *P. lucidum* was shown in Figure 1c. The TP concentrations showed a gradual increase with decreasing N concentration in the growth media. The highest TP concentration

23.74±0.01 mg/L was recorded in cyanobacteria grown in the media containing 0.25N g/L. Lipid concentrations showed a slight variation depending on the N content in the growth medium. There was gradual increase in the chl-a concentration with decreasing N concentraion. The highest (2.265±0.068 µg/L) and lowest (0.807±0.112 µg/L) chl-a concentrations were observed in groups grown at 1.5N g/L and 0.25N g/L, respectively.

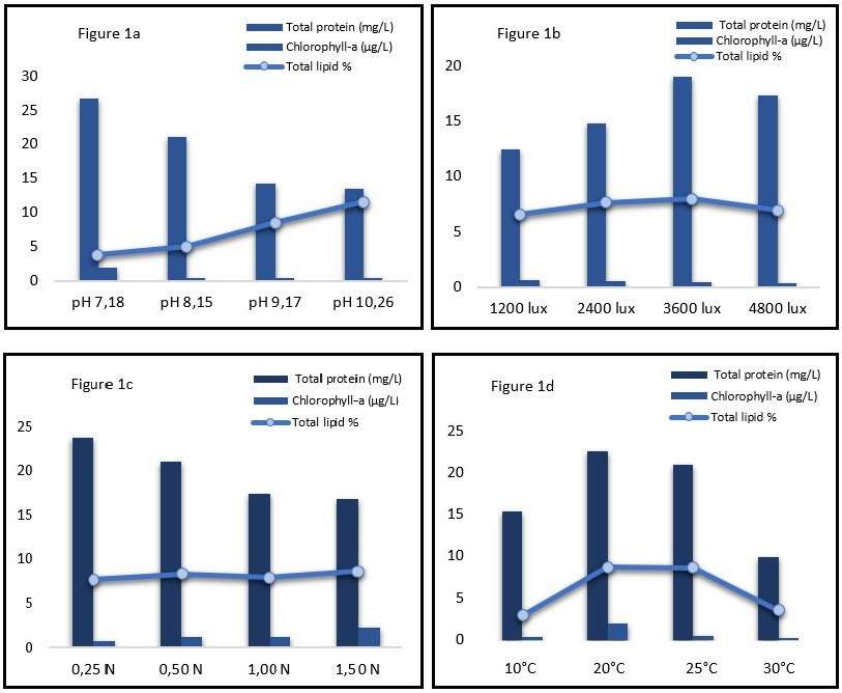

FIGURE 1. The other parameters applied while determining TP, TL and Chl-a amount in the stress conditions experiment, respectively; a: 2400 lux, 1N, 23 ± 2°C, b: pH 7.18, 1N, 23 ± 2°C, c: pH 7.18, 3600 lux, 23 ± 2°C, d: pH 7.18, 0.25 N, 3600 lux.

3.3.4. Temperature

In order to test the effects of increasing temperature on *P. lucidum*, the ambient temperature range was set to four different temperatures ranging from 10 to 30°C. Both TP and TL concentrations showed a gradual increase up to 30°C, but decreased at 25°C and reached to min levels at 30°C (Figure 1d). A similar trend was observed for chl-a levels with a highest level at 20°C (1.921±0.130 µg/L) and lowest at 30°C (0.171±0.072 µg/L).

According to the results mentioned above the optimum growth conditions for *P. lucidum* was set to 7.18 for pH, 3600 lux for light intensity, 0.25 g/L for N and 20°C for temperature. Bioexperiments for *D. magna* were carried out with *P. lucidum* grown in optimum growth conditions.

3.3.5. Evaluation of diet composition on the growth rates of *Daphnia magna*

Daphnia magna were fed with five different nutrient groups indlucing various concentrations of microalgae *C. vulgaris* and cyanobacteria *P. lucidum* to determine the optimum growth rates for *D. magna*.

4. DISCUSSION

The number of adult females and juveniles were calculated separately and it was found that there was no recruitment of juveniles up to the first 5 days of the experiments (Table 1). Similarly, there was no changes in the number of adult females during this initial phase. It was observed that the number of juveniles increased with the addition of *P. lucidum* to the as diet, while for adult females it increased when *C. vulgaris* was added to the as diet. In this study, it was seen that the use of PL as a diet supported the increase in the number of new individuals depending on the algae rate and it was seen that it gave weight to development and maturation depending on the adult female CV rate. However, a sharp increase was noted on the 13th day in all diet groups. The highest recruitment was observed in daphniids fed with 100% *P. lucidum* (Figure 2). An identical pattern was also observed for the number of adult females on the 13th day.

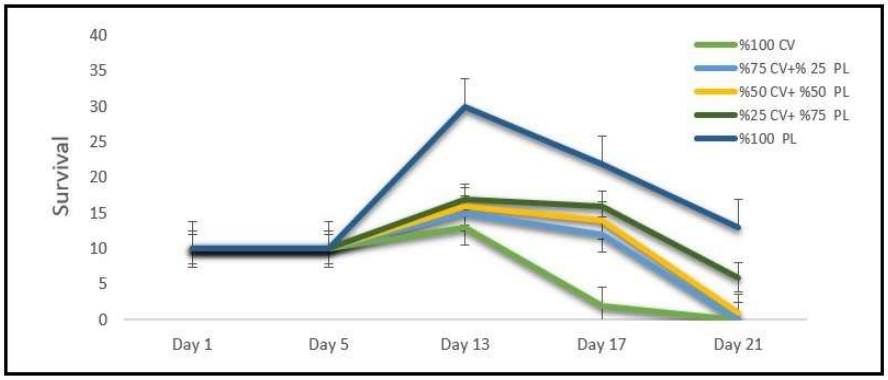

FIGURE 2. Number of individuals surviving different diets for 21 days experimental period

Bolluk Lake is an extreme habitat due to its high sodium sulphate and located in Tuz Lake Basin which is home to several endemic species, such as *Silene salsuginea* and *Saponaria halophila* [47-48]. Thus, have a potential to be home to new isolates of microalgae and cyanobacteria which might have a nutritional value for zooplankton cultures. Therefore, in this study we aimed to determine i. the optimum growth conditions (pH, light intensity, nitrogen and temperature) for the cyanobacteria, *Phormidium lucidum*, isolated from Bolluk Lake and ii. its

potential to be used as an alternative food source for *Daphnia magna*. Several studies indicated that the optimum growth rates and thus the biomass of various algal species depend on several environmental parameters including light intensity, temperature, nutrient composition, salinity and pH [5-11].

TABLE 1. *Daphnia magna* growth rate, adult females and juveniles individuals

Days /diets	**100% CV**	**75% CV + 25% PL**	**50% CV+ 50% PL**	**25% CV + 75% PL**	**100% PL**
Juveniles					
Day 1	0	0	0	0	0
Day 5	0	0	0	0	0
Day 13	5.333±1.528	8.333±3.055	9.667±2.082	9.667±2.309	21.333±4.163
Day 17	2±2	7.333±2.082	8.333±4.726	10.667±2.517	15± 6.083
Day 21	0	0	0.667±0.577	4.333±2.517	8.333±1.528
Adult females					
Day 1	10	10	10	10	10
Day 5	10	10	10	10	10
Day 13	12.524±0.577	6.667±0.577	6.333±1.528	7.333±1.528	9.333±0.577
Day 17	0.333±0.57	4.333±0.577	5.333±2.082	5.667±1.155	6.667±1.528
Day 21	0	0	0.333±0.577	1.667±0.577	4.667±2.517
Growth rate %					
Day 1	0	0	0	0	0
Day 5	0	0	0	0	0
Day 13	12.231	14.231	15.231	16.231	30.231
Day 17	1.411	11.411	13.411	15.411	21.411
Day 21	0	0	0.524	5.524	12.524

pH is considered as one of the most important factors affecting the growth rates, distribution and diversity of cyanobacteria species [14-18]. We found that TP and chl-a concentrations showed a gradual increase with decreasing pH levels with being highest at pH 7.18. An opposite pattern was observed for TL concentraions reaching maximum levels at pH 10.26. Chandra and Rajashekhar [18] reported that the optimum pH level ranges between 5.5-10 for *P. lucidum* and they observed highest growth rate at pH 7.5 with a maximum chl-a concentration. Other reports are also available; indicating optimum pH was 9 for *Spirulina platensis* with a considerably higher TP and chl-a concentration or demonstrating favorable pH levels were approximately 8 for *Halomicronema hongdechloris* [49]. Yadav et al. [11], found no differences for TL concentrations and biomass production among two different *Phormidium* sp. grown under different pH levels.

Several studies are available indicating the effect of light intensity on the growth of cyanobacteria species [11,19-22]. In this study we found that the highest TP and TL concentrations are observed in *P. lucidum* grown under 3600 lux. Yadav et al. [11], reported a similar pattern demonstrating that the increasing light intensity had a positive effect on lipid content in *Phormidium* sp. However, an opposite pattern was observed for chl-a concentrations with increasing light intensity, being highest at 1200 lux in our study. Similar results are available for

Phormidium sp. with a positive effect on biomass production and a decrease in chl-a concentrations with increasing light intensity had [21] and for *Spirulina platensis* with a decrease in chl-a concentration and an increase in TP concentrations with increasing light intensity [49] and for *Phormidium* sp. with the highest chl-a concentration at lowest light intensity [10]. Several studies report that higher light intensity is associated with lower chl-a concentrations, higher lipid content in algal cells [10,19], there are also studies showing increasing light intensity did not lead to significant alterations in *Nostoc spongiarforme* and *Phormidium corium* [50]. Furthermore, it is also known that color and photoperiod may affect the production of fatty acids in cyanobacteria [10,17,20-21,51-53]. It has been shown that optimum growth rates, chl-a, TP and TL concentrations vary depending on the species-specific light intensity and color requirements of the algal species including *Phormidium* sp., *Spirulina platensis, Nostoc sphaeroides, Scenedesmus abundans, Chlorella sorokiniana, Pseudanabaena galeata, Microcystis aeruginosa, Synechococcus* sp., *Cyanobium* sp., *Oscillatoria* sp. [10-11,19,49,54-58].

Nitrogen content is one of the cheapest and easiest methods used to increase lipid content in algal cells [59]. We found that the increase in nitrogen amount in the growth medium led to an increase on chl-a concentration of *P. lucidum*. On the other hand, we observed an opposite pattern for TP concentrations. Essential nutrients such as nitrogen are vital for growth and synthesis of proteins, nucleic acids and celular components in algal cells [60]. Cyanobacteria can use a variety of nitrogen sources, including nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) [23-24]. However, any changes in the concentrations of nitrogenous compounds or the sources used for nitrogen may lead to changes in the growth rate, development, biomass production, N_2 fixation rates, presence of heterocysts, chl-a, polysaccharide composition, secondary metabolite levels, TP and TL concentrations in cyanobacterial strains [56-57,60-64].

The changes in the ambient temperature affects the rate of cell metabolism and biochemical composition of cells [9]. Several studies have shown the effects of temperature are species speficic [9,16,65-66] and may lead to changes in protein production, biomass, growth rate and lipid content in algal cells [11,19,22]. In our experiments, we found that the highest growth rates and TP, TL and chl-a concentrations were observed in *P. lucidum* grown at 20°C. Yadav et al. [11], reported that TL content in *Phormidium* sp. increased with increasing ambient temperature. We also observed that P. lucidum was also able to tolerate temperatures up to 25°C. Similar findings were also reported by Hotos [10] who stated that optimum range is between 21 and 22.5 °C for *Phormidium* sp.

Each zooplankton species has its own specific requirements for suitable food type and quantity in order to obtain a high growth rate [67-69]. Several natural or commercially produced nutrient types are available for daphniids. *Ankistrodesmus* sp., *Botrycoccus* sp., *Chlorella* sp., *Cylindrospermopsis* sp., *Nannochloropsis* sp., *Scenedesmus* sp. and *Stephanodiscus* sp. are among the

most used natural food sources for *Daphnia* spp. [26-31,68]. However, no information exists on the use of *P. lucidum* as a food source for *D. magna*. Choi et al. [30], found that *C. vulgaris* was not effective to obtain a larger Dapniid size when used alone. However, they reported a larger size when *D. magna* was fed with *Stephanodiscus hantzschii-Chlorella vulgaris* or *Stephanodiscus hantzschii* alone. *Ölmez et al*. [29], found that highest growth rates for *D. magna* were recorded when they are fed with a containing *Scenedesmus acuminatus*. On the other hand, in another study where the mixtures of *Clamydomonas* sp. and *Chlorella* sp. were tested to feed *D. magna*, highest growth rates were observed when they are fed with single *Chlorella* sp. cell cultures [70]. Bednarska et al. [71], in their study with *C. raciborskii* and *S. obliquus*, stated that the first reproductive age increased in clones fed with *C. raciborskii* due to temperature and *D. magna* clones would gain durability in adapting to the environment. They found that feeding green algae promoted early maturation, increase in body size and egg size.

4. CONCLUSIONS

Daphnia magna is a valuable zooplankton species which is widely used in fish production. Although, several microrganisms including *Scenedesmus* spp., *Chlorella* spp. and yeast generally have been used for Daphniid culture [26-31,68-69] testing new microalgal strains for their nutritional value is vital for aquaculture development. In this study, the effects of the mixtures of *C. vulgaris* and the cyanobacterial isolate *P. lucidum*, on the growth rates of *D. magna* were investigated. We found that *Phormidium lucidum* is a productive species in terms of biomass under optimum growth conditions and has a potential to be used as an alternative feed for *Daphnia magna* cultures.

Acknowledgement. This work was financially supported by the Coordination of Scientific Research Projects of Ankara University (Research Project No: 21L0430011) in Türkiye. We would like to thank Ankara University Department of Hydrobiology for their support in the conduct of this study.

Author Contribution Statements SA-supervising, resources, conceptualization, writing–review. TA-conceptualization, investigation. MBE-data curation, formal analysis, manuscript editing. KME-project development, manuscript editing. KK-data analysis, manuscript writing and manuscript editing. All authors have read and approved the manuscript.

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

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