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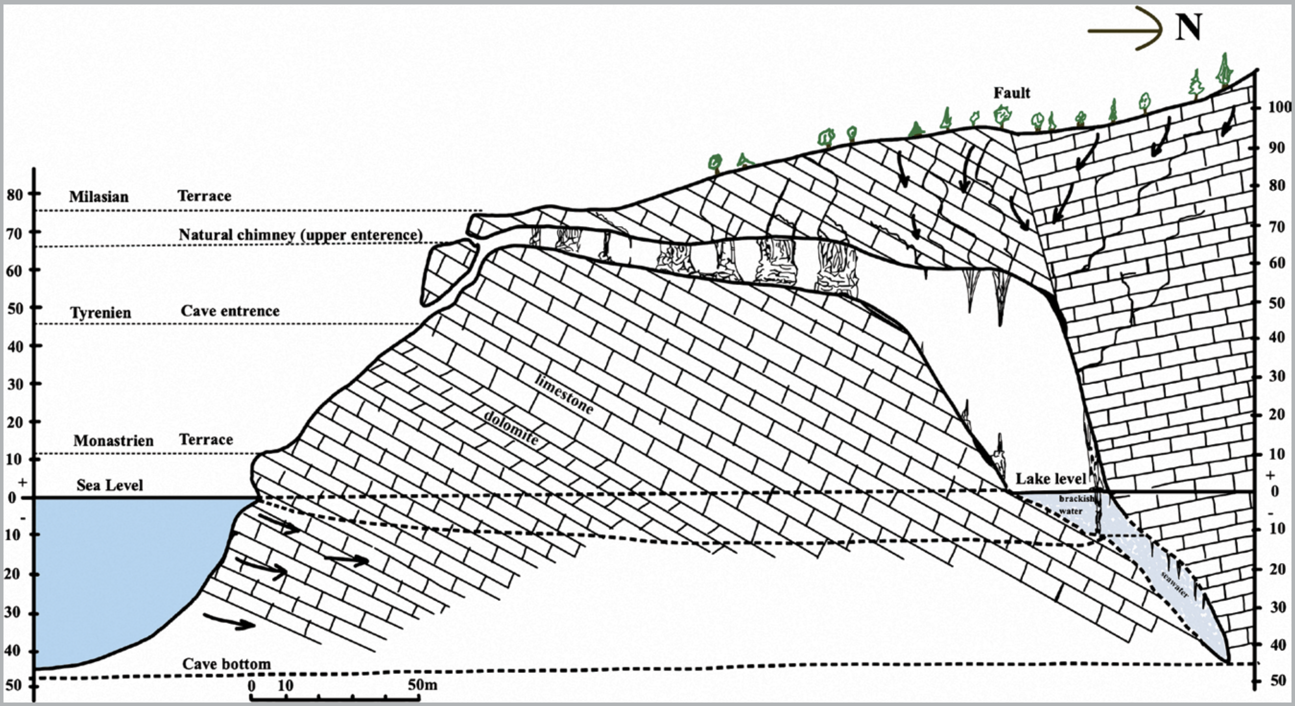

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Front cover: Cross Section of Gilindire Cave. See Deliceirmak and Karahan in this issue.

Joshua B. Mouser, David C. Ashley, Douglas L. Zentner, and Shannon K. Brewer. Seasonal context of Bristly Cave Crayfish *Cambarus setosus* habitat use and life history. *Journal of Cave and Karst Studies*, v. 84, no. 3, p. 85-95. DOI:10.4311/2021LSC0110

SEASONAL CONTEXT OF BRISTLY CAVE CRAYFISH *CAMBARUS SETOSUS* HABITAT USE AND LIFE HISTORY

Joshua B. Mouser[1], David C. Ashley[2], Douglas L. Zentner[1], and Shannon K. Brewer[3,4,C]

Abstract

Cave crayfishes are important members of groundwater communities, but many cave crayfishes are threatened or endangered. Unfortunately, we lack basic life history and ecological data that are needed for developing conservation plans for most cave crayfishes, especially the role of seasonal and annual fluctuations in structuring populations. Therefore, we determined the seasonal life history and habitat use of *Cambarus setosus* in Smallin Civil War Cave, Christian County, Missouri, United States. We conducted visual crayfish surveys over a 400 m section of the cave from 2006 to 2019. We used multinomial logit, multiple linear regression, and logistic regression models to estimate crayfish substrate, water depth, and water velocity use, respectively. All models included sex, carapace length, season, distance into the cave, and interactions between all variables and sex as predictor terms. We also used *t*-tests to assess morphometric differences between male and female crayfish. Six mark-recapture events (2010 to 2019) were used to estimate population sizes using a nil-recapture model. We attempted to age eight individuals using gastric mill bands, but annual bands were not discernable. We found reproductively active males during all seasons. We captured one ovigerous female during the spring, though ovigerous females were observed during show cave tours during spring, summer, and autumn. Male *C. setosus* were more likely to use homogenous and heterogeneous rock substrates and shallower and calmer water when compared to females; however, these relationships varied based on distance into the cave and season. Females sampled were significantly larger than males, and males regenerated chelae more often. Minimum population size estimates ranged from 9 to 159 individuals and indicated the population was relatively stable. Our data provide both a baseline population estimate for comparison with future studies and valuable trait information that is often lacking but useful for developing conservation efforts.

INTRODUCTION

There is broad recognition that cave crayfishes play an important role in groundwater ecosystems, and many populations are at risk of extinction. Crayfishes are keystone species that shape the structure and function of aquatic ecosystems (Paine, 1969). For example, crayfishes serve as aquatic nutrient cyclers (Momot, 1995) and are food for many species (e.g., >200 in the Ozark Highlands ecoregion (DiStefano, 2005)). In groundwater systems specifically, cave crayfishes are part of stygobiont communities that support clean water that is used for drinking and crop irrigation (Danielopol and Griebler, 2008; Boulton et al., 2008; Griebler et al., 2014). Cave crayfishes typically have narrow distributions (Larson and Olden, 2010) and K-selected life histories (e.g., long life span (Venarsky et al., 2012)), resulting in an intrinsically high risk of extinction. For example, approximately 70 % of stygobiont crayfishes are at risk of extinction (Taylor et al., 2007). The persistence of cave crayfish populations is threatened by water pollution, recreational caving, and invasive species (Graening et al., 2006; Mouser et al., 2019).

Effective conservation and management strategies for cave crayfish populations requires an understanding of their life history and habitat use (Moore et al., 2013; DiStefano et al., 2016; Taylor et al., 2019). We lack a basic understanding of both the biology and ecology of many crayfishes, especially cave-dwelling species (Taylor et al. 2019). The lack of basic biological and ecological knowledge is a major impediment in developing meaningful conservation efforts for subterranean organisms (Mammola et al., 2019). Life history data (e.g., age, fecundity, and recruitment) can be used to predict at-risk and invasive crayfish species (Larson and Olden, 2010), to help managers determine appropriate sampling techniques (Crandall, 2016), and to develop habitat restoration strategies that target life-stage requirements (Dyer et al., 2016). Management decisions also benefit from an understanding of a species' habitat requirements. For example, species-habitat associations are particularly useful to help direct restoration efforts (Smith et al., 1996), to control invasive crayfishes (Light, 2003), and to determine potential reintroduction sites (Renai et al., 2006).

The bristly cave crayfish *Cambarus setosus* is the most common cave crayfish of the Ozark Highlands ecoregion. *Cambarus setosus* has been documented at 48 sites (i.e., caves, wells, or springs) in Missouri and two sites in Arkan-

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sas with 164 individuals being reported from all sites (Graening et al., 2006). *Cambarus setosus* is currently listed as stable by the American Fisheries Society (Taylor et al., 2007), near threatened by the International Union for Conservation of Nature (DiStefano et al., 2021), and vulnerable by NatureServe (NatureServe, 2009) and by the Missouri Department of Conservation (Missouri Natural Heritage Program, 2021). Gardner (1986) noted that *C. setosus* was more abundant in stream sections characterized by a sandy, muddy substrate with scattered breakdown and abundant bat guano. Similarly, Marquart (1979) found *C. setosus* in silty substrates with rock, gravel, and organic debris. *Cambarus setosus* can reach 120 mm total length (TL); males can be reproductively active (i.e., form I) at 53 mm TL, and there is little difference in size between the sexes (Pflieger, 1996).

The previous studies of *C. setosus* habitat use and life history provide important insight but are mostly qualitative observations or short-term studies limited to a few caves (but see Marquart (1979)). These studies do not capture seasonal or annual changes within a cave and these dynamics (e.g., changing water levels or flow) may be quite important in structuring the life history and ecology of cave organisms (Jegla and Poulson, 1970; DiStefano et al., 2020). Therefore, the goal of our paper was to provide basic population information for *C. setosus* in a seasonal context. We specifically focused on basic life history (i.e., reproductive timing and size), habitat use (i.e., substrate, water depth, and water flow) and estimating minimum population size of *C. setosus*. Collectively, these metrics are useful for determining growth, mortality, and recruitment (Panfili et al., 2002) and can be used to protect or restore key habitat within caves.

METHODS

Study Area

We focused our study on *Cambarus setosus* within Smallin Civil War Cave (Smallin Cave), Christian County, Missouri, US. Smallin Cave is a show cave located within the Ozark Highlands ecoregion. The Ozark Highlands ecoregion is characterized by karst topography (Unklesbay and Vineyard, 1992), moderate climate (e.g., rainfall of 97–122 cm and average temperature of 13–16 °C (Adamski, 2000)), and mixed forest interspersed with lowland agricultural use (Woods et al., 2005). Smallin Cave has a handicap-accessible tour route elevated 1–2 m above the cave floor, which extends approximately 200 m into the cave from the entrance. From the end of the tour route, the cave extends an additional 700 m. A shallow stream with occasional pools meanders along the floor of the cave. Smallin Cave is home to one of the largest known populations of *C. setosus* with at least 47 individuals (Graening et al., 2006) and a relatively small population of bats (D.C. Ashley, personal observation).

Crayfish and Habitat Surveys

We conducted 38 visual surveys from November 19, 2006 to June 6, 2019, and we also report some opportunistic observations that were noted during cave tours in 2010. We sampled during spring (March–May, $n = 18$ surveys), summer (June–August, $n = 8$), autumn (September–November, $n = 5$), and winter (December–February, $n = 7$). Each survey covered approximately 400 m of the cave and consisted of 2 to 6 observers walking slowly from the cave entrance to the end of the study area visually searching for crayfish on the substrate. The remaining cave passage beyond the study area is a tight crawl passage and was not surveyed. We captured crayfish via hand nets, recorded distance into the cave from the dripline (i.e., cave entrance), and placed a 900 cm^2 frame at the capture location to estimate microhabitat use. Water depth (±1.0 cm) was measured in the center of each occupied grid and water velocity was visually estimated as: calm ($n = 161$), slow flow ($n = 51$), moderate flow ($n = 17$), or fast flow ($n = 4$). We combined slow, moderate, and fast flows into a single category (flowing) because these data were naturally bimodal when compared to calm water. Substrate was visually estimated as the proportion of the quadrat comprising mud/silt (particle diameter <0.01 cm), sand (0.01–0.2 cm), pebble (>0.2–6 cm), cobble (>6–20 cm), large rock (>20–26 cm), or bedrock (>26 cm). For each crayfish, we measured carapace length (±1.0 mm) and length of both chelae (±1.0 mm) using a ruler. We also recorded sex and whether crayfish were reproductively active (i.e., form I males or ovigerous females) or not (i.e., form II males or non-ovigerous females) using visual observation. Males were considered form I if the tip of the gonopod was corneous and pointed, and females were considered ovigerous if they were carrying eggs on their telson. The cave manager also recorded ovigerous females during show cave tours in 2010. On six surveys, a permanent marker was used to label the carapace with a unique number and a repeat survey was conducted 2–3 days later to count the number of marked and unmarked individuals (Table 1). We chose to use permanent marker because it is effective and safe for short-term recapture events (Ramalho et al., 2010) while being cheaper than other methods. Distance into the cave, habitat data, and crayfish morphometrics were not collected during most of the six repeat surveys.

We condensed the substrate estimates into four categories that we hypothesized to be ecologically relevant. We classified quadrats comprising 100 % bedrock, sand, silt, or clay as “bedrock/fine” because they serve as poor shelter for a crayfish (i.e., crayfish cannot burrow into bedrock and too much fine substrate results in suffocation; (Dyer et al., 2015)). Quadrats comprising a mixture of fines and other substrates were categorized as heterogeneous fine because excess fine substrates can suffocate crayfish (Dyer et al., 2015); however, this substrate would present usable shelter, and *C. setosus* has been shown to use this habitat (Marquart, 1979; Gardner, 1986). All substrate mixtures containing size distributions larger than sand, but excluding bedrock, were classified as "homogenous rock." Lastly, we classified quadrats with a mix of bedrock and homogenous rock as "heterogenous rock."

Analyses

We chose variables hypothesized to influence habitat use as predictor terms in our models (described in the next three paragraphs). Crayfish habitat use often differs depending on sex and season (e.g., DiStefano et al., 2013) and size (e.g., Dyer et al., 2016). Therefore, we hypothesized *C. setosus* habitat use depended on sex (female or male), size (i.e., carapace length (CL)), and season (i.e., spring, summer, autumn, or winter). Distance into the cave was included as a predictor term because habitat visibly changed throughout the cave. Lastly, we included interaction terms between sex and all other variables to account for possible differences in habitat use between males and females. Our analyses were conducted using the statistical software R (R Core Team, 2020), and $\alpha \leq 0.1$ was chosen *a priori* as our cutoff for significance.

We built a multinomial logit model to estimate the probability that crayfish were found in each of our four substrate categories (i.e., bedrock/fine, heterogeneous rock, homogenous rock, and heterogeneous fine). Sex, CL, season, distance into the cave, and interactions between sex and all other variables were included as predictor terms. Sex and season were treated as categorical variables with female and spring as reference categories. Our response variable was the probability that crayfish were found in each of the four substrate categories. We used the multinom function within the nnet package (Venables and Ripley, 2002) to fit our model as described by Faraway (2005). We started with the full model and removed predictor terms one at a time that most decreased Akaike information criterion adjusted for a small sample size (AICc; Burnham and Anderson, 2001) until removing terms no longer decreased AICc (Faraway 2005). We assessed model fit using a χ^2 test to compare the observed probability that crayfish were found in each substrate category to the probability predicted by the model (Yau, 2013).

We built a multiple linear regression model to predict water depth used by bristly cave crayfish. Sex, CL, season, distance into the cave, and interactions between sex and the other variables were included as predictor terms in the model. Sex and season were treated as categorical variables with female and spring as reference categories. Our response variable was water depth used by the crayfish. Water depth was natural-log transformed due to a right-skewed distribution (Zar, 1999). Two crayfish were found out of the water and those data were removed due to high influence on the model (i.e., Cook's distance > 0.5). We selected the best model using the same approach described above. Model assumptions and fit were determined via visual analysis of the quantile-quantile plot (Q-Q plot) and plotting residual versus predicted values (Freund and Wilson, 2003).

We built a logistic regression model to determine whether crayfish were more likely to be found in calm or flowing water. Sex, CL, season, distance into the cave, and interactions between sex and the other predictor variables were included in the model. Sex and season were treated as categorical variables with female and spring as reference categories. Our response variable was calm (0) or flowing water (1). We selected the best model using the selection approach previously described. We assessed model fit using binned residual plots because traditional residual plots are uninformative for models with binary response variables (Gelman and Hill, 2007).

We assessed morphometric and gender differences using *t*-tests ($\alpha \leq 0.1$). We used Welch's *t*-tests to compare groups with unequal variances as indicated by an *F*-test. We used unpaired *t*-tests to determine if there were significant differences in male and female crayfish for both average CL and average left and right chelae size divided by CL (i.e., adjusted for overall size). We hypothesized that a larger difference in left and right chelae size would reflect loss and partial chela regeneration resulting from aggressive behavior. Therefore, we also compared the left and right chelae size via paired *t*-tests for both males and females.

A nil-recapture model was used to estimate the minimum expected population size of bristly cave crayfish in Smallin Cave. The nil-recapture model was selected because recaptures during each event ranged from 0 to 2 and because Smallin Cave can be classified as an environment that is difficult to sample (Bell, 1974; Friedenberg et al., 2018). Using Bayesian methods improves the interpretation of the resulting model by allowing statements about the probability that abundance exceeds some lower bound. Because spatial data were not available, the spatial distribution of crayfish was assumed uniform and population estimates were obtained from the posterior gamma distribution where the shape and rate parameters were defined using:

$$\alpha = \alpha_0 + R$$

$$\beta = \beta_0 + \sum_{t=1}^{i=I} c_i m_i$$

where α is the shape parameter defining gamma distribution, α_0 is the prior for the shape parameter, R is the total number of recaptured individuals, β_0 is the rate parameter defining gamma distribution, c_i is the prior for the rate parameter, is the number of individuals captured in sample event *i*, m_i is the estimated number of individuals marked during sample event *i*, and *I* is the maximum number of sample events (Friedenberg et al., 2018).

This formulation of the nil-recapture model allows an estimate of minimum expected population size (Edwards, 1974) at various probability levels using the reciprocal obtained from the gamma quantile function. To provide a range of minimum expected population size, we *a priori* selected probability levels of 0.99, 0.95, and 0.75. This allowed us to be 99 %, 95 %, and 75 % confident, respectively, that the actual population was greater than or equal to our estimates. Every estimate was obtained using both uniform and Jefferys priors as they are convenient priors that produce equivalent estimates to lower bounds from frequentist and likelihood approaches, respectively. The use of two different priors also allowed us to better understand the effect of prior choice on our minimum expected population size estimates given our limited number of recaptures (Friedenberg et al., 2018).

Crayfish aging

We attempted to age *C. setosus* via the gastric mill to determine the longevity of the species following Mouser et al. (2020). Eight *C. setosus* that died naturally in Smallin Cave during summer 2018 were collected. We extracted the gastric mills, thinly sectioned them, and mounted them on microscope slides. We aged sections from multiple ossicles for each crayfish because the recovered crayfish were in various states of decomposition and not all of the ossicles could be located. The best section from each zygocardiac ossicle and pterocardiac ossicle, and the two best sections from the mesocardiac ossicle were mounted on each slide. Two readers attempted to age the slides together.

RESULTS

Cambarus setosus CLs ranged 3.0–45.0 mm (*n* = 399, mean = 22.7 ± 7.2 mm) and were captured during all seasons from a variety of habitats (Table 1). Location of capture ranged between 23–420 m from the dripline. Both male (*n* = 167, mean CL = 22.0 ± 6.0 mm) and female (*n* = 133, mean CL = 24.7 ± 7.5 mm) crayfish were collected during all seasons. Reproductively active males were collected during spring (*n* = 8), summer (*n* = 6), autumn (*n* = 5), and winter (*n* = 7), whereas a single ovigerous female was captured during the spring; all other crayfish collected were not reproductively active. However, ovigerous females were observed during cave tours in May, June, July, and November. The smallest form I male had an 18 mm CL and the smallest ovigerous female had a 30 mm CL. Crayfish were observed using a variety of microhabitats characterized by different substrate compositions: bedrock/fine substrate (*n* = 31), heterogeneous fine (*n* = 31), heterogeneous rock (*n* = 28), and homogenous rock (*n* = 142). Crayfish were found in 0–106 cm of water (mean = 20.4 ± 17.8 cm) and more often in calm water (*n* = 161) than flowing water (*n* = 72).

The results of the multinomial model indicated that distance into cave and sex were predictors of substrate use (Fig. 1 and Table 2). Male *C. setosus* were more likely to use homogenous and heterogeneous rock substrates than females, whereas female *C. setosus* were more likely to use bedrock/fine substrate than males. The magnitude of the difference was greater near the entrance of the cave for bedrock/fine and homogenous rock substrates. Results of the χ^2 goodness-of-fit test indicated multinomial model fit was appropriate ($\chi^2_6 = 8$, $p = 0.24$).

The results of the multiple linear regression model indicated that water depth use was related to season and distance into the cave, depending on sex (Fig. 2 and Table 3). Male *C. setosus* were more likely to use deeper water than females near the entrance of the cave, but more likely to use shallower water than females farther in the cave. Crayfish were found in shallower water more often in autumn compared to spring. The Q-Q plot and the residual plot showed no concerning trends, suggesting adequate linear model fit.

Similar to the multiple linear regression model, the results of the logistic regression model indicated that water depth use was related to season and distance into the cave depending on sex Fig. 3 and Table 4). Males were more likely than females to use flowing water near the entrance and less likely to use flowing water farther in the cave compared to females. Crayfish were less likely to use flowing water in the summer and autumn (i.e., negative relationship) compared to spring, when high flows are more common. The binned residual plot indicated good generalized linear model fit because 95 % of the binned residual were contained in theoretical error bounds, and the plot did not reveal any concerning trends.

We also found morphological differences between male and female *C. setosus*. Female crayfish were significantly larger than males ($t_{243.69} = 3.29$, $p < 0.01$). There was not a significant difference between male and female crayfish chelae size when adjusted for carapace length ($t_{286} = 0.31$, $p = 0.76$). Right and left chelae were not significantly different for male ($t_{157} = 1.09$, $p = 0.60$) or female crayfish ($t_{129} = -0.41$, $p = 0.68$). Although more males had regenerated or missing chelae (*n* = 37) than females (*n* = 29), this represented 22 %.

Our minimum population estimates of *C. setosus* were generally low but appear relatively stable through time (Fig. 4). The largest variability in minimum population size estimates was observed when a Jeffreys prior and a probability of 0.75 were used to estimate the minimum population size (range = 32–159). The smallest variability in minimum population size estimates was observed when a uniform prior and a probability of 0.99 was used to estimate the minimum population size (range = 9–23). The Jeffreys and uniform priors gave similar minimum population size estimates when

Table 1. Crayfish counts from visual surveys in Smallin Civil War Cave, Christian County, Missouri, United States. Female crayfish (Fem; *n* = 133) captured ranged from a minimum (Min) carapace length of 7 mm to a maximum (Max) of 40 mm. Male crayfish (*n* = 167) ranged from 8–45 mm. Sex and length were not determined (ND) for some crayfish (Unknown, *n* = 91).

Trip	Date	Female			Male			Unknown			Total
		Count	Min	Max	Count	Min	Max	Count	Min	Max	
1	19 Nov 2006	6	12	33	2	11	20	2	10	12	10
2	28 Jan 2007	4	7	21	5	15	24	2	ND	ND	11
3	18 May 2007	2	18	19	4	19	34	0	···	···	6
4	18 Nov 2007	2	19	27	6	14	30	3	3	ND	11
5	29 May 2008	3	27	40	3	12	30	0	···	···	6
6	20 Jul 2008	4	23	40	1	26	26	2	ND	ND	7
7	02 Nov 2008	5	21	28	4	28	35	2	ND	ND	11
8	15 Feb 2009	3	24	31	0	···	···	0	···	···	3
9	28 May 2009	5	23	40	1	33	33	0	···	···	6
10[a]	24 May 2010	5	20	28	3	26	30	4	ND	ND	12 (11)
11[b]	27 May 2010	3	22	35	2	10	13	3	ND	ND	8 (6,1)
12	28 May 2010	3	31	35	5	19	30	0	ND	ND	8
13	14 Aug 2010	4	7	19	6	12	30	2	ND	ND	12
14	14 Nov 2010	1	26	26	1	19	19	2	ND	ND	4
15	30 May 2011	0	···	···	1	19	19	0	···	···	1
16	19 Dec 2011	2	24	25	4	21	38	2	ND	ND	8
17[a]	23 May 2012	7	7	27	14	10	43	5	ND	ND	26 (26)
18[b]	25 May 2012	2	ND	ND	4	ND	ND	0	···	···	6 (6,1)
19	03 Jan 2013	4	15	33	4	15	24	1	15	15	9
20[a]	22 May 2013	7	25	35	1	35	35	0	···	···	8 (8)
21[b]	24 May 2013	0	···	···	4	20	25	1	ND	ND	5 (5,0)
22	21 May 2014	6	10	30	5	16	29	2	ND	ND	13
23	24 Jul 2014	5	12	26	9	14	25	5	ND	ND	19
24	16 Jan 2015	0	···	···	5	14	29	2	ND	ND	7
25	18 Jan 2015	2	22	25	2	19	33	3	ND	ND	7
26[a]	20 May 2015	7	15	30	8	12	30	2	ND	ND	17 (15)
27[b]	22 May 2015	1	ND	ND	5	ND	ND	4	···	···	10 (7,0)
28	25 Ma. 2016	4	25	32	4	16	27	4	ND	ND	12
29[a]	16 May 2016	4	20	34	6	20	26	0	···	···	10 (10)
30[b]	18 May 2016	1	37	37	0	···	···	12	ND	ND	13 (13,1)
31	26 Aug 2016	7	13	40	9	8	26	1	ND	ND	17
32	27 Feb 2017	3	29	32	8	12	30	0	···	···	11
33	24 May 2017	1	16	16	1	30	30	0	···	···	2
34	02 Aug 2017	8	17	29	5	21	30	1	ND	ND	14
35	26 June 2018	5	19	30	9	16	30	9	ND	ND	23
36	27 Nov 2018	4	11	20	7	18	28	7	ND	ND	18
37[a]	04 Jun 2019	3	18	45	9	16	29	3	ND	ND	15 (12)
38[b]	06 Jun 2019	0	···	···	0	···	···	13	ND	ND	13 (13,2)

[1] Crayfish were marked on this trip. Number in parentheses indicate the number marked.

[2] Crayfish were recaptured on this trip. Numbers in parentheses indicate the number of crayfish captured (first number) and how many of those were previously marked (if a second number is provided).

using a probability of 0.95 (Jeffreys = 17–55, uniform = 13–35) or 0.99 (Jeffrey's = 12–32, uniform = 9–23). The range of minimum population estimates (i.e., 10–32) during the initial mark-recapture period (May 2010) overlapped with the range of minimum population estimates (19–47) in the final mark-recapture period (June 2019), demonstrating relative stability through time.

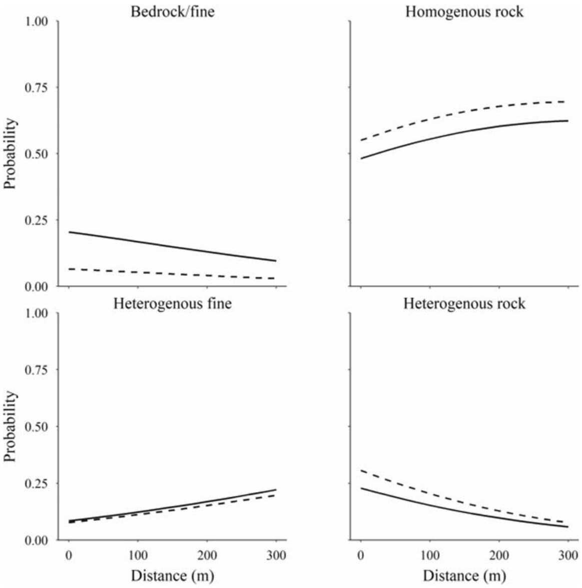

Figure 1. Predicted probabilities from our multinomial model of bristly cave crayfish *Cambarus setosus* using different substrate types in Smallin Civil War Cave, Christian County, Missouri, United States. A significant interaction between sex and distance into the cave indicates that male (dashed line) and female (solid line) crayfish have differing habitat uses depending on location within the cave.

Table 2. Mean and standard error (SE) estimates for variables included in the top multinomial model used to predict the probability of substrate category used by bristly cave crayfish *Cambarus setosus* in Smallin Civil War Cave, Christian County, Missouri, United States. Sex and season were treated as categorical variables with female, and spring as reference categories, respectively. Distance (±1 m) was a continuous variable representing the distance a sample was taken from the cave entrance. Probability of substrate use was modeled with respect to bedrock/fine substrate and parameter estimates of the environmental variables are given for each of the other categories.

Parameter	Mean	SE
Heterogenous fine-intercept	−0.88	0.91
Heterogenous rock-intercept	0.11	0.99
Homogenous rock-intercept	0.86	0.79
Heterogenous fine-male	1.05	0.72
Heterogenous rock-male	1.44	0.73
Homogenous rock-male	1.27	0.62
Heterogenous fine-distance	5.72×10^{-3}	4.92×10^{-3}
Heterogenous rock-distance	-2.03×10^{-3}	5.71×10^{-3}
Homogenous rock-distance	3.38×10^{-3}	4.48×10^{-3}

We used gastric mill sections in an attempt to age eight *C. setosus*, but our results were inconclusive. The carapace length of the aged crayfish ranged from 13.0–31.0 mm (mean = 19.1 ± 5.7 mm). None of the crayfish displayed clear yearly growth bands (Fig. 5). However, many gastric mills displayed hypothesized sub-yearly bands (Fig. 5).

DISCUSSION

In this study, we provide detailed information on the seasonal habitat use and life history of *C. setosus*. Caves are relatively stable environments in some ways (e.g., temperature); however, they can also be quite dynamic (e.g., flooding) (Simon, 2012). Many studies have shown that seasonal dynamics are important for structuring how cave ecosystems function (e.g., food flux during floods (Poulson, 2012), controlling life history of stygobionts (DiStefano et al., 2020)). Despite this knowledge, most data for *C. setosus* are limited to a short time frame. We found that seasonal dynamics are important for the reproduction and habitat use of *C. setosus* and may affect the formation of gastric mill bands.

Cambarus setosus reproduction appears to follow seasonal patterns, but shifts in seasonal patterns (e.g., rainfall) may alter reproduction timing. We found that crayfish reproduction in Smallin Cave occurred primarily in the spring and early summer (i.e., when the majority of ovigerous females were found); however, form-I males were found during all seasons. These results are similar to reproductive patterns found for surface crayfishes and other cave crayfishes. For example, many species of lotic crayfish in the Ozark Highlands ecoregion molt twice annually for reproductive purposes and lay eggs during the spring (Pflieger, 1996). Less is known about the reproductive habits of cave crayfishes; however, it appears cave crayfish or surface-dwelling crayfish that invade caves may reproduce more often under some circumstances and changes in seasonal patterns may shift reproductive timing. For example, Mouser et al. (2019) found that surface crayfish reproduced all year in a cave; however, most reproduction occurred during spring. Jegla (1966) observed that *Orconectes pellucidus inermis* also followed reproductive patterns similar to surface species; however, flood events were noted by Jegla and Poulson (1970) to shift reproductive timing. Similarly, DiStefano et al. (2020) observed ovigerous *O. stygocaneyi* in August a few months after heavy rainfall. Shifts in seasonal environmental patterns may explain why we found reproductively active crayfish during seasons not typically associated with reproduction.

We did not observe hypothesized yearly bands on *C. setosus* gastric mills, which is contrary to work on *Faxonius neglectus* found in surface streams (Mouser et al., 2020) and caves (Mouser et al., 2019). Although the mechanism is unknown, the lack of yearly bands may be due to the absence of seasonal changes in temperature that typically influence the formation of growth marks on hard structures (Wright et al., 2002), including gastric mills (Leland et al., 2015; Mouser et al., 2020). Caves have relatively stable temperatures; therefore, the absence of yearly bands may be due to constant growth in those systems or extremely limited growth occurring over much shorter intervals. Limited growth over shorter intervals may also explain the presence of sub-yearly bands, as sub-yearly rings have been observed on fish otoliths due to feeding changes (Wright et al., 2002). In contrast to our findings, Mouser et al. (2019) found that epigean *F. neglectus* in caves still displayed bands that seemed to reflect annual conditions, but crayfish

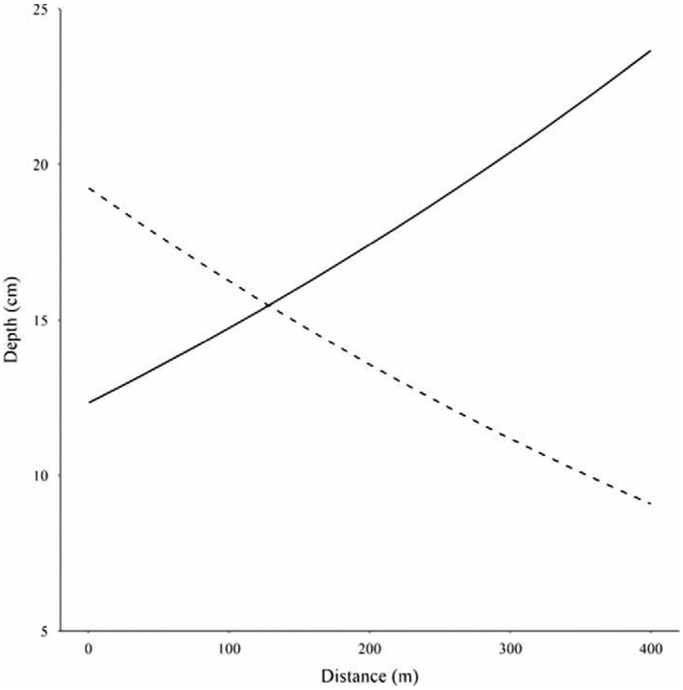

Figure 2. Relationship between water depth use by bristly cave crayfish *Cambarus setosus* and distance into Smallin Civil War Cave, Christian County, Missouri, United States. A significant interaction between sex and distance into the cave indicates that male (dashed line) and female (solid line) crayfish have differing habitat uses depending on location within the cave.

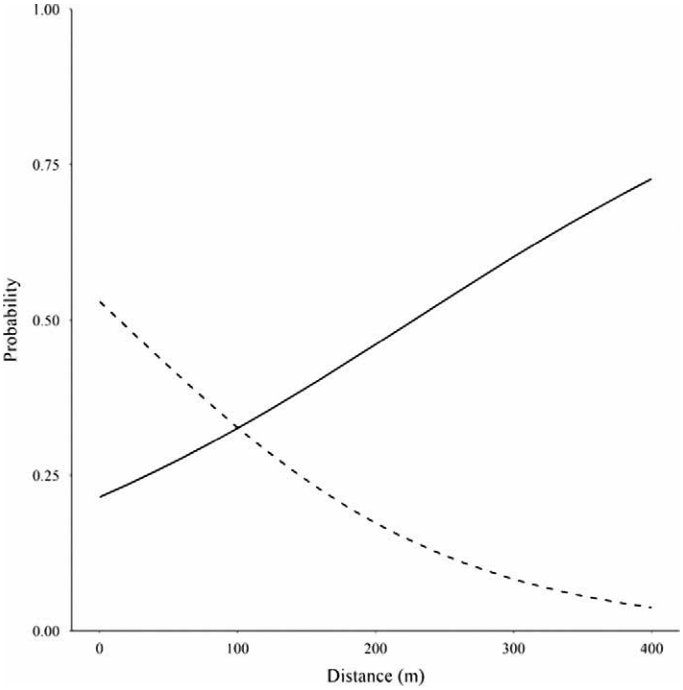

Figure 3. Predicted probability of flowing water use by bristly cave crayfish *Cambarus setosus* in Smallin Civil War Cave, Christian County, Missouri, United States. A significant interaction between sex and distance into the cave indicates that male (dashed line) and female (solid line) crayfish have differing habitat uses depending on location within the cave.

Table 3. Mean and standard error (SE) estimates along with *p* values for variables included in the top multiple linear regression model used to determine the association between bristly cave crayfish *Cambarus setos*us and water depth in Smallin Civil War Cave, Christian County, Missouri, United States. Sex and season were treated as categorical variables with female and spring as reference categories. Distance (±1 m) was a continuous variable representing the distance of the sample from the cave entranc**e.**

Parameter	Mean	SE	*p* value
Intercept	2.52	0.28	<0.01
Male	0.45	0.34	0.19
Distance	1.71×10^{-3}	1.42×10^{-3}	0.23
Summer	0.10	0.17	0.53
Autumn	−0.42	0.22	0.06
Winter	−0.29	0.21	0.18
Male × distance	-3.50×10^{-3}	1.88×10^{-3}	0.06

Table 4. Mean and standard error (SE) estimates along with *p* values for variables included in the top logistic regression model used to predict the association between bristly cave crayfish *Cambarus setosus* and flowing water in Small Civil War Cave, Christian County, Mi ssouri, United States. Sex and season were treated as categorical variables with female and spring as reference categories. Distance (±1 m) was a continuous variable representing the distance of the sample from the cave entrance.

Parameter	Mean	SE	*p* value
Intercept	−1.29	0.72	0.07
Male	1.41	1.07	0.19
Distance	5.69×10^{-3}	3.68×10^{-3}	0.12
Summer	−0.96	0.49	0.05
Autumn	−2.28	1.08	0.04
Winter	0.14	0.50	0.29
Male × distance	−0.01	6.79×10^{-3}	0.03

movement out of the cave or food fluctuations derived from maternal colonies of gray bats *Myotis grisescens* may be contributing factors. Using the gastric mill to age cave crayfish could be valuable because it is difficult to determine the longevity of cave crayfish through traditional methods (Venarsky et al., 2012). However, this technique might not be useful for populations with low abundances unless natural mortality is observed. Further, more laboratory work needs to be completed on band formation before the technique could be considered for subterranean crayfishes.

Male and female *C. setosus* are found in different habitats depending on the location within the cave. The observed differences in habitat use between the sexes could be explained by more dominant males excluding females from ideal habitats (Fero and Moore, 2008). However, chelae size was not significantly different in male and female crayfish, but other forms of behavior (e.g., pheromone releases (Schneider et al., 1999)) may cause females to avoid males. Females were typically larger than male crayfish, and body size can influence cover use (Streissl and Hödl, 2002; Dyer et

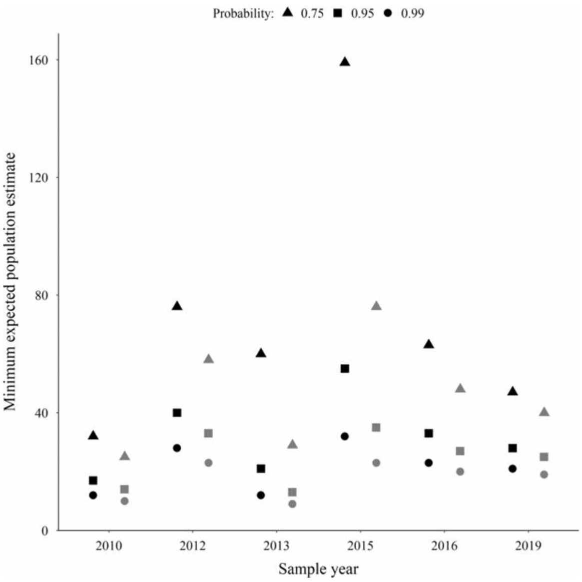

Figure 4. Minimum expected population estimates from our nil-recapture model for the bristly cave crayfish *Cambarus setosus* in Smallin Civil War Cave, Christian County, Missouri, United States. Points represent the minimum population estimate when there is a 75 %, 95 %, or 99 % chance that the true population is greater than or equal to the population estimate when using a Jefferys (gray shapes) or uniform (black shapes) prior.

al., 2016). For example, male *C. setosus* may avoid faster water because smaller crayfish are more likely to be swept downstream (Hobbs, 1978; Caine, 1978). Female crayfish could be associated with bedrock because they are too large to take refuge under other substrates and crevices in the bedrock that may provide cover.

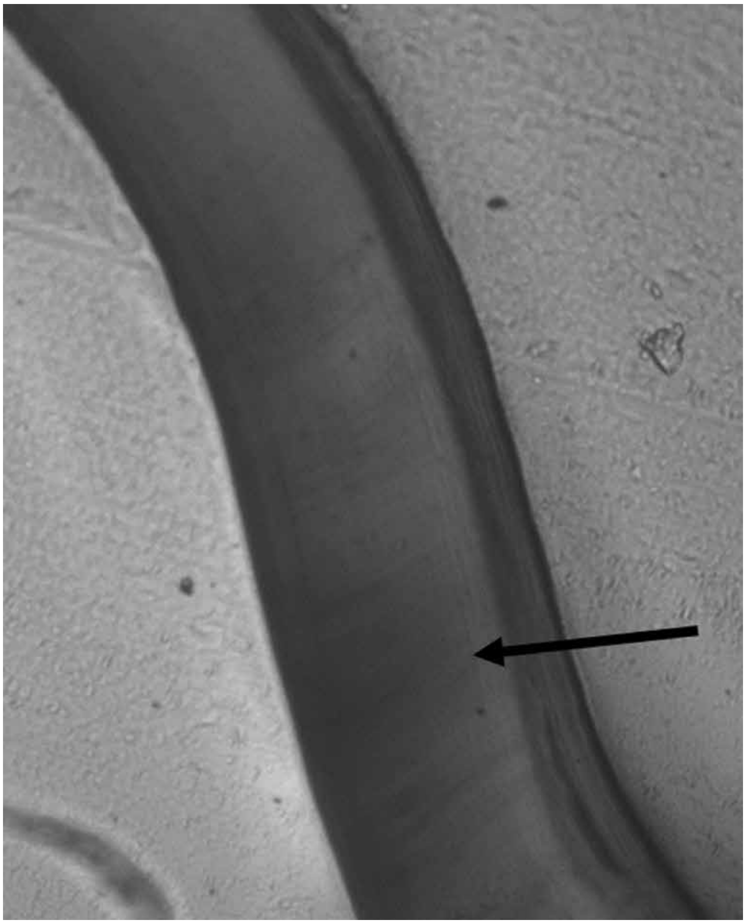

Figure 5. Photomicrograph of a bristly cave crayfish *Cambarus setosus* gastric mill section from a 30 mm carapace length individual collected from Smallin Civil War Cave, Christian County, Missouri, United States. No clear yearly bands are visible; however, faint bands can be seen and are denoted by the arrow. We hypothesize that these bands are sub-yearly in nature and may represent unidentified cycles such as feeding or temperature (Wright et al., 2002; Mouser et al., 2020).

Our minimum population size estimate of *C. setosus* fluctuated over the 6 mark-recapture events. Our observed fluctuations were greater than the minimum population estimates; thus, our results suggest the population may be vulnerable to extirpation given catastrophic events. However, vulnerability is common when populations are isolated (Bland, 2017). Despite the general fragility of isolated populations, our minimum population size in 2010 overlapped with our estimate from 2019 suggesting some population stability that has been demonstrated in other cave-dwelling populations (e.g., Hobbs, 1978). Seasonal variation in the relative abundance of cave organisms is typical (Barr, 1967) and high numbers of individuals can be difficult to obtain (Cooper and Cooper, 1997; Miller and Niemiller, 2008), making longer term studies of cave species valuable. If future population estimates are desired for comparison to our estimates, studies would benefit from the increasing array of diminutive tags available for recapture studies over longer time periods (e.g., passive integrated transponders (Musselman et al., 2017), visible implant elastomer tags (Bolland et al., 2009; Venarsky et al., 2012), and p-Chips (Tenczar et al., 2014; Moore and Brewer, 2021)). Moreover, quantitative advancements that facilitate more robust mark-recapture designs would be beneficial to improving our understanding of population fluctuations (e.g., Royle-Nichols (Nakashima, 2020)). Lastly, estimating detection bias in population estimates would be desirable (Royle, 2004), but we were unable to do so because habitat was not measured on most of the repeat surveys.

Our results reflect the life history and basic ecology of a single population of *C. setosus* but adds to the growing body of literature necessary to conserve cave crayfishes and overcome our limited knowledge of species traits (Mammola et al., 2019). It is important to recognize that populations have genetic differences, which may translate into phenotypic or life history differences. Therefore, it is beneficial if future studies consider investigating cave crayfish population traits to assess generalizations that can be extended to other cave systems. We found that males can reproduce when their CLs reach 18 mm, and these data can be used in population models to predict changes when different management options are applied (e.g., Crouse et al., 1987). Conservation efforts focused on maintaining the natural habitat within the cave would be advantageous given the crayfish appear to use many different habitat components depending on their sex, the time of year, and location within the cave. Knowing the reproduction timing of crayfish can also aid in management decisions. For example, environmental DNA surveys would be most effective when they coincide with reproductive periods (e.g., de Souza et al., 2016). In contrast, it may be beneficial to avoid recreational caving during reproduction to avoid crushing crayfish which is a significant source of mortality (Graening et al., 2006).

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16S rRNA DIVERSITY OF MIRROR LAKE IN GILINDIRE CAVE (TURKEY) SHOWS ABUNDANT *NITROSPIRA*

Selin Deliceirmak[1,2,3,C] and Arzu Karahan[3]

Abstract

We present the prokaryotic microbial diversity of Mirror Lake, located at the end of Gilindire Cave (Turkey), whose geomorphology shows development in multiple geologic periods and by multiple mechanisms. The lake comprises brackish water with both fresh and seawater inputs. In total, 5 liters of water was sampled from Mirror Lake and was filtered through a 0.22 µm membrane, and after the DNA isolation, 16S amplicon sequencing was conducted to get whole prokaryotic diversity. The bacterial community of this system is predominately composed of nitrite-oxidizing *Nitrospira* with a relative abundance of 28 %. We hypothesize that *Nitrospira* recovered in our samples mediates nitrification by reciprocal feeding with ammonia-oxidizing archaea (*Nitrososphaeria*). We found *Nitrospira* had a close association with Planctomycetes CL500-3 clade and Marinimicrobia (SAR406) in the cave habitat, with a relative abundance of 8.3 % and 5.7 %, respectively. To our knowledge, this is the first time that the presence of marine clade SAR324 has been reported from brackish cave waters.

INTRODUCTION

Gilindire Cave, also known as Aynalıgöl Cave (Mirror Lake Cave, is located in the Aydıncık district, part of the Mersin province of Turkey (Fig. 1A). The name Aynalıgöl comes from the lake located at sea level in the deepest part of the cave that reflects images like a mirror (Fig. 1B). A shepherd discovered the cave by chance in 1999. He noticed a hedgehog in the steep rocky slopes of Aydıncık when he was trying to protect himself from the scorching Mediterranean sun. He followed the hedgehog disappearing among the rocks and found the cave entrance. After baseline characterization of the environment, the cave opened for visitors. It receives approximately 50,000 visitors annually (personal communication with Aydıncık Municipality). Visitation is limited to between 8:00 a.m. and 5:00 p.m. from November to April, and 8:00 a.m. and 7:00 p.m. in the other months. Visitors go down 560 steps to reach the lake. Safety measures include cage-like railings around the stairs (Fig. 1C). Visitors are directed to a balcony by the lake by the steel path with

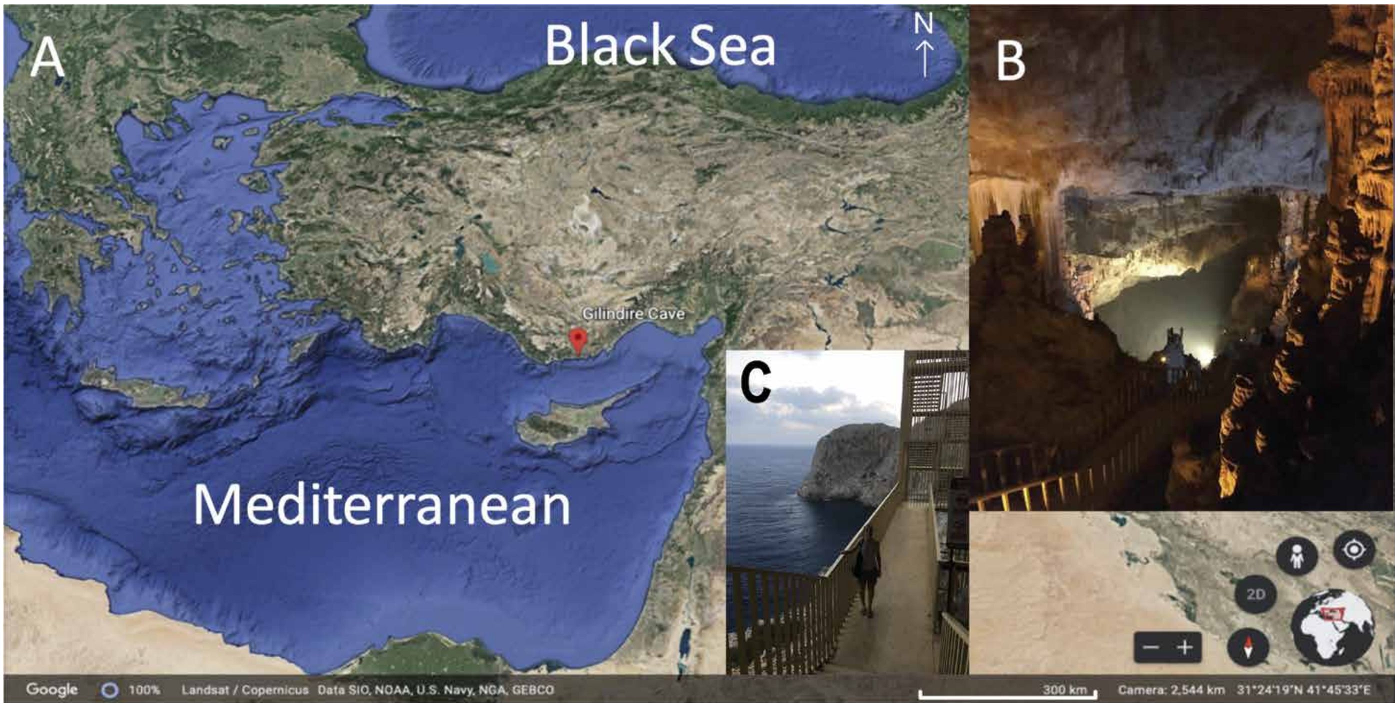

Figure 1. (A) Location of the Gilindire Cave (source: GoogleEarth https://earth.google.com/web). (B) A picture from inside the cave with lake view, in 2017. (C) A picture from the entrance section of the cave, 2017.

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handrails from the cave entrance, but they cannot come in contact with the lake water. There are artificial light sources along with the stairs around the lake, and the lights are on only if there is a visitor.

The first comprehensive study of the geological and hydrological features of Gilindire Cave was conducted by Nazik et al. (2001). The cave contains stalagmites, stalactites, and pillars that divide the interior into many small chambers. There are three main chambers in the cave: 1) the entrance, 2) the main gallery, and 3) the gallery with Mirror Lake. The lake is located at the northeast end of the cave (Fig. 2), 46 m below the cave entrance. The cave formed during the Würm glacial stage that began about 70,000 years ago when the Mediterranean Sea was at its lowest level.

Prokaryotes show high resilience coping with changing environmental conditions; they evolved different strategies from heterotrophy to autotrophy and survived under various conditions, both anoxic to oxic. Recent advances in molecular techniques, such as next generation sequencing, enable scientists to discover microbial diversity in various habitats without the need to culture them.

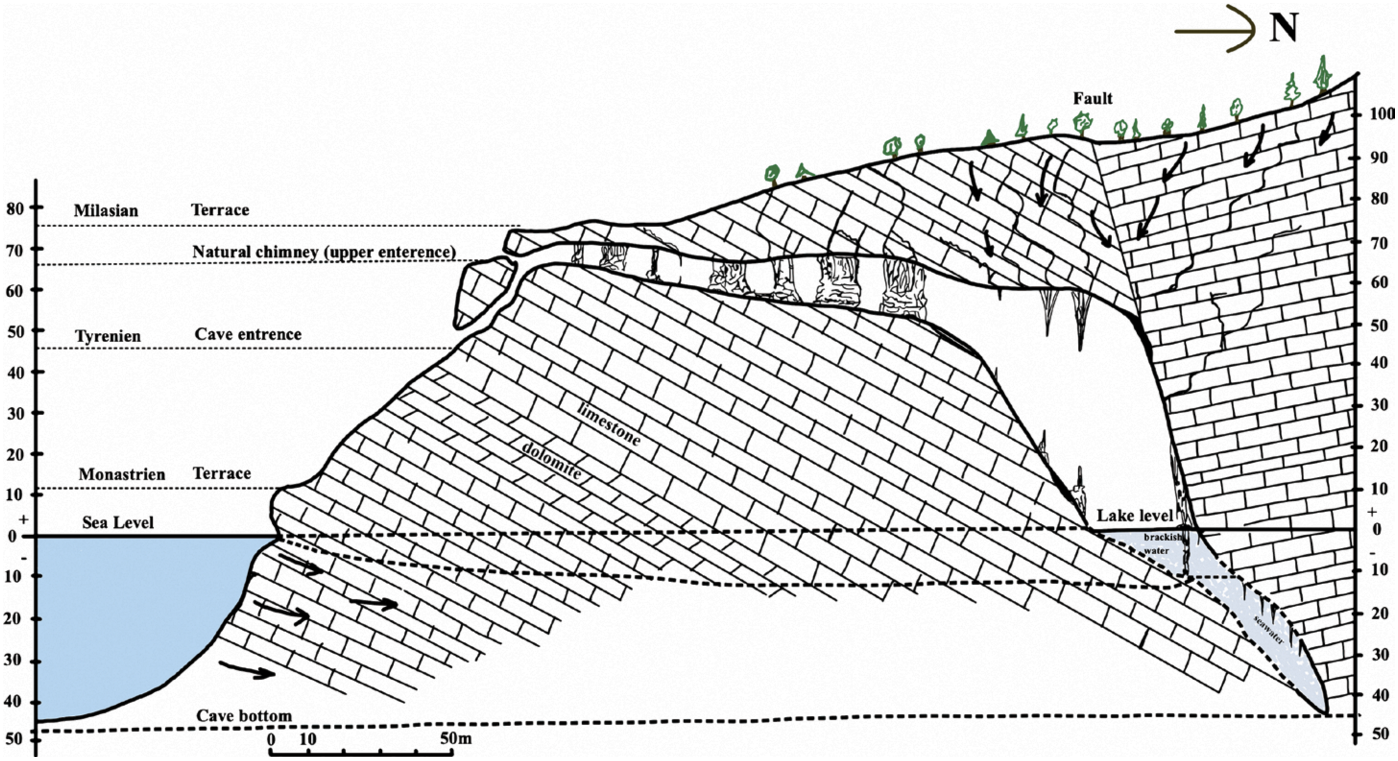

Figure 2. Geomorphological section of the Gilindire Cave (redrawn from Nazik et al. 2001). Arrows indicate infiltrating water.

Here, we present the first culture-independent amplicon sequencing study from the surface water of Mirror Lake in Gilindire Cave to identify the microbiota of the brackish lake. We recognize the nitrification potential of the cave environment, because the most dominant bacteria are the nitrite-oxidizing *Nitrospira*.

MATERIALS AND METHODS

Sampling Site

Gilindire Cave is located at 36°07ʹ58.08″’ north latitude and 33°24ʹ11.04″ east longitude. The area exhibits a typical Mediterranean climate, and the temperature reaches around 40 ºC during the summer with an average of 80 % humidity. The surface vegetation of the study area is dominated by maquis. The cave was developed within Cambrian limestone or dolomitic limestone as a result of two faults intersecting each other in a northeast-southwest direction. The cave developed in multiple geologic periods and by multiple mechanisms. Erosion surfaces belonging to the Monastrien-I, Thyrrenian, and Milazzian periods are observed in the cave. The main chambers in the cave were formed by different processes. The entrance of the cave is located on the steep rocky slopes 46 m above present-day sea level. The first part of the cave might have been connected to the surface during the Thyrrenian period. The second part (the main gallery) of the cave is dominated by the speleothems developed during the Pliocene. The lake chamber is the youngest part of the cave and developed along a prominent fault during the Würm glaciation due to the 90 m lowering of the sea. This part initially developed as a result of karstification and then filled with seawater due to the rising sea level again. Mirror Lake is located at the end of the cave, 46 m below the cave entrance and 250 meters inland from the coast.

The total depth of the lake is 46 m. While the first 10 m are brackish, salty seawater can be found below this depth from intrusion along fracture zones (Nazik et al., 2001). According to physicochemical measurements, the salinity increases with depth (2.4 ppt at the surface and 31.7 ppt at a depth of 27 m), whereas temperature is observed to be relatively constant (about 21 °C). Saturated oxygen concentrations also decreased throughout the depth (86.6 % at the surface and 43.5 % at a depth of 27 m) (Nazik et al., 2001). Physicochemical measurements from the deepest part of the lake (46m) were not reported elsewhere.

Sampling

The water sample was collected from Mirror Lake on July 31, 2017. In total, 5 L of water were collected using a sterile bottle from the lake's surface (approximately the first 15 cm of depth). The bottle's lid was closed immediately to avoid contamination, and the bottle was carried out in a sterile plastic bag. The water sample was then transported to the Institute of Marine Sciences, Middle East Technical University (IMS-METU) laboratory, which is an hour away, and then filtered through a 0.22 µm-pore MoBio polyethersulfone membrane.

DNA Extraction, Sequencing, and Analysis

Total DNA extraction from the filter was processed in the IMS-METU genetic laboratory. DNA extractions were carried out using the protocol of Paz et al. (2003). The filter was placed into 2 mL vial and 1000 µL of lysis solution (0.25 M Tris Borate, pH 8.2, 0.1 M EDTA, 2 % sodium dodecyl sulfate, 0.1 M NaCl) and the same volume of phenol/chloroform/isoamyl alcohol solution (25:24:1 v:v:v) was added. The sample was incubated for a week at room temperature and then mixed thoroughly (1 min.) before centrifugation (14,000 rpm, 10,000 *g*, 5 min.). The aqueous phase was collected, added to the same volume of chloroform-isoamyl alcohol solution (24:1 v:v), thoroughly mixed (1 min.), and centrifuged (14,000 rpm, 10,000 *g*, 5 min.). Genomic DNA was then precipitated by further centrifugation with cold 100 % ethanol (14,000 rpm, 10,000 *g*, 15 min.). The alcohol was removed and the DNA was twice washed with 2 mL of 70 % ethanol, then centrifuged again (14,000 rpm, 10,000 *g*, 15 min.). The DNA pellet was then dried in a ventilated hood, dissolved in 50 µL sterile, molecular-grade water, and quantified using a nano drop spectrophotometer. DNA samples were kept at −20 °C until sending to the sequencing company. PCR amplification and amplicon sequencing were performed by Macrogen Inc. (Macrogen-Europe) for both directions (forward and reverse) by using the Illumina MiSeq platform (300 bp paired-end sequencing). Herlemann et al. (2011) primer pairs (341F- CCTACGGGNGGCWGCAG and 805R-GACTACHVGGGTATCTAATCC) were used to amplify two variable sites (V3-V4) of the 16S rRNA gene. All sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al., 2013). Each read was aligned using the SILVA Incremental Aligner version 1.2.10 for ARB SVN (revision 21008) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). Reads shorter than 50 aligned nucleotides and reads with more than 2 % of ambiguities or 2 % of homopolymers were excluded from further processing.

Reads were dereplicated and the unique reads were clustered into operational taxonomic units (OTUs), and the reference read of each OTU was classified. Dereplication and clustering were done using cd-hit-est (version 3.1.2; http://www. bioinformatics.org/cd-hit) (Li and Godzik, 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.95, respectively. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 132) using BLASTN (version 2.2.30+) with standard settings (Camacho et al., 2009). Reads without any BLAST hits or reads with weak BLAST hits remain unclassified. These reads were assigned to the meta group "no relative" in the SILVAngs fingerprint and Krona charts (Ondov et al., 2011). The associated sequencing data was deposited in the NCBI BioProject under the accession ID PRJNA789137.

RESULTS AND DISCUSSION

In total, 162,559 sequences were recovered from the brackish water of Mirror Lake's surface. The number of OTUs was 12,839, and 94.9 % of all sequence reads were classified. At the phylum level, the three dominant taxa in our sample were Proteobacteria (36 %), *Nitrospirae* (28 %), and Planctomycetes (12 %). Phylum Marinimicrobia (SAR406 clade), Bacteroidetes, Patescibacteria, and Verrucomicrobia were represented by relative abundances between 2.7 % and 5.7 % (Fig. 3). In total, 35 phyla were detected, of which 3 were archaeal. The relative abundances of the rest of the taxa recovered from our sample were each less than 1 %. The water sample was taken from the surface of the brackish zone, which extends to a depth of 10 m. The groundwater, which reaches the lake through rainfall, is mostly mixed with seawater at the surface of the lake (Nazik et al., 2001). We recorded the presence of many marine isolates in our samples. The presence of marine isolates might be as a result of seawater inflow from the fracture zones.

At the phylum level, Proteobacteria were the most abundant (36 %) taxa in our study and were represented by Alphaproteobacteria (20 %), Gammaproteobacteria (10 %) and Deltaproteobacteria (6 %). The genus *Gemmobacter* is represented by 4.2 % relative abundance in the recovered OTUs. The utilization of methanol and formate by various

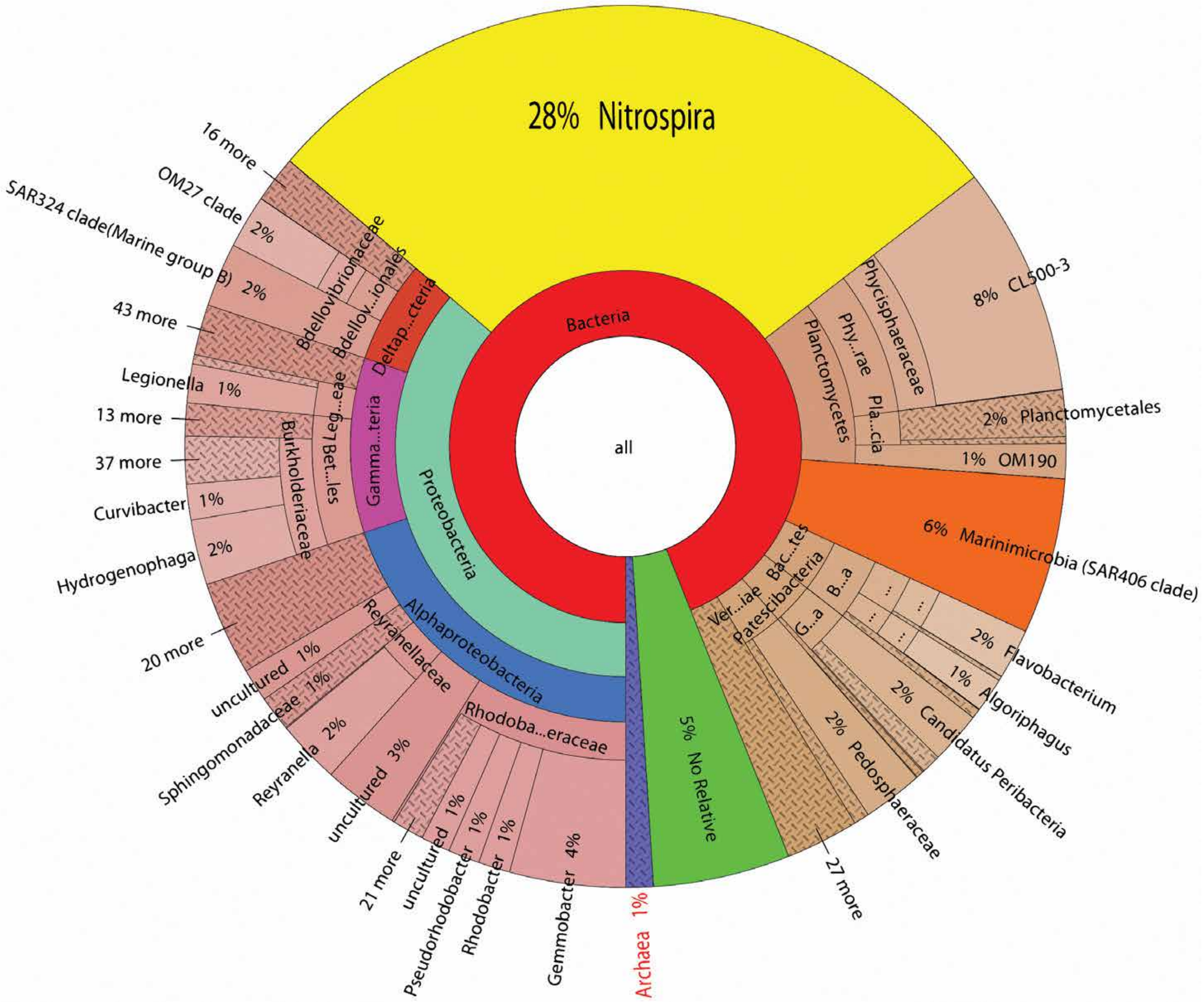

Figure 3. Taxonomic abundances of phyla observed in the Gilindire Cave. Dark blue colored pie slice at the bottom represents the Archaea (relative abundance of 1 %). OTUs belonging to the archaeal sequences were affiliated with Thaumarchaeota and Nanoarchaeaeota.

species of *Gemmobacter* genus has been reported previously (Chen et al., 2013; Hameed et al., 2020; Kröber et al., 2021). While they comprise a relatively small portion of total abundance in Gilindire Cave's Mirror Lake, the degree to which cave ecosystems contribute to the global carbon sink should be further studied. The genus *Hydrogenophaga*, which is capable of using hydrogen as an energy source (Willems et al., 1989), was represented by 2.3 % of relative abundance. We also recovered OTUs which belong to deltaproteobacterial SAR324 clade (marine group B) with a relative abundance of 2.3 %. The clade SAR324 is ubiquitous in the marine environment and has been reported from various depths of marine waters (Wright et al., 2012). Their versatile metabolism has been attributed to sulfur oxidation, carbon fixation, hydrocarbon utilization, and heterotrophy (Wright et al., 2012; Haroon et al., 2016). This study is the first time clade SAR324 has been reported from any brackish water system, to the best of the authors' knowledge

We recorded the dominance of globally-distributed, nitrite-oxidizing bacteria (Bock and Wagner, 2013); *Nitrospira* was the dominant genus in our sample (Table 1). The success of *Nitrospira* has been associated with nitrite oxidation (Lücker et al., 2010; Bock and Wagner, 2013), metabolic diversity (Watson et al., 1986; Daims et al., 2001; Lücker et al., 2010; Koch et al., 2015), nitrification by reciprocal feeding with ammonia-oxidizing microbes (Koch et al., 2015; Palatinszky et al., 2015), chemolithoautotrophic aerobic hydrogen oxidation (Koch et al., 2014), and complete nitrification (complete ammonia oxidation, or "comammox") (Daims et al., 2015). We did not measure any of the nutrient (ammonia or nitrate) concentrations in our study, so we are not able to conclude if the *Nitrospira* representative in our sample performs nitrite oxidation or comammox.

Table 1. The relative abundances of recorded taxa.

Relative Abundance	Taxonomy
28.3	*Nitrospirae; Nitrospira; Nitrospirales; Nitrospiraceae; Nitrospira*
8.4	*Planctomycetes; Phycisphaerae; Phycisphaerales; Phycisphaeraceae; CL500-3*
5.7	*Marinimicrobia (SAR406 clade)*
5.0	*No Relative*[a]
4.2	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Gemmobacter*
2.8	*Proteobacteria; Alphaproteobacteria; uncultured*
2.4	*Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Hydrogenophaga*
2.3	*Proteobacteria; Deltaproteobacteria; SAR324 clade (Marine group B)*
2.3	*Proteobacteria; Alphaproteobacteria; Reyranellales; Reyranellaceae; Reyranella*
2.2	*Verrucomicrobia; Verrucomicrobiae; Pedosphaerales; Pedosphaeraceae*
2.0	*Proteobacteria; Deltaproteobacteria; Bdellovibrionales; Bdellovibrionaceae; OM27 clade;*
1.9	*Patescibacteria; Gracilibacteria; Candidatus Peribacteria*
1.8	*Bacteroidetes; Bacteroidia; Flavobacteriales; Flavobacteriaceae; Flavobacterium*
1.4	*Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; Legionella*
1.3	*Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Curvibacter*
1.3	*Planctomycetes; OM190*
1.3	*Bacteroidetes; Bacteroidia; Cytophagales; Cyclobacteriaceae; Algoriphagus*
1.2	*Proteobacteria; Alphaproteobacteria; Acetobacterales; Acetobacterales Incertae Sedis; uncultured*
1.2	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter*
1.2	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Pseudorhodobacter*
1.1	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; uncultured*
20.7	Other (≤1 %)[b]

[a] Reads without any BLAST hits or reads with weak BLAST hits remain unclassified and assigned to the meta group “No Relative.”

[b] Taxa having relative abundance less than 1% grouped together and annotated as “Other (<1 %).”

The other dominant taxon recovered in our sample was CL500-3, belonging to the Planctomycetes and represented by 8.3 % relative abundance. According to 16S amplicon sequencing studies, Planctomycetes CL500-3 clade was reported from the deeper water columns of ultra-oligotrophic Crater Lake in Oregon, United States (Urbach et al., 2001) and of the oxygenated hypolimnion deep lakes in Japan (Okazaki et al., 2017). In contrast with the earlier studies, we recovered CL500-3 from the surface of the Gilindire Cave’s Mirror Lake. However, environmental conditions at the surface of our sampling site (such as low light, low saline, and oxygenated water) might be similar to the conditions in earlier studies’ deeper water-column lake conditions. According to Urbach et al. (2001), who first described the CL500-3 in the Crater Lake, Oregon, the distribution of relative abundance throughout the water column suggests that this clade functions in the remineralization of detrital particles or processes associated with sediments or hydrothermal waters. Here, we suggest that the products of the remineralization process mediated by the CL500-3 might be used by the dominant *Nitrospira* and linking carbon and nitrogen cycles in the brackish waters of Mirror Lake.

The deep-branching bacterial phylum Marinimicrobia (SAR406) comprised 5.7 % of all 16S rRNA affiliated sequence reads at Mirror Lake. A recent study showed that most of the *Marinimicrobia* clades participate in the biogeochemical cycling of sulfur and nitrogen. Additionally, two of these clades use nitrous oxide and act as a global sink for the greenhouse gas nitrous oxide (Hawley et al., 2017). We speculate that Marinimicrobia representatives recorded at Mirror Lake might also use nitrous oxide, which is a by-product of nitrification.

The uncultured OM190 Planctomycetes (Silva taxonomy) was also recovered from the brackish waters of Mirror Lake at 1 % of relative abundance. The OM190 sequence was reported previously from a variety of environments like kelp surfaces (Bengtsson and Øvreås, 2010), seawater (Rappé et al., 2003), soil (Elshahed et al., 2008), and sponges (Mohamed et al., 2010). The taxon OM190 was reported to have a negative correlation with salinity, and some representative sequences were distantly related to anammox-like environmental sequences (Ye et al., 2016). Environmental conditions in our sampling area, such as low salinity and oxygenated water, support those findings.

Archaea sequences were only represented by Thermoplasmata, Woesearchaeia, and *Nitrososphaeria* in our sample, and they constituted only 1% of relative abundance of all OTUs. It is critical to stress here that the primer pairs used in our study have limited coverage over the Archaea domain (Fischer et al., 2016). This is the possible reason for such low coverage. Among the archaeal sequences, *Nitrososphaeria* constituted the highest relative abundance (0.96 %). *Nitrososphaeria* is one of the two genera of Thaumarchaeota mediating ammonium oxidation and is known as an ammonia-oxidizing Archaea (AOA). The presence of *Nitrososphaeria* (Candidatus *nitrosoarchaeum*, Candidatus *nitrosopumilus*, Candidatus *nitrosotenuis*) in the brackish waters of Gilindire Cave's Mirror Lake is consistent with other studies conducted in freshwater sediments (Xie et al., 2014). Methanomassiliicoccales belonging to the Thermoplasmata is one of the archaeal lineages that retain the methanogenesis pathway (Evans et al., 2019). However, Zinke et al. (2021) analyzed twelve metagenome-assembled genomes (MAGs) belonging to the Methanomassiliicoccales. They further recovered 16S rRNA from three of the MAGs and showed that if these sequences were recovered by 16S rRNA-based approaches, they were most likely classified as Methanomassiliicoccales*,* which leads to wrong assumptions that these sequences represent methanogens (Zinke et al., 2021). We recovered archaeal sequences from the lake waters of Gilindire Cave that are classified as Methanomassiliicoccales*.* However, when Zinke et al. (2021) is considered, their assignment to a methanogenesis mediating group is uncertain. Marine group II Archaea (Thermoplasmata) were also recovered from the brackish waters of Gilindire Cave. They are known to be ubiquitous in marine surface waters (Rinke et al., 2019) and have different salinity preferences (Xie et al., 2018). Marine Benthic Group D, which belongs to Thermoplasmata and plays a role in protein remineralization in anoxic marine sediments (Lloyd et al., 2013), was also recovered in our study. Our findings regarding the archaeal lineage suggest further investigation of metabolic diversities in Gilindire Cave using Archaea-specific primers or metagenomic approaches. Lastly, *Nitrososphaeria* (which are AOA) and *Nitrospira* recorded in the Gilindire Cave's Mirror Lake might mediate nitrification by reciprocal feeding.

Gilindire Cave's Mirror Lake shows some major features of anchialine caves, which have both freshwater and seawater influences due to their sea and groundwater connections, may form in limestone (Bishop et al., 2015; Sawicki, 2003), and have similar prokaryotic communities (Kajan et al., 2022). Anchialine caves have sinkholes where they connect directly with the surface (Sawicki, 2003, figure 1). However, the non-sinkhole entrance to Gilindire Cave is its only connection to the surface. For that reason, even if Gilindire Cave shares some features with anchialine caves, we hesitate to define it as an anchialine cave.

Kajan et al. (2022) studied the diversity of four anchialine caves in the Mediterranean and identified the phyla Proteobacteria as the most abundant taxa. In the present study, we found that Nitrososphaeria dominated. This AOA was also recovered with high abundance above and in the halocline of two of the caves studied by Kajan et al. (2022). A 5-year study of the cave waters of the Emilia Romagna region, Italy, has demonstrated that the microbial community variation depends on location. Additionally, that study showed seasonality is responsible for the community variation (D'Angeli et al., 2017). Based on the findings of Kajan et al. (2021) and D'Angeli et al. (2017), we can say that the composition of Gilindire Cave's microbial community should be further investigated by including water column diversity and seasonality.

CONCLUSION

In this study, we evaluated the prokaryotic microbial diversity in the brackish surface waters of Mirror Lake in Gilindire Cave, which formed when the Mediterranean Sea was at its lowest level. One methodological drawback to our study is the lack of physicochemical measurements, which limits our conclusion. Keeping in mind the methodological limitation, our results point to the nitrification by reciprocal feeding between *Nitrospira* and *Nitrososphaeria,* which are ammonia-oxidizing *Archaea.* Investigating microbial diversity in such a unique environment showed the close relationship between *Nitrospira*, Planctomycetes CL500-3 clade, and Marinimicrobia (SAR406), which dominate the cave's surface water.

This is the first study conducted in Mirror Lake. However, more comprehensive and integrated studies should be carried out in the lake to uncover the complex relationship between recorded taxa and their small-scale contribution compared to global-scale biogeochemical cycles.

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CONTENTS

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