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Nitrogen Isotope Sclerochronology—Insights Into Coastal Environmental Conditions and *Pinna nobilis* Ecology

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Pinna nobilis is a large bivalve endemic to the Mediterranean Sea that lives in shallow coastal areas. Due to its size and relatively fast shell growth rates, it is an interesting taxon for high-resolution study of nitrogen isotopes of carbonate bound organic matter ($\delta^{15}N_{CBOM}$). In this study we tested if *P. nobilis* shells can be used as an indicator of the nitrogen isotope baseline of the system, if it can provide high-resolution data on environmental $\delta^{15}N$ variability, and if the chemical properties of the shell and biomineralization process change in response to mass mortality events spreading in the Mediterranean. Shells were opportunistically collected during 2019 and 2020 by skin diving, as a part of a project on mortality monitoring, from four shallow coastal localities in the eastern Adriatic. Shell powder for $\delta^{15}N_{CBOM}$ analysis was collected by milling sample swaths from the internal (low-resolution) and external (high resolution) shell surface. Significant differences in $\delta^{15}N_{CBOM}$, obtained from the internal shell surface, were observed between sampling localities with different anthropogenic influences, with lowest values (~3–4‰) recorded for shells obtained from Pag Bay, and highest (~6–8‰) for shells sampled in Lim and Kaštela Bays. High-resolution samples from the external shell surface of *Pinna nobilis* showed spatial and temporal variations in $\delta^{15}N_{CBOM}$ values, with temporal resolution of 1–3 weeks. High-resolution $\delta^{15}N_{CBOM}$ data obtained from the shell Kas1 corresponded to a time interval from spring 2018 to summer 2019 and had a pronounced increase of $\delta^{15}N_{CBOM}$ values closest to the shell margin coupled with a decrease in $\delta^{13}C_{shell}$ values, indicating that this animal was experiencing stressful conditions several months prior to its death. According to our findings, $\delta^{15}N_{CBOM}$ values from *P. nobilis* shells can serve as an indicator of the isotopic baseline of the ecosystem potentially as a powerful tool to study bivalve physiology.

Keywords: bivalve, stable isotope, shell, Mediterranean, Adriatic

INTRODUCTION

Pinna nobilis is a large bivalve endemic to the Mediterranean Sea. In early autumn of 2016, *P. nobilis* mass mortality event was detected in the Spanish part of the Mediterranean Sea (Vázquez-Luis et al., 2017), which soon spread to other parts of this enclosed sea (Cabanellas-Reboredo et al., 2019), including the Adriatic Sea (Čižmek et al., 2020; Šarić et al., 2020). By the end of 2020, all *P. nobilis* populations in the Croatian part of the Adriatic Sea have been affected by the mass mortality and very few individuals survived (Šarić, personal observation). At the first occurrence of a mass mortality event in Spain, the parasite *Haplosporidium pinnae* was considered. as the likely cause (Darriba, 2017; Catanese et al., 2018). However, further research has indicated that other pathogens, such as bacteria from the genus *Mycobacterium* and *Vibrio*, were also involved (Carella et al., 2019; Šarić et al., 2020; Scarpa et al., 2020; Lattos et al., 2021). Moreover, according to these reports, the mass mortality events were presumably caused by polymicrobial disease associated with various abiotic factors, and the pathogenesis has not yet been fully elucidated.

Conservation efforts require comprehensive knowledge on the biology and ecology of species as well as environmental variations, including those related to nitrogen in coastal marine habitats. *Pinna nobilis* was listed as an endangered and protected species under the European Council Directive 92/43/EEC (EEC, 1992), is recorded in the ANNEX II of the Barcelona Convention, and is under local law protection in all European Union Mediterranean countries. Concerns for the status of *P. nobilis* were raised even before 2016 and the start of mass mortality event, because it declined due to exposure to a number of cumulative stressors including habitat degradation, food web alteration and contaminant burden (Basso et al., 2015). Due to the devastating effects of mass mortality event on the population of *P. nobilis*, IUCN changed the status of this species to "critically endangered" (Kersting et al., 2019).

Over the past decade, methods for the analysis of nitrogen stable isotopes in carbonate-bound organic matter ($\delta^{15}N_{CBOM}$) using direct combustion of carbonates have been developed and optimized enabling the investigation of $\delta^{15}N_{CBOM}$ in bivalve shells from freshwater, estuarine, and marine habitats (e.g., Versteegh et al., 2011; Darrow et al., 2017; Gillikin et al., 2017; Graniero et al., 2021; Kukolich and Dettman, 2021; Schöne and Huang, 2021). Nitrogen isotopes measured in carbonate-bound organic matter are a function of $\delta^{15}N$ values of dissolved nitrogen in the ambient water as well as the physiology and diet of the bivalve (e.g., Gillikin et al., 2017; Whitney et al., 2019; Das et al., 2021; Graniero et al., 2021). Bivalves are considered as low-level consumers, and as such, they can provide information on the nitrogen isotope baseline of the environment (e.g., Jennings and Warr, 2003; Thibault et al., 2020). In many previous studies, $\delta^{15}N_{CBOM}$ was measured in marine bivalve species including *Mercenaria* sp. (O'Donnell et al., 2003), *Mercenaria mercenaria* (Carmichael et al., 2008; Oczkowski et al., 2016), *Ruditapes philippinarum* (Watanabe et al., 2009), *Mytilus edulis* (Versteegh et al., 2011), *Crassostrea virginica* (Kovacs et al., 2010; Oczkowski et al., 2016; Black et al., 2017; Darrow et al., 2017), *Pecten maximus* (Gillikin et al., 2017), *Rangia cuneata* (Graniero et al., 2021), *Spisula solidissima* (Das et al., 2021), and *Arctica islandica* (Whitney et al., 2019; Schöne and Huang, 2021). An increase in $\delta^{15}N_{CBOM}$ has been related to nutrient enrichment reflecting anthropogenic perturbation (e.g., Carmichael et al., 2008; Kovacs et al., 2010; Oczkowski et al., 2016). As yet, only a limited number of bivalves were sampled at high-resolution to generate $\delta^{15}N_{CBOM}$ time-series, e.g., *Pecten maximus* (Gillikin et al., 2017), *Spisula solidissima* (Das et al., 2021), and *Arctica islandica* (Schöne and Huang, 2021). Due to its size (>1 m, Zavodnik et al., 1991) and very fast shell growth rate (Richardson et al., 2004), *Pinna nobilis* presents an interesting taxon for high-resolution $\delta^{15}N_{CBOM}$ research. High-resolution $\delta^{15}N_{CBOM}$ can provide an important insight into temporal variation in bivalve diet, physiology (including ontogenetic changes), as well as background nitrogen isotopic signatures (Gillikin et al., 2017; Das et al., 2021; Schöne and Huang, 2021).

Previous studies on the chemical properties of *P. nobilis* shells include research on oxygen ($\delta^{18}O_{shell}$) and carbon ($\delta^{13}C_{shell}$) stable isotopes (Richardson et al., 1999, 2004; Kennedy et al., 2001a; Freitas et al., 2005; García-March et al., 2011), as well as element ratios including Mg/Ca and Sr/Ca (Richardson et al., 2004; Freitas et al., 2005). García-March et al. (2011) suggested that *P. nobilis* can be used to reconstruct environmental, ecological and climate changes in the Mediterranean Sea. Following previous analyses of $\delta^{18}O$ and $\delta^{13}C$ in *P. nobilis* shells, the present study focuses on $\delta^{15}N_{CBOM}$ values of this species. The main objectives were to test if *P. nobilis* shells (i) can be used as an indicator of the nitrogen isotope baseline of the system, and (ii) provides high-resolution data on environmental $\delta^{15}N$ variability. Due to the multiple mass mortality events spreading throughout the Mediterranean, including the Adriatic Sea, we also tested if (iii) the chemical properties of *P. nobilis* and shell deposition process changed in response to the diseases.

MATERIALS AND METHODS

A preliminary study was conducted in 2018 to test the feasibility to obtain $\delta^{15}N_{CBOM}$ data from shells of several bivalve species from the Adriatic Sea using the standard combustion technique on an elemental analyzer (e.g., Versteegh et al., 2011).

Sample Collection

For the purpose of this study, *P. nobilis* shells were opportunistically collected by skin diving from four shallow coastal localities in the eastern Adriatic (**Figure 1**), as a part of a project on mortality monitoring. More information on sample collection and epidemiological status of live-collected specimens is available in Šarić et al. (2020). Three specimens from Lim Bay (October 2019; 45°07′55″N, 13°43′55″E), Kaštela Bay (January 2020, 43°33′01″N, 16°21′36″E), and Mali Ston Bay (November 2019, 42°52′01″N, 17°41′51″E) were collected alive, while in Pag Bay (44°26′35″N, 15°03′01″E), shells of three recently dead specimens were collected in September 2020. In addition, one dead empty shell collected from Kaštela Bay in January 2020 was also analyzed. Lim Bay and Mali

FIGURE 1 | Map of the Adriatic Sea showing sample localities (red dots).

Ston Bay are important bivalve aquaculture areas. Lim Bay is located in the North of the eastern Adriatic and Mali Ston Bay in the South. Kaštela Bay is semi-enclosed bay that is under strong anthropogenic influence including agriculture as well as municipal and industrial effluents (Anđelić et al., 2020). Of the four localities, Pag Bay is the most pristine and least polluted area (Kušpilić, personal communication).

Sample Preparation and Stable Isotope Analysis

In the laboratory, tissue and epibionts were physically removed and shells carefully cleaned and air-dried, and shell heights were measured. Due to the *P. nobilis* mass mortality event and opportunistic sampling strategy it was not possible to target specific shell sizes. The height of the studied *P. nobilis* shells ranged from 15 cm (Pag Bay, Pag3) to 58 cm (Mali Ston Bay, Sto1) (**Table 1**). The average shell height was 50.7 ± 4.0, 30.3 ± 14.6, 39.8 ± 7.9, and 56.7 ± 1.5 cm for Lim Bay, Pag Bay, Kaštela Bay, and Mali Ston Bay, respectively. Muscle tissue samples from Lim Bay and Kaštela Bay were frozen for later isotope analysis.

Shell powder (∼20–30 mg) was collected by milling sample swaths by hand using a DREMEL Fortiflex drill equipped with a 300 μm tungsten carbide drill bit. For the analysis of spatial $\delta^{15}N_{CBOM}$ variations, three samples were collected from each of these shells by milling three shallow lines parallel to the growth axis from the internal shell surface (**Figure 2A** and **Table 1**). Sample tracks measured 130 ± 37 mm in length. These sample swaths started, on average, 40 ± 11 mm from the shell edge. For the analysis of temporal variability, high-resolution $\delta^{15}N_{CBOM}$ data were collected for the last deposited shell material from two shells collected at three localities including Lim Bay, Kaštela Bay, and Mali Ston Bay. Milling swaths were arranged perpendicular to the major growth axis on the external shell surface (**Figure 2B**). For shell Kas1 from Kaštela Bay, a total of 40 samples were collected, while between 14 and 17 samples were obtained from each of the five other shells. Shell powder (∼80 μg) was also used for $\delta^{18}O_{shell}$ and $\delta^{13}C_{shell}$ analysis. An overview of shells and samples is given in **Table 1**. Gillikin et al. (2017) previously established that vigorous chemical cleaning of bivalve shell carbonate is not required for CBOM analysis in bivalves due to the dense structure of the shell.

Soft tissues were dried for ∼24 h at 55°C, then ground and homogenized using a mortar and pestle. ∼1 mg powdered samples were packed into tin capsules. 20–30 mg of shell carbonate were packed into tin capsules (signal intensities on the mass spectrometer were similar between soft tissues and shell). Samples were analyzed on a Thermo Delta V Advantage isotope ratio mass spectrometer (IRMS) in continuous flow mode connected to a Costech Elemental Analyzer (EA) via ConFlo IV at Union College (Schenectady, NY) following methods in previous studies (Versteegh et al., 2011; Graniero et al., 2016, 2021; Black et al., 2017; Darrow et al., 2017; Gillikin et al., 2017; Whitney et al., 2019). An in-house acetanilide ($\delta^{15}N$ = –0.96‰), ammonium sulfate (IAEA-N-2) ($\delta^{15}N$ = + 20.3‰), and caffeine (IAEA-600) ($\delta^{15}N$ = + 1.0‰) reference standards were used for regression-based corrections, and to assign the data to the appropriate isotopic scale. Percent N was calculated using additional acetanilide standards of varying mass. Corrections were done using a regression-based method. The reproducibility for $\delta^{15}N$ (AIR) was ± 0.04‰, based on 12 acetanilide standards over three analytical sessions. Stable carbon isotope analysis used similar techniques, see Graniero et al. (2021) for complete details.

Oxygen and carbon isotopes of carbonate powders (∼80 μg) were analyzed on a Gas Bench II coupled to the aforementioned

TABLE 1 | Overview of *Pinna nobilis* specimens and number of samples per shell used in this study.

Location	Shell ID	Shell height (cm)	Low resolution $\delta^{15}N_{CBOM}$	High resolution $\delta^{15}N_{CBOM}$	High resolution $\delta^{18}O$ and $\delta^{13}C$
Lim Bay	Lim1	47	3	16	16
	Lim2	50	3	14	14
	Lim3	55	3	–	–
Pag Bay	Pag1†	44	3	–	–
	Pag2†	32	3	–	–
	Pag3†	15	3	–	–
Kaštela Bay	Kas1	49	3	40	46
	Kas2	38	3	–	–
	Kas3	30	3	–	–
	Kas4†	42	3	16	16
Mali Ston Bay	Sto1	58	3	17	17
	Sto2	55	3	–	–
	Sto3	57	3	17	17
Total			39	120	129

†*Dead collected shells.*

mass-spectrometer. Powders were reacted with > 100% phosphoric acid in helium-flushed Exetainer vials at 55°C for at least 3 h. Carbonate isotope results are reported in δ notation (‰) relative to the VPDB carbonate standard. NBS-18 and IAEA 603 were used for regression-based corrections; LSVEC was used as a check standard. The following values (VPDB) were used for $\delta^{13}C$ and $\delta^{18}O$, respectively: IAEA 603: + 2.46‰, −2.37‰, NBS-18: −5.014‰, −23.2‰, and LSVEC: −46.6‰, −26.7‰. The reproducibility (1σ) for $\delta^{13}C_{shell}$ and $\delta^{18}O_{shell}$ was ± 0.03 and ± 0.04‰, respectively, based on eight IAEA 603 standards over two analytical sessions.

Environmental Data and Statistical Analysis

In this study we used results based on Regional Ocean Modeling System (ROMS), a 3D numerical ocean model for the Adriatic Sea with a spatial resolution of 2 km and 20 vertical levels in terrain following coordinates (Janeković et al., 2014). The model was previously validated in studies using available observations including CTD profiles and satellite SST data (e.g., Janeković et al., 2010, 2014; Vilibić et al., 2016) and was used to estimate daily bottom salinity and temperature data—corresponding to the first vertical level in the model. These data were used to calculate predicted $\delta^{18}O_{shell}$ values and temporally align the measured $\delta^{18}O_{shell}$ data of Kaštela Bay. An earlier study by García-March et al. (2011) on *Pinna nobilis* used Friedman and O'Neil (1977) paleothermometry equation for calcite and water and observed ∼1.4‰ offset relative to measured seawater temperature. To enable aligning of data obtained from shell with expected $\delta^{18}O_{calcite}$, in this study we used the paleothermometry equation by Killingley and Newman (1982) (Eq. 1).

$$T = 22.14 - 4.37\left(\delta^{18}O_{calcite} - \delta^{18}O_{water}\right) + 0.07(\delta^{18}O_{calcite} - \delta^{18}O_{water})^2 \quad (1)$$

$\delta^{18}O_{water}$ values were estimated from the modeled salinity values using the equation of Purroy et al. (2018) for the coastal areas in the eastern Adriatic Sea:

$$\delta^{18}O_{water} = 0.23 \times salinity - 7.54 \quad (2)$$

including a VPDB-SMOW scale correction for $\delta^{18}O_{water}$ of -0.27‰ (Gonfiantini et al., 1995). Calculated $\delta^{18}O$ values were converted from SMOW to VPDB scale using the equation from Coplen et al. (1983) to temporally align $\delta^{18}O_{shell}$ values with predicted shell oxygen isotope values obtained from the ocean model:

$$\delta^{18}O_{PDB} = 0.97002\ \delta^{18}O_{V-SMOW} - 29.98 \quad (3)$$

Differences in $\delta^{15}N_{CBOM}$ between localities were tested using one-way ANOVA. A Shapiro-Wilk test was used to test for normality, and the Brown-Forsythe test for equal variances. The Holm-Sidak method was applied for pairwise multiple comparison. Kruskal-Wallis test was applied for the analysis of percent nitrogen. Spearman correlation coefficient was applied for testing correlation between different variables.

RESULTS

A preliminary study of seven bivalve species from the Adriatic Sea, showed pronounced interspecies variations in $\%Ns_{hell}$ (**Table 2**). The lowest values (<0.03%) were noted for *Venus verrucosa*, and *Glycymeris pilosa* specimens. Shells of the commercially important European flat oyster (*Ostrea edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*), had among highest values, 0.22 and 0.16%, respectively. However, the highest value (0.47%) was obtained for a *Pinna nobilis* shell, clearly indicating potential of this species for the analysis of $\delta^{15}N_{CBOM}$ data.

Spatial Variations in $\delta^{15}N_{CBOM}$ Data

Values of $\delta^{15}N_{CBOM}$ in samples milled from the internal shell surface of *Pinna nobilis* shells had pronounced spatial

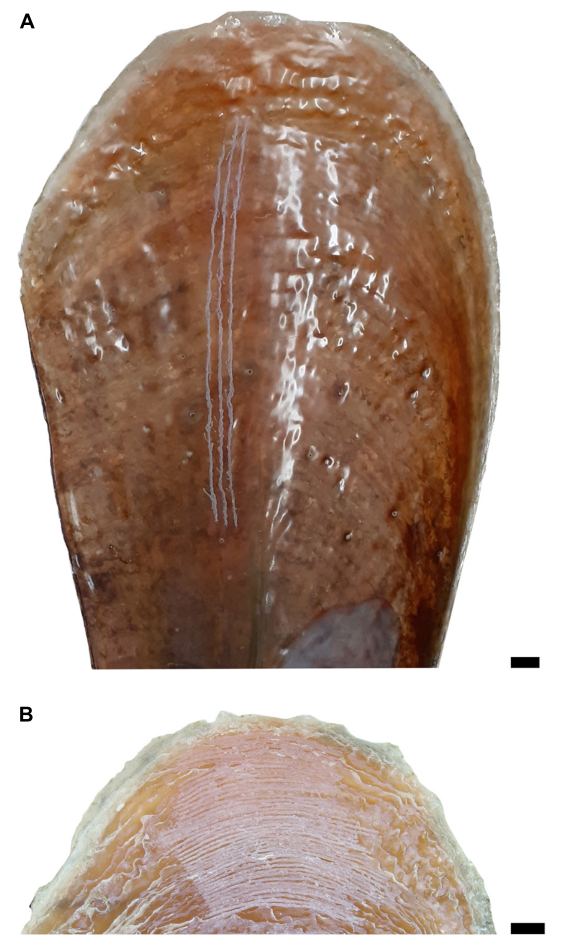

FIGURE 2 | Illustration of milling lines on **(A)** inner shell surface and **(B)** external shell surface of *Pinna nobilis* Kas1 collected at Kaštela Bay. Scale bar 1 cm.

TABLE 2 | Results of preliminary study of $\delta^{15}N_{CBOM}$ on bivalve species from the Adriatic Sea.

Species	Specimen ID	Locality	Carbonate (mg)	$\delta^{15}N$	$\%N_{shell}$
Venus verrucosa	S3V3	Istria	41.993	6.45	0.027
Venus verrucosa	S3V2	Istria	39.863	6.70	0.025
Callista chione	S3C38	Istria	46.987	3.15	0.040
Callista chione	S3C56	Istria	40.349	2.93	0.040
Callista chione	S1C53	Pašman	41.235	2.77	0.047
Callista chione	S1C52	Pašman	43.995	4.71	0.047
Glycymeris pilosa	BG14-1	Pašman	41.185	4.16	0.022
Ostrea edulis	Lim-1	Lim Bay	43.017	8.79	0.216
Mytilus galloprovincialis	Nov-1	Novigrad	39.522	7.23	0.164
Crassostrea gigas	Lim-1	Lim Bay	43.849	8.00	0.081
Pinna nobilis	Kas0	Kaštela Bay	47.449	5.49	0.469

variations (**Figure 3** and **Supplementary Material 1**). Lowest values were obtained for shells from Pag Bay, with a range of 3.06–.47‰ (average ± 1σ = 3.72 ± 0.41‰). Samples from Mali Ston Bay had intermediate values ranging from 5.43 to 5.96‰ (5.72 ± 0.22‰), while values between 6 and 8‰ were characteristic for *P. nobilis* from Lim Bay and Kaštela Bay. Observed differences were statistically significant between localities (one-way Anova $F = 27.36$, $p < 0.001$). According to pairwise comparison tests between locations, differences were not statistically significant, except for Lim Bay and Kaštela Bay. Percent N ranged from 0.2 to 0.7%. Percent nitrogen was higher in shells from Lim Bay (0.43 ± 0.16%), while Pag Bay shells had intermediate amounts (0.35 ± 0.06%), and Mali Ston Bay (0.30 ± 0.03%) and Kaštela Bay (0.28 ± 0.05%) had lower amounts. Observed differences were statistically significant between localities [Kruskal-Wallis test, H (chi^2): 13.3, $p < 0.005$]. According to pairwise comparison tests, samples from Lim Bay had significantly higher percent nitrogen than samples from Mali Ston Bay and Kaštela Bay. Statistically significant difference was also noted between Kaštela Bay and Pag Bay. The localities with highest $\delta^{15}N_{CBOM}$ had both the highest (Lim Bay) and lowest percent N (Kaštela Bay).

High-Resolution $\delta^{15}N_{CBOM}$ Data

Samples from the external shell surface of *Pinna nobilis* show spatial and temporal variations in $\delta^{15}N_{CBOM}$ values. The largest number of samples came from the shell Kas1 (Kaštela Bay) and corresponded to the last 6 cm of shell growth (**Figure 4**). Earlier deposited shell material had $\delta^{15}N_{CBOM}$ values between approx. 4.5 and 5.5‰ and showed cyclic variations, while an increase in $\delta^{15}N_{CBOM}$ values (up to approx. 10‰) was identified closer to the shell margin. This increase in $\delta^{15}N_{CBOM}$ was accompanied by a pronounced $\delta^{13}C_{shell}$ decrease. The $\delta^{18}O_{shell}$ provided an insight into the timing and rate of shell growth. Accordingly, the sampled shell portion was formed between spring 2018 and summer 2019 (**Figure 5**). As this shell was collected alive in January 2020 and the last sample was milled 1.6 mm from the shell margin, these results indicate that the shell Kas1 slowed down its growth or even ceased to grow for several months prior to collection. The increase in $\delta^{15}N_{CBOM}$ values occurred in spring and summer of 2019. Temporal resolution of $\delta^{15}N_{CBOM}$ data was ~10 days, with seasonal variations ranging between 1 and 3 weeks.

The $\delta^{18}O_{shell}$ values from shell Kas1 ranged between −0.21 and 3.10‰, while those for $\delta^{13}C_{shell}$ values varied between −1.48 and 0.55‰ ($N = 46$). There was a statistically significant positive correlation between $\delta^{18}O_{shell}$ and $\delta^{13}C_{shell}$ (Spearman $r = 0.74$, $p < 0.001$). Shell $\delta^{15}N$ values were negatively correlated to both $\delta^{18}O_{shell}$ ($r = -0.344$, $p = 0.03$) and $\delta^{13}C_{shell}$ ($r = -0.438$, $p = 0.005$). However, this relationship was largely driven by the last eight samples. Without these data points, the only statistically significant correlation exists between $\delta^{18}O_{shell}$ and $\delta^{15}N_{CBOM}$ values ($r = 0.667$, $p < 0.001$).

We also completed a high-resolution analysis of five additional *P. nobilis* shells, two live-collected specimens from Lim Bay (Lim1 and Lim2) and Ston Bay (Sto1 and Sto3), and one dead, empty shell from Kaštela Bay (Kas4). In these shells, 15 mm (Lim2) to 23 mm (Sto3) transects were sampled, with the last samples coming from approx. 10 mm before the shell margin. In these specimens, sampling closer to the shell margin was not possible

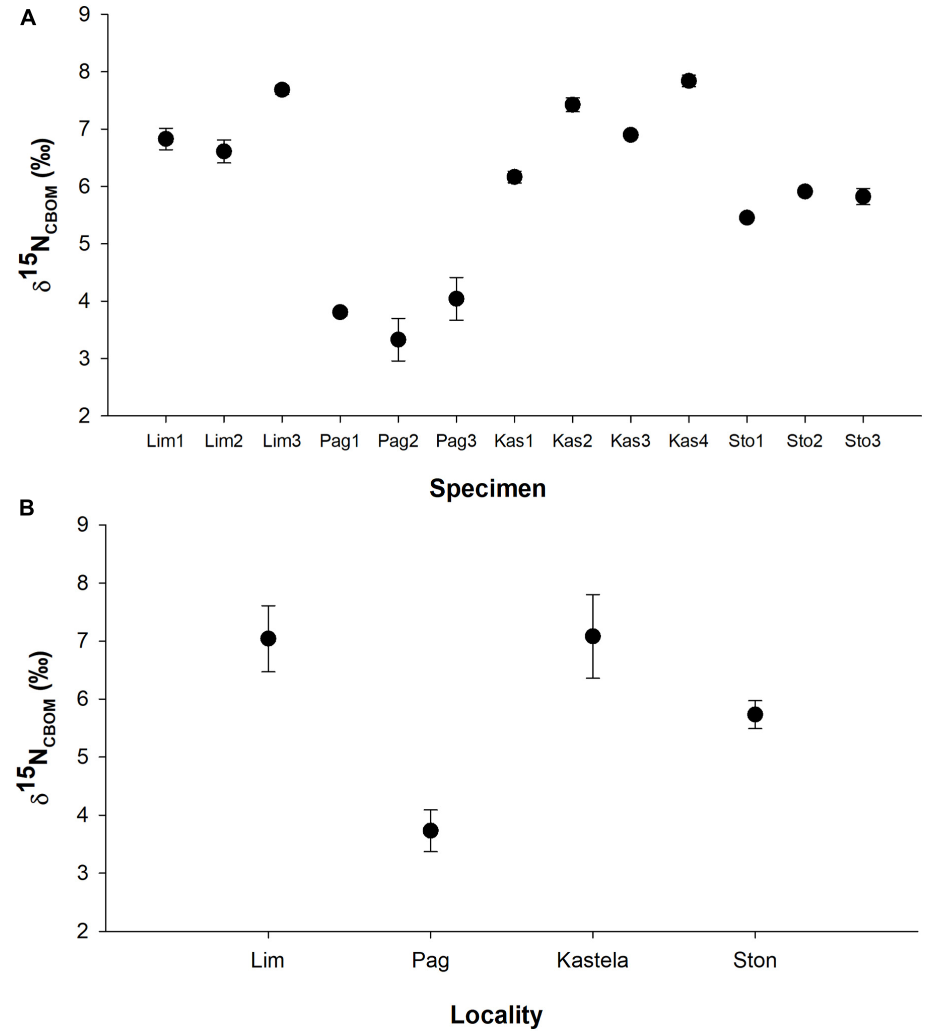

FIGURE 3 | $\delta^{15}N_{CBOM}$ values of the inner shell surface of *Pinna nobilis*. **(A)** Data of the three milling lines per specimen, **(B)** differences between locality averages. Mean values ± 1 standard deviation (often smaller than the symbol).

because of contamination: foreign particles, mostly sediment, were trapped in the shell.

$\delta^{15}N_{CBOM}$ values ranged from 3.43 to 7.84‰ (**Figure 6**). Lowest values were characteristic for shells from Mali Ston Bay, with an average value of 4.30 ± 0.42‰ for shell Sto1 4.09 ± 0.52‰ and for shell Sto3. Shells from Lim Bay had highest mean values, i.e., 7.00 ± 0.57‰ (Lim1) and 6.24 ± 0.36‰ (Lim2). These spatial variations are consistent with $\delta^{15}N_{CBOM}$ values of the inner shell surface. Shell Kas4 from Kaštela Bay showed intermediate average values for samples obtained from the outer shell (5.46 ± 0.16‰), and the least variable range of 5.22–5.74‰. In the case of this shell, average $\delta^{15}N_{CBOM}$ values obtained from samples milled from the outer shell were lower than those obtained from the inner shell (7.84 ± 0.10‰).

As noted above, muscle tissues were only available for shells from Lim Bay and Kaštela Bay (**Figure 7**). The $\delta^{15}N_{tissue}$ data of these two localities varied from each other, with higher values for Lim Bay (range: 7.22–7.28‰) and lower values for Kaštela Bay (5.74–6.19‰). In the present study, $\delta^{13}C_{tissue}$ data had a very narrow range at both localities (Lim Bay: −19.88 to −19.70‰; Kaštela Bay: −20.06 to −19.57‰).

DISCUSSION

Shell $\delta^{15}N$ Values

Results of this study clearly indicate that *Pinna nobilis* shells contain a sufficient amount of N-bearing organic matter to enable reliable analyses of $\delta^{15}N_{CBOM}$ values using direct combustion. For the analysis of site-specific/temporal $\delta^{15}N_{CBOM}$ variations of *P. nobilis*, carbonate material can be milled from the inner or outer shell surface (**Figures 3**, **6**). Pronounced site-specific $\delta^{15}N_{CBOM}$ variations were observed in specimens collected in the eastern Adriatic Sea. $\delta^{15}N_{CBOM}$ values from the inner shell surface of specimens from Lim Bay and Kaštela Bay were almost two times higher than those from *P. nobilis* from the Pag Bay

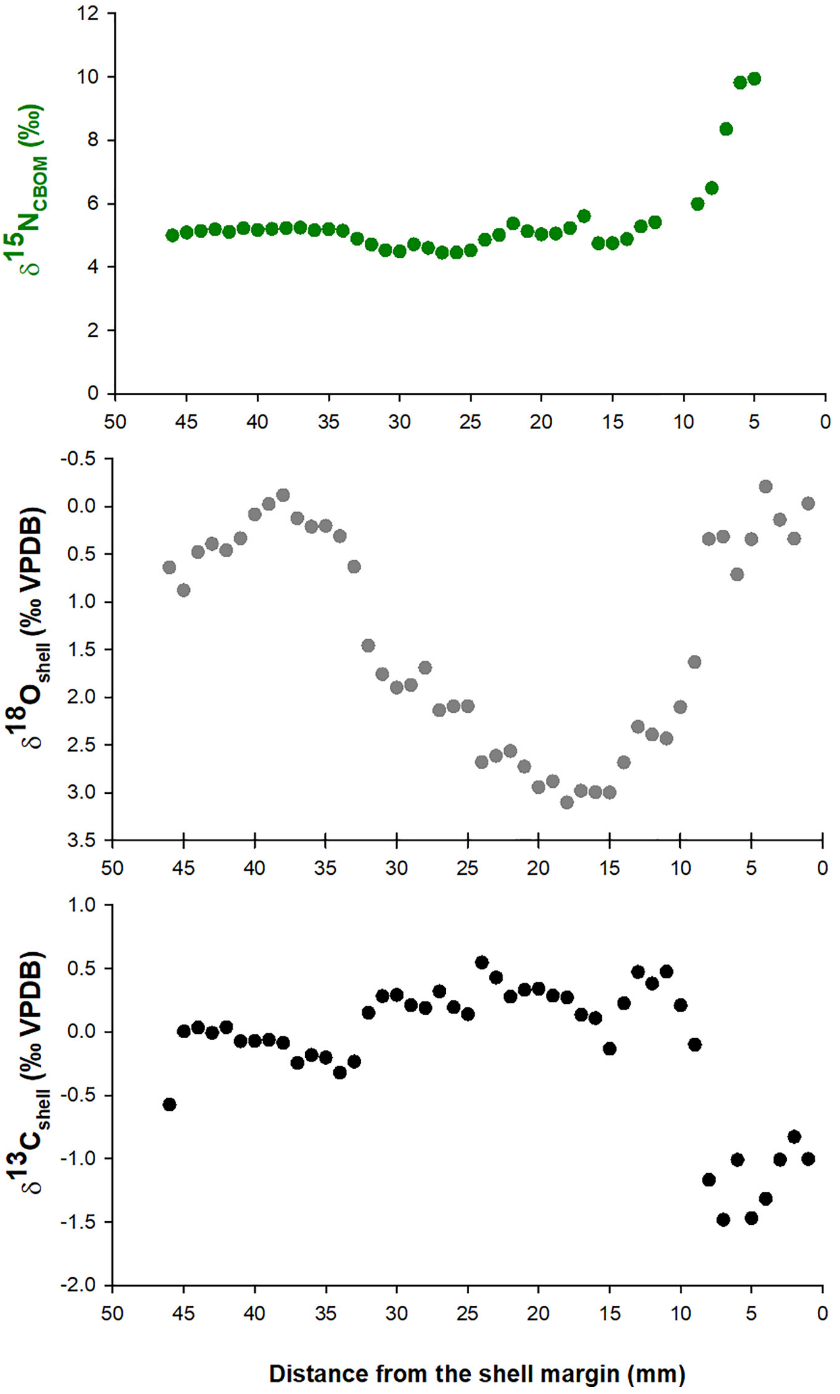

FIGURE 4 | High resolution $\delta^{15}N_{CBOM}$, $\delta^{18}O_{shell}$, and $\delta^{13}C_{shell}$ data of *Pinna nobilis* specimen Kas1 collected alive at Kaštela Bay in January 2020.

(**Figure 3**). Similar site-specific $\delta^{15}N_{CBOM}$ differences existed in samples from the external shell surface of the specimens collected from three localities.

Kaštela is a semi enclosed coastal bay that was identified as one of the most polluted coastal areas in the eastern Adriatic in the late 20th century (Barić et al., 1992). The situation has improved since 1990 onward when most chemical industries located in the area were closed and again after mid-2000 when a modern wastewater treatment plant was completed (Kušilić et al., 2009). However, due to large urban population in the area, including cities of Split, Kaštela and Trogir, Kaštela bay is still one of the areas with the highest anthropogenic impacts in the eastern Adriatic. Due to the number of freshwater springs, Lim Bay, an important bivalve aquaculture site, is characterized

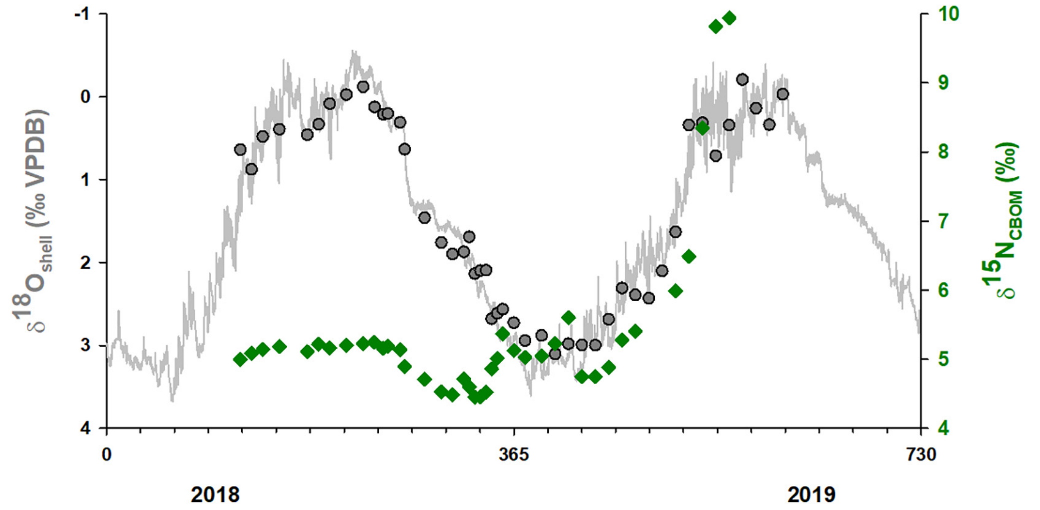

FIGURE 5 | Predicted shell oxygen isotope values (gray line) obtained from the temperature (ROMS model) and $\delta^{18}O_{water}$ estimates based on salinity (ocean model) using the paleothermometry equation by Killingley and Newman (1982). Gray circles represent temporally aligned $\delta^{18}O_{shell}$ values of *Pinna nobilis* specimen Kas1 collected alive at Kaštela Bay in January 2020; green diamonds represent corresponding $\delta^{15}N_{CBOM}$ values. Scale on y1-axes is reversed.

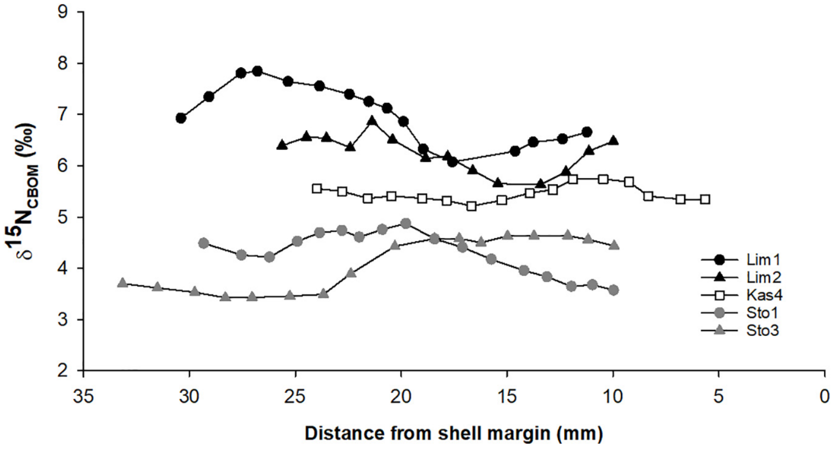

FIGURE 6 | High-resolution $\delta^{15}N_{CBOM}$ values of *Pinna nobilis* shells collected at Lim Bay (Lim1 and Lim2), Kaštela Bay (Kas4), and Mali Ston Bay (Sto1 and Sto3).

with high nutrient input (Bosak et al., 2009). Other than impacts associated with aquaculture activities, anthropogenic influences in this area are limited. A similar situation exists in Mali Ston Bay, as it is also relatively sparsely populated area. The Mali Ston Bay may be classified as a moderate natural eutrophicated system based on the phytoplankton community structure (Viličić et al., 1998). The least data are available for Pag Bay. The Island of Pag, including the Pag Bay area, is characterized by bare rocks, and scarce terrestrial vegetation. There is no industrial activity in the area, and agriculture is very limited. Previous studies (e.g., Carmichael et al., 2008; Kovacs et al., 2010; Gillikin et al., 2017) showed that $\delta^{15}N_{CBOM}$ data relate to nitrogen in the environment and can be used for reconstructing environmental biochemical conditions. Our results are consistent with this, as samples from the more pristine Pag Bay had the lowest $\delta^{15}N_{CBOM}$ values.

According to Gillikin et al. (2017) bivalve shells can provide high-resolution $\delta^{15}N_{CBOM}$ data because they are capable of recording the $\delta^{15}N$ signal of metabolically-acquired N with suitably short time-averaging. The target species of the present study, *P. nobilis* is the largest marine bivalve in the Mediterranean Sea (Vicente, 1990; Zavodnik et al., 1991) and is characterized by rapid shell growth during early ontogeny. It attains a shell height of 20–30 cm during first 2 years of life (Richardson et al., 2004; Kožul et al., 2012; García-March et al., 2020). In this study, average temporal resolution of $\delta^{15}N_{CBOM}$ from the external shell samples was 10 days, and this was obtained by milling ∼20–30 mg of carbonate material. Resolution was estimated from temporal alignment of measured $\delta^{18}O_{shell}$ values with predicted $\delta^{18}O_{shell}$ values obtained from the ocean model. The sample mass of milled carbonate in this study was rather large, as we targeted an easy to analyze mass with a robust

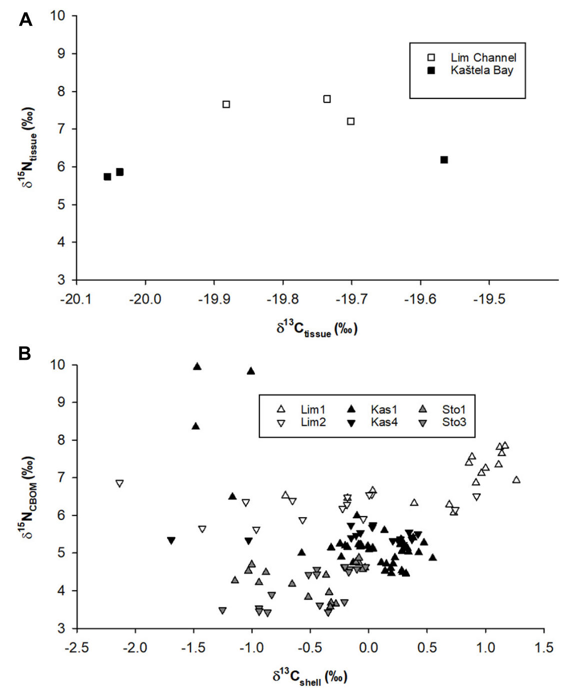

FIGURE 7 | Relationship between $\delta^{13}C$ and $\delta^{15}N$ in **(A)** muscle tissue and **(B)** shell of *Pinna nobilis*.

signal to noise ratio. The sample mass could be smaller (e.g. Gillikin et al., 2017; Graniero et al., 2021) in future studies, which would greatly increase temporal resolution; down to a few days in early ontogeny and during seasons of faster shell growth. Such extremely highly resolved $\delta^{15}N_{CBOM}$ has great potential for reconstructing environmental changes in coastal marine ecosystems. Gillikin et al. (2017) reported a resolution of $\delta^{15}N_{CBOM}$ down to 2 days in *Pecten maximus* shells, and *P. nobilis* has more than three times the N content and faster growth rates, so daily resolved $\delta^{15}N_{CBOM}$ should be attainable using this technique.

In this study, we conducted high-resolution analysis of $\delta^{15}N_{CBOM}$ on specimens ranging in size from 42 to 58 cm. Due to the mass mortality event it was not possible to collect more shells and select animals of similar size and age limiting comparison and temporal alignment of geochemical data. As *Pinna nobilis* does not show periodic growth marks on its shell margin, adductor muscle scars have been used to estimate its age (Richardson et al., 1999). However, later studies pointed out bias and limitation of this method including the difficulty in determining first and second scar and obscuring of scars by nacre in older shells (Richardson et al., 2004; Garcia-March and Márquez-Aliaga, 2007). Furthermore, shells from Lim Bay and Mali Ston Bay did not have distinct muscle scars on the inner shell surface (Peharda, personal observation). Hence, estimation of their age was beyond the scope of the present study. However, it is interesting to note that largest shells came from the Mali Ston Bay, and they appear to have a larger number of muscle scars than those sampled from Kaštela Bay (see **Supplementary Material 3**). Furthermore, shell Lim1 from Lim Bay had a similar size (47 cm) as shell Kas1 from Kaštela Bay (49 cm), but based on muscle scars visible on the internal shell surface, this shell was older and slower growing thereby likely leading to more time averaging in data from stable nitrogen isotope analysis this shells. In addition, shell layering near the shell margin was the most fragile and friable part of the shell, which resulted in trapping of particles from the environment preventing proper sampling for isotope analysis.

Taking into account the limitations described above, high-resolution $\delta^{15}N_{CBOM}$ data were presented according to sample distance from the shell margin (**Figure 6**). These data revealed strong temporal variations in specimens from same site (Lim2: Δ = 1.24‰; Lim 1: Δ = 1.76‰). In a recent study, Das et al. (2021) also observed inter- and intra- specimen variations in *S. solidisima* shells. Gillikin et al. (2017) analyzed *Pecten maximus* and noted differences of up to 2.5‰ between shells growing at the same site and time. Despite variations between specimens, data presented here and in previous studies seem to reflect that the nitrogen isotope baseline changes seasonally. This change is most likely caused by seasonal variation in nutrients and food sources available to bivalves, including *P. nobilis*, in shallow coastal marine habitats. According to Gillikin et al. (2017) and Das et al. (2021), bivalve shells have a high potential to reveal short-term nitrogen cycle dynamics and this is confirmed in our study.

Precise temporal alignment was only performed for one specimen (Kas1) collected from Kaštela Bay. In this specimen, it was possible to collect carbonate samples from very close to the shell margin, i.e., the last formed shell portion prior to collection. Based on modeled temperature and salinity it was possible to calculate expected $\delta^{18}O_{shell}$ values. In January 2020, when sampling was conducted, *Pinna nobilis* population in Kaštela Bay has been impacted by a mass mortality event, and strong infiltrative inflammation that included *Haplosporidium pinnae* and *Mycrobacterium* was observed in Kas1 specimen (Šarić et al., 2020). The $\delta^{18}O_{shell}$ data indicated that shell material was deposited until late summer (see **Figure 5**). According to results of previous studies (Kennedy et al., 2001a; Richardson et al., 2004; Freitas et al., 2005; García-March et al., 2020), *P. nobilis* deposits shell material in late summer and autumn period, what was not the case with our shell Kas1. As this specimen was collected in January, it is possible that it was experiencing starvation and/or infection for several months.

An increase in $\delta^{15}N_{CBOM}$ by up to approx. 10‰ most likely occurred in early summer and was coupled with a decrease in $\delta^{13}C_{shell}$ likely indicating a stressed animal and an increase in the amount of metabolic-sourced carbon in the shell (see Lorrain et al., 2004; McConnaughey and Gillikin, 2008). Changes in $\delta^{15}N$ have been linked to variations in diet and physiological state of the animals, and according to a recent experimental study by Prado et al. (2021), on feeding of *P. nobilis*. The increase in $\delta^{15}N$ can be a consequence of starvation. As the nitrogen balance of bivalves drops below nitrogen demands, they start to break down their own tissues, thereby preferentially losing ^{14}N and retaining ^{15}N (see Fry, 2006 for examples). Both $\delta^{13}C_{shell}$ and $^{15}N_{CBOM}$ suggest the animal was sick. This is further supported by the lack of shell growth in the second half of 2019 (**Figure 5**).

δ^{15}N in *Pinna nobilis* Tissue

Previous studies conducted on other bivalve species found that bulk $\delta^{15}N_{CBOM}$ correlates with δ^{15}N of soft tissues (e.g., O'Donnell et al., 2003; Carmichael et al., 2008; Kovacs et al., 2010; Gillikin et al., 2017; Graniero et al., 2021). In the present study, only a limited number of samples was available for tissue analysis, and due to mass mortality event and conservation status of the species, it was not possible to collect additional samples. Hence, a direct comparison of shell and tissue δ^{15}N was not possible, and the tissue data need to be interpreted with care. Yet, the tissue δ^{15}N values were much higher than such determined in the few previous studies on *P. nobilis* from relatively undisturbed settings suggesting increased anthropogenic and/or natural inputs at the study localities.

To the best of our knowledge, Kennedy et al. (2001b) provided the first stable isotope data of *Pinna nobilis* muscle tissue. Respective specimens came from the southeastern Spanish Mediterranean coast. These authors reported a mean value of −18.3‰ for $\delta^{13}C_{tissue}$ and 3.3‰ for $\delta^{15}N_{tissue}$. In a study by Cabanellas-Reboredo et al. (2009), average stable isotope values for *P. nobilis* muscle tissue samples were −19.30‰ for $\delta^{13}C_{tissue}$ and 3.51‰ for $\delta^{15}N_{tissue}$. According to data presented in their study, $\delta^{15}N_{tissue}$ values higher than 5‰ were found only at one sampling site. Similar results were found in a third study conducted in Spain by Alomar et al. (2015). According to their results, *P. nobilis* tissue samples from non-Marine Protected Areas were characterized by higher $\delta^{15}N_{tissue}$ and $\delta^{13}C_{tissue}$, than those from Marine Protected Areas. Deudero et al. (2017) analyzed muscle tissue of monthly collected *P. nobilis* and found a 0.93‰ annual valuation in $\delta^{15}N_{tissue}$, with minima recorded in March (on average, 2.54‰) and maxima in May (3.47‰). All the above studies were conducted prior to mass mortality events and their values are lower than those obtained for $\delta^{15}N_{tissue}$ in the present study (5.74–7.28‰). Possible explanations for observed differences are variations in anthropogenic perturbations that suggest eutrophication and/or physiological response of animal to the mass mortality event. From the conservation perspective, it would be informative if future studies are conducted on the disease spread involving live-collected bivalves and analysis of stable isotopes in different *P. nobilis* tissues.

Pinnindae and Shell Chemistry

According to Huber (2010), the cosmopolitan family Pinnidae comprises more than 50 species, inhabiting tropical and temperate seas. Pinnids likewise inhabit shallow coastal regions (e.g., *P. nobilis*, Zavodnik et al., 1991) and deep waters (e.g., *Pinna cellophana,* Matsukuma and Okutani, 1986). Despite their characteristic shape and size, new species are still being discovered, e.g., in 2016, Araya and Osorio (2016) described *Pinna rapanui* from Easter Island, South Pacific Ocean.

Results of this study as well as earlier works conducted on the shell chemistry of *P. nobilis* (e.g., Richardson et al., 1999, 2004; García-March et al., 2011; Gilbert et al., 2017), clearly indicate the great potential of *P. nobilis* as well as related taxa for sclerochronological research. Over the past decade, research on shell chemistry has been conducted on several species of Pinnidae including *Atrina fragilis* (Valentine et al., 2011), *Atrina rigida* (Gilbert et al., 2017), and *Pinna carnea* (Gilbert et al., 2017). Respective studies have been conducted on modern and fossil specimens presenting possibilities to reconstruct past environmental conditions. High-resolution analysis of $\delta^{15}N_{CBOM}$ in different pinnids from coastal environments would provide powerful insights into anthropogenic caused perturbations of the environment, especially in areas with limited instrumental data.

CONCLUSION AND RECOMMENDATIONS

Results of this study clearly indicate the potential of *Pinna nobilis* to provide reliable high-resolution $\delta^{15}N_{CBOM}$ time-series. Such analyses can be conducted on museum stored specimens for which the collection date is known, thereby extending valuable δ^{15}N reconstructions further into the past. Furthermore, *P. nobilis* shells collected in the mid twentieth century at different locations within the Adriatic Sea, as well as probably in other parts of the Mediterranean, are available from private collections. Although for many of such specimens exact date of collection is not known, data on approximate year of collection is often available. Challenges of high-resolution $\delta^{15}N_{CBOM}$ analysis of *P. nobilis* shells include the crowding of growth patterns in shell material deposited during late ontogeny that can result in large time averaging, trapping of different particles from the environment (organic and inorganic) within the shell, and difficulties associated with milling samples from the margin where shell is very thin and fragile. Spatial $\delta^{15}N_{CBOM}$ variations can be a valuable indicator of the nitrogen isotope baseline. However, due to the current *P. nobilis* mass mortality event in the Mediterranean, further research on this species is very restricted and attempts should be made to identify other bivalve taxa that are relatively fast growing and whose shells contain sufficient amounts of N-bearing organic matter to enable high-resolution $\delta^{15}N_{CBOM}$ analyses. Results of our preliminary study indicate that two commercially important and relatively fast growing bivalves, the European flat oyster (*Ostrea edulis*) and Mediterranean mussel (*Mytilus galloprovincialis*), have high shell nitrogen content, making them interesting taxa for high resolution $\delta^{15}N_{CBOM}$ analysis in the Mediterranean. At a global level, other species of the Pinnidae likely serve as valuable $\delta^{15}N_{CBOM}$ archives as well.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MP: obtained the funding, designed the experiment, analyzed the data, and wrote the manuscript. DG: designed the experiment, conducted laboratory analysis of stable isotopes in soft tissues and shell carbonate material, analyzed the data, and wrote the manuscript. BS: designed the experiment, analyzed the data, and wrote the manuscript. AV: conducted laboratory analysis of stable isotopes in soft tissues and shell carbonate material, helped with writing, and revision of the manuscript. HU: milling of carbonate material, help with data analysis, and writing and revision of the manuscript. KM: milling of carbonate material, help with writing and revision of the manuscript. TŠ and IŽ: field work, help with writing and revision of the manuscript. IJ: 3D numerical ocean model data, help with writing and revision of the manuscript. All authors gave final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.816879/full#supplementary-material

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