Not all nudibranchs are carnivorous: trophic ecology of *Polycerella emertoni* in the Ebro Delta

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ABSTRACT: Nudibranchs have always been regarded as a group of specialized predators. In Alfacs Bay (Ebro Delta, Spain), the cryptic nudibranch *Polycerella emertoni* is found associated with the pseudo-indigenous bryozoan *Amathia verticillata*, which has been thought to be its target prey. Here, we assessed the trophic ecology of *P. emertoni* using a combination of methods, including stable isotope analyses coupled with Bayesian mixing models, examination of stomach contents, and video recording of feeding activities. A morphological analysis of the radula was also conducted to explore a possible relationship with feeding behavior. Finally, we investigated the seasonal abundance of both species throughout an entire year in order to assess the nature and strength of their association. Contrary to current belief, our results show that *P. emertoni* is a micro-herbivore that feeds on the periphyton covering *A. verticillata* (up to 99% of the diet, according to mixing models). In particular, a diverse diatom community was observed on *A. verticillata*, and several of these taxa were found within the stomach contents of *P. emertoni*. Our results evidence a distinctive radular formula and morphology compared to other species and suggest the presence of multiple feeding strategies in nudibranchs. The high seasonal association observed between *P. emertoni* and *A. verticillata* may be attributed to mimicking habitat features as a mechanism to avoid predation. The possible effects of periphyton removal in the survival of detached fragments of *A. verticillata* may have implications for the dispersal of fouling species and functioning of ecosystems.

KEY WORDS: Nudibranch · Herbivory · Stable isotopes · Trophic ecology · Gut contents · Diatoms · Bryozoan · Mimicry

1. INTRODUCTION

All nudibranchs are considered carnivorous (Clark 1975, Wagner et al. 2009) and to be amongst the most specialized predators in benthic marine ecosystems (Megina & Cervera 2003). Nudibranchs are frequently associated with sessile benthic species such as sponges, hydrozoans, or bryozoans that they have become adapted to consume as slow-moving predators (Todd 1981). For this reason, nudibranchs have traditionally been organized into 4 major feeding categories: sponge-grazers, bryozoan-grazers, hydroid-grazers, and a 'miscellaneous' group that includes other animals in their diets such as other nudibranchs and tunicates (Todd 1981). Knowledge on the trophic ecology of nudibranchs is mostly based on casual observational records, whereas experimental and quantitative information is scarce and has largely been overlooked (Chadwick & Thorpe 1981, Megina & Cervera 2003). This is partly due to their small size, but mostly due to their scarcity (Todd 1981), compared to other common benthic invertebrate preda-

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tors such as decapods and asteroideans. For instance, stomach contents and food assays have only been described for larger species of nudibranchs that measure more than 10 mm, like *Platydoris argo*, *Roboastra europaea*, and *Phyllodesmium poindimiei*, in which manipulation and direct observation is easier (Megina et al. 2002, Wagner et al. 2009). Also, several observations of nudibranchs feeding on bryozoan and sponge substrates have been reported (Franz & Clark 1972, Harvell 1984). However, to our knowledge, no study has yet used stable isotope analysis for a long-term dietary assessment in nudibranchs. Furthermore, although nudibranchs are supposedly highly specialized predators, there is very little information about adaptations of the radular morphology to their feeding strategy (Nybakken & McDonald 1981). Therefore, several general perceptions about the trophic ecology and biology of the group require a major re-evaluation (Todd et al. 2001).

Polycerella emertoni is a small nudibranch originally described from the Atlantic coast of North America (Verrill 1881). Many authors have suggested an amphi-Atlantic distribution of the species, including the Mediterranean Sea (García-Gómez & Bobo 1986, Moro et al. 2017). Reported locations include areas of the Western Atlantic region such as Brazil (Marcus 1957), Cuba (Espinosa et al. 2005), and Venezuela (Gutiérrez et al. 2015); and locations in the Eastern Atlantic region, including south-western Spain (García-Gómez & Bobo 1986), the Azores Islands (Amat & Tempera 2009), Morocco (Moro et al. 2017) and the Canary Islands (Ortea & Moro 2019). In the Mediterranean Sea, it was first described in 1946 in the Fusaro coastal lagoon (Italy) (Schmekel 1965), and later reported in Malta (Sammut & Perrone 1998), Greece (Koutsoubas et al. 2000), Tunisia (Antit et al. 2011), and Spain (Camps & Prado 2018). In the Atlantic Ocean, *P. emertoni* has been observed associated with *Zostera* spp. (Verrill 1881), filamentous algae (Verrill 1881), hydroids (Chambers 1934), and soft-bodied bryozoans such as *Amathia distans* (Marcus 1957), *A. gracilis* (reported as *Bowerbankia gracilis* by Franz & Clark 1972) and *A. verticillata* (Ortea & Moro 2019). However, in the Mediterranean Sea it has only been observed associated with the widespread bryozoan *A. verticillata* (Camps & Prado 2018), which prompted Zenetos et al. (2004) to propose *P. emertoni* as an invasive species in the Mediterranean. Yet, although the putative alien status of *A. verticillata* has been debated in the recent literature (Galil & Gevili 2014), there are no molecular studies to confirm this issue; thus in the meantime, the species should be regarded as being of unknown

origin (Floerl et al. 2009). Similarly for *P. emertoni*, the number of verified observations is considerably higher in the Mediterranean than along the Western Atlantic coast, so in the absence of molecular data to support its alien status, the species is considered as cryptogenic in this paper.

A. verticillata is a soft-bodied, stoloniferous ctenostomate capable of forming large branching colonies. The species is widely distributed in temperate and tropical waters of the Western Atlantic, where it thrives in natural habitats such as seagrass meadows, mangroves, oyster reefs, and rocky shores (Galil & Gevili 2014). However, it was first described in human-modified Mediterranean environments (delle Chiaje 1822) such as harbors and marinas, where it causes undesired fouling (Rizgalla et al. 2019). According to Micael et al. (2018), *A. verticillata* is a seasonal species; its growth declines considerably once temperature drops below 18°C (in laboratory conditions), and it moves into a senescent cryptic phase in autumn and winter (Zabala 1986). This species can produce new colonies that are capable of surviving the cold-water period and settle on a variety of natural and artificial substrates until environmental conditions become favorable again (McKinney 1983, Robinson 2004).

Franz & Clark (1972) described *P. emertoni* as a specialized bryozoan-grazer, based on its strong association with *A. verticillata* in the Mediterranean Sea. However, no feeding observations, manipulative experiments, or biochemical assessments have been conducted to support this idea, which is also questionable given the records of *P. emertoni* on other substrates (Verrill 1881, Chambers 1934, Marcus 1957, Franz & Clark 1972). Overall, there is a lack of understanding about the relationship between the seasonal abundance of *P. emertoni* and that of its potential prey.

The aim of this study was to investigate the trophic ecology of *P. emertoni.* Our initial hypothesis, supported by all work on nudibranchs conducted previously, was that *P. emertoni* is a predator and eats the bryozoan *A. verticillata*, on which it occurs in the Ebro Delta. However, in the course of testing this hypothesis using stable isotope analysis, we found a major discrepancy between the $\delta^{13}C$ signature of the nudibranch and that of its host. This led us to investigate a second hypothesis, that *P. emertoni* does not feed on the bryozoan itself, but rather on its microalgal biofilm, which is available in large quantities. To this end, we video-recorded feeding activities and conducted further stable isotope analyses coupled with Bayesian mixing models and stomach content analy-

sis, to evaluate the diet of *P. emertoni* at different time scales. In addition, we conducted a morphological assessment of the radula of *P. emertoni* and compared it with available information from other species to evaluate possible differences in their trophic ecology. A second objective was to understand the association between *P. emertoni* and the seasonal availability of *A. verticillata*. For this, we performed seasonal estimates of the abundance of both *P. emertoni* and *A. verticillata* aimed at investigating the nature and the strength of the relationship between them.

2. MATERIALS AND METHODS

2.1. Study site

Alfacs Bay (40° 36′ N, 0° 43′ E) and Fangar Bay (40° 47′ N, 0° 46′ E) are semi-confined estuarine areas located respectively on the southern and northern hemi-deltas of the Ebro River (Catalonia, NW Mediterranean; Fig. 1). Both bays receive recurrent discharges of freshwater rich in nutrients and organic matter from rice fields. Furthermore, they are subject to strong seasonal salinity gradients, particularly Fangar Bay, due to its smaller size. The southern shore of Alfacs Bay (the 'Banya' sandspit) was included in the Ebro Delta Natural Park in 1986 and belongs to the Natura 2000 network of the European Union, because of habitats containing the seagrass *Cymodocea nodosa* and the presence of a large population of the fan mussel *Pinna nobilis* (Prado et al. 2020).

2.2. Morpho-anatomical examination and video recording of feeding habits

The external morphology of specimens was examined under a dissecting binocular microscope (Olympus SZX10). The buccal mass was extracted and soaked in 10% potassium hydroxide (KOH) to remove surrounding tissue. The radula and jaws were then rinsed in distilled water. These structures were finally mounted on aluminum stubs with carbon adhesive discs, sputter-coated with gold in a Fisons Instrument SC 510, and examined using a Zeiss DSM 940A scanning electron microscope (SEM) at 15 kV at the Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB). We also recorded nudibranch feeding habits with a camera (Nikon DS-Ri1) coupled to a dissecting microscope (Nikon SMZ1500). Trials were repeated over 1–2 h until a reasonably good quality and illustrative video was obtained.
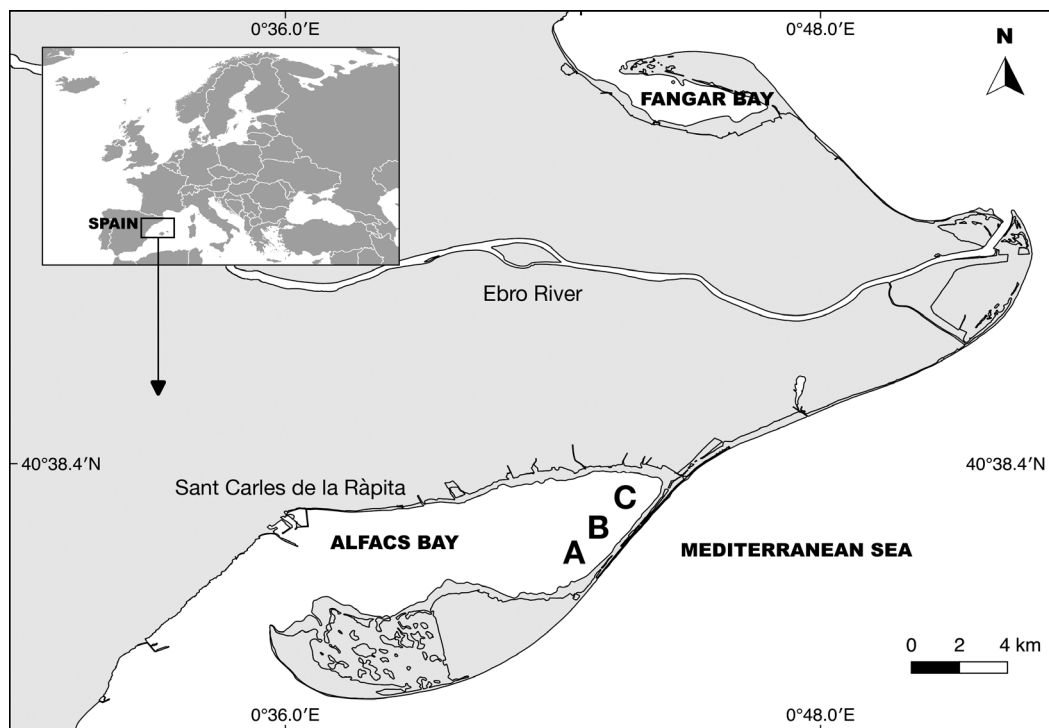

Fig. 1. Ebro Delta (Catalonia, NW Mediterranean), showing Alfacs and Fangar Bays and the 3 sampling sites (A, B, and C). Inset shows the location of the Ebro Delta in Europe

2.3. Stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) and elemental contents

In a first examination of individuals of *Polycerella emertoni* and *Amathia verticillata* collected in August 2018 in Alfacs Bay, a large deviation of 2.3‰ between the $\delta^{13}C$ signature of the consumer and the supposed diet (*A. verticillata*) was measured. This result was discordant with the general consideration that consumers have negligible values of ^{13}C fractionation (i.e. ~0 ‰) compared to their prey (Post 2002), and pointed to the possibility of an alternative diet. Hence, in August 2019 samples of *P. emertoni* were collected together with samples of *A. verticillata* and the associated microalgal biofilm. The collection of the biofilm was conducted by gently shaking the bryozoan colony apart within a zip-lock plastic bag filled with seawater, and then filtering the detached material through a 100 μm mesh net to remove small broken bryozoan fragments. The remaining material was filtered again through 1 μm mesh net in order to retain the diatom fraction (size ranges from 2 μm up to several millimeters, although only a few species are larger than 200 μm; see Round et al. 1990). This process was conducted 5 times, and the associated bryozoan fragments were also kept for stable isotope analysis. For *P. emertoni*, whole individuals (N = 100) found on the bryozoan colony fragments were pooled in groups of 20 to obtain enough material for 5 replicates.

All samples were dried separately at 60°C over 24 h, and then ground to fine homogeneous powder in a ceramic mortar. Samples were analyzed with a Flash 112 IRMS delta C series EA Thermo Finnigan mass spectrometer connected to an elemental analyzer for the determination of C and N contents (at the isotopic ratio mass spectrometry facility in CCi-TUB). Isotope ratios in samples were calculated from linear calibration curves constructed with standard reference materials of known composition and a blank correction. The difference in isotopic composition between the sample and reference materials was determined by:

$$\delta(‰) = (R_{sample} / R_{standard} - 1) \times 1000 \quad (1)$$

where R_{sample} is the isotopic ratio of the sample, $R_{standard}$ is that of the standard reference material, and δ (‰) is the difference in isotopic composition of the sample relative to that of the reference (Vienna PeeDee Belemnite and atmospheric nitrogen for carbon and nitrogen, respectively). The reproducibility of the stable isotope measurements was ~0.1‰.

2.4. Stable isotope mixing models

The MixSiar Bayesian mixing model was used to identify the long-term biomass contributions of food resources to the diet of *P. emertoni*. This model was outlined by Moore & Semmens (2008) and incorporates uncertainty and prior information into stable isotope mixing models. MixSiar v.1.0.4 uses stable isotope signatures with their standard error (SE) and tissue–diet discrimination factor input variables to estimate the probability distributions (5th, 25th, 50th, 75th, and 95th percentiles) of each food item to the mixture and accounts for uncertainty associated with multiple sources. The estimated median contribution (i.e. the 50th percentile) for each food source is usually given for comparative purposes. All stable isotope data of the samples collected from Alfacs Bay in August 2019 were pooled and input into the MixSiar Bayesian mixing model. For $\delta^{15}N$, we fed the model with the 3.40 ± 0.18‰ (mean ± SE) fractionation value indicated for consumers by Vander Zanden & Rasmussen (2001), whereas for $\delta^{13}C$, we assumed the commonly accepted view of no fractionation (Post 2002). Since these fractionation rates constitute a theoretical approximation, additional runs were conducted using 3.4 ± 1‰ for $\delta^{15}N$ and 0 ± 0.5‰ for $\delta^{13}C$ in order to assess the importance of possible deviations.

2.5. Stomach contents and biofilm community analysis

For molecular biology, the *P. emertoni* specimens collected from Alfacs Bay in October 2019 were preserved individually in Eppendorf tubes of 0.20 ml capacity, with 0.10 ml of ultrapure water, and stored at −80°C until further processing. We analyzed the stomach contents of 15 nudibranchs. To do this, we used an adapted protocol from Trobajo & Mann (2019) to digest remains of organic matter in the samples and investigate the diatom community within the stomach contents of the nudibranchs. Briefly, nudibranchs were placed individually on clean cover slips. Once the coverslips were fully dry, they were placed on a ceramic hotplate within a fume cabinet. When the temperature reached 90°C, a drop of HNO_3 (65–70%) was added to each coverslip and allowed to evaporate for 2–3 min. This operation was repeated several times until there was no visible colored residue. Coverslips were mounted with Naphrax (Brunel Microscopes) for light microscopy (Nikon Eclipse 90i) or attached to stubs for SEM examination.

For evaluation of the periphyton covering the bryozoan, samples from the same mats of *A. verticillata* where nudibranchs were found were processed as previously indicated for isotopic analyses and then subjected to the same acid digestion steps mentioned above. In addition, some bryozoan samples (from August 2019) were also prepared for SEM observation. Diatoms were identified to species wherever possible using mainly identification keys by Witkowski et al. (2000) and Álvarez-Blanco & Blanco (2014).

2.6. Seasonal abundance of *A. verticillata* and *P. emertoni*

In Alfacs Bay, the monthly biomass (g dry weight [DW] m^{-2}) of the bryozoan *A. verticillata* (August 2018 to August 2019) was estimated using random 5 × 5 m quadrats (N = 5) deployed in 3 different random sites at depths ranging from 60 to 80 cm (Site A: 40° 36′ N, 0° 43′ E; Site B: 40° 36′ N, 0° 43′ E; Site C: 40° 37′ N, 0° 44′ E, see Fig. 1). The bryozoan colonies within each quadrat were collected in plastic bags and transported to the laboratory for further processing. All samples were dried separately at 60°C until they reached constant weight (24 h), then weighed (0.01 g accuracy). Surface seawater temperature (°C), salinity (psu), oxygen (mg l^{-1}), and pH were recorded monthly at the 3 sampling sites using a multiparameter YSI 556 MPS instrument.

The abundance of *P. emertoni* individuals on the bryozoan *A. verticillata* (ind. g^{-1} wet weight [WW]) and its egg masses (no. of egg masses g^{-1} WW) were estimated every month in which the bryozoan was found. Specifically, random samples of free-floating colonies of *A. verticillata* were collected and preserved within ice-coolers to prevent death by overheating. Once in the laboratory, a total of 30 subsamples were inspected for the presence of individuals and egg masses of *P. emertoni* under a dissecting binocular microscope (Nikon SMZ1500). Subsamples were then blotted dry for ~3 min and weighed to the nearest 0.001 g. In addition, the entire biomass of the bryozoan was surveyed for additional individuals, which tended to detach and accumulate on the edges of the container, where they could easily be collected. All individuals found were kept in absolute ethanol for later studies.

In Fangar Bay, in a single site randomly selected, *A. verticillata* was collected in October 2018 and July 2019, at ~40 cm depth. In this bay, only the abundance of *P. emertoni* and its egg masses were assessed due to logistic difficulties in reaching the area. Sample collection and processing were conducted as in Alfacs Bay.

2.7. Statistical analyses

2.7.1. Abundance of *A. verticillata* and *P. emertoni*

Patterns in the abundance of *A. verticillata* (g DW m^{-2}) among sites (random factor, 3 levels) and months (fixed factor, 13 levels) were investigated with a 1-way repeated measures ANOVA. Multiple regression analysis was used to establish the relationship between the abundance of *A. verticillata* and environmental variables. Similarly, differences in the abundance of *P. emertoni* (ind. g^{-1} WW of *A. verticillata*) and their egg masses across the months of presence (fixed factor, 3 levels) and between bays (fixed factor, 2 levels) were investigated with a Student's *t*-test for independent samples.

2.7.2. Stable isotopes and elemental contents

Differences in the δ^{13}C and δ^{15}N composition and in the elemental contents of *P. emertoni* and *A. verticillata* in Alfacs Bay between years (2018 vs. 2019) were tested with a 2-tailed unpaired *t*-test with Welch's correction. Stable isotope signatures and elemental contents of potential food items in August 2019 were analyzed with a 1-way ANOVA.

For all parametric analyses, homogeneity of variance and normality assumptions were tested by Cochran's test and the Kolmogorov-Smirnov distribution-fitting test of the residuals, respectively. The critical level of significance was fixed at α = 0.05. However, in some instances the assumptions were not met by transformation, and the level of significance was fixed at α = 0.01 to minimize the possibility of making a Type II error. Student-Newman-Keuls (SNK) post hoc comparisons were used when necessary to identify significant differences in the interaction between sites and months. All analyses were performed using the software package STATISTICA v.13 (StatSoft).

3. RESULTS

3.1. Morpho-anatomical features and video recording of feeding habits

Examined individuals of *Polycerella emertoni* featured a small (maximum length 5–6 mm, typically 3–4 mm), translucent body with bright yellowish tones and small scattered dark brown and/or green spots (Fig. 2a–d). The small size and color patterns camouflage this species very effectively on the bry-

ozoan where it lives. Egg masses were ca. 1.8 mm length, and over 100 eggs were found within detached mats of *Amathia verticillata* (Fig. 2e).

The jaws of the animals were broadly rounded with prominent anterior wings and smooth masticatory borders. The radula was completely translucent, elongate, and narrow (Fig. 3a,e), had a radular formula 28–30 × 2.1.0.1.2, and lacked any rachidian teeth (Fig. 3c,e,f). The lateral tooth was ca. 2 times larger than the marginal ones and had an elongated and narrow base that widened in its upper area where it had 2 hook-shaped cusps, the upper one being wider and more developed (Fig. 3b,c,d). The 2 marginal teeth had a broad rectangular base and a pointed cusp (Fig. 3b,c,e,f).

Results obtained from the video recording of the nudibranch feeding habits showed an individual of *P. emertoni* repeatedly sucking a kenozooid of *A. verticillata*, without visible loss of, or damage to, the bryozoan tissue (see Video S1 in the Supplement at www.int-res.com/articles/suppl/m645p067_supp/).

3.2. Stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) and elemental contents

$\delta^{15}N$ values of *P. emertoni* in Alfacs Bay were significantly higher (df = 5.27, t = 18.64, p < 0.05) in 2018 than in 2019 (mean ± SE: 12.59 ± 0.03 and 11.02 ± 0.07‰, respectively), and $\delta^{13}C$ values also significantly differed (df = 4.67, t = 0.77, p < 0.05) between years (−17.40 ± 0.03 and −16.61 ± 0.13‰, respectively) (Fig. 4). In contrast, for elemental contents, no significant differences were observed between 2018 and 2019 (%C: 40–01 ± 1.22 and 39.03 ± 0.35%; %N: 12.01 ± 0.02 and 11.16 ± 0.01%, respectively, for each year).

For *A. verticillata*, $\delta^{15}N$ values in Alfacs Bay were not significantly different between 2018 and 2019 ($\delta^{15}N$: 7.88 ± 0.04 and 7.96 ± 0.09‰, respectively), but $\delta^{13}C$ values did differ (df = 6.70, t = 19.77, p < 0.05) (−15.11 ± 0.09 and −18.60 ± 0.14‰, respectively). For elemental contents, no significant differences between years were observed (%C: 13.25 ± 0.39 and 12.2 ± 0.46%; %N: 2.72 ± 0.06 and 2.76 ± 0.11%).

When the 2 potential diets (biofilm of microalgae and *A. verticillata*) and the consumer (*P. emertoni*) were investigated together for Alfacs Bay in 2019, results showed the presence of significant effects for both stable isotope signatures and elemental contents (Table 1, Fig. 4). The $\delta^{13}C$ signature of the nudibranch *P. emertoni* (−16.61 ± 0.12‰) was very similar to that of the biofilm (−16.76 ± 0.06‰), whereas that of *A. verticillata* was significantly lower (−18.60 ± 0.15‰) (Fig. 4). The $\delta^{15}N$ signature was significantly higher in *P. emertoni* (11.02 ± 0.07‰), followed by the biofilm
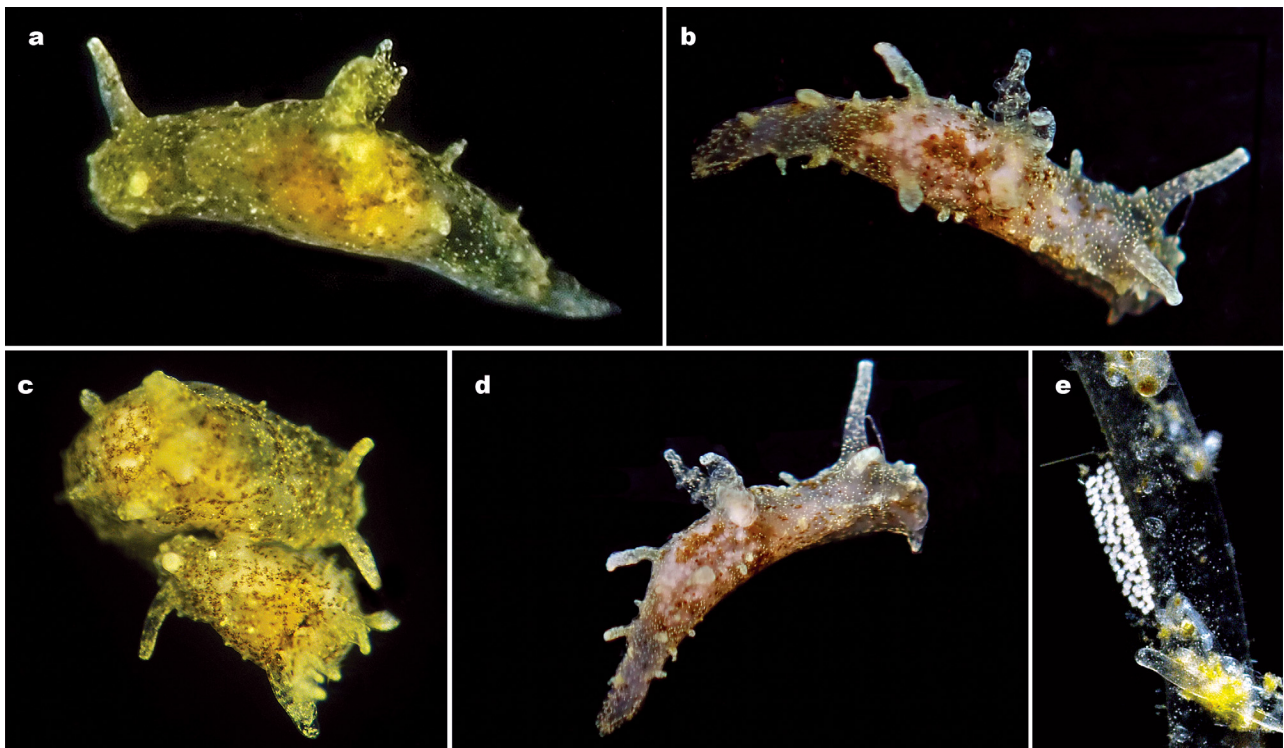

Fig. 2. *Polycerella emertoni* individuals between 1 and 3 mm: (a,b) dorsal view; (c) copulation between 2 individuals; (d) lateral view; (e) egg mass attached to bryozoan branches
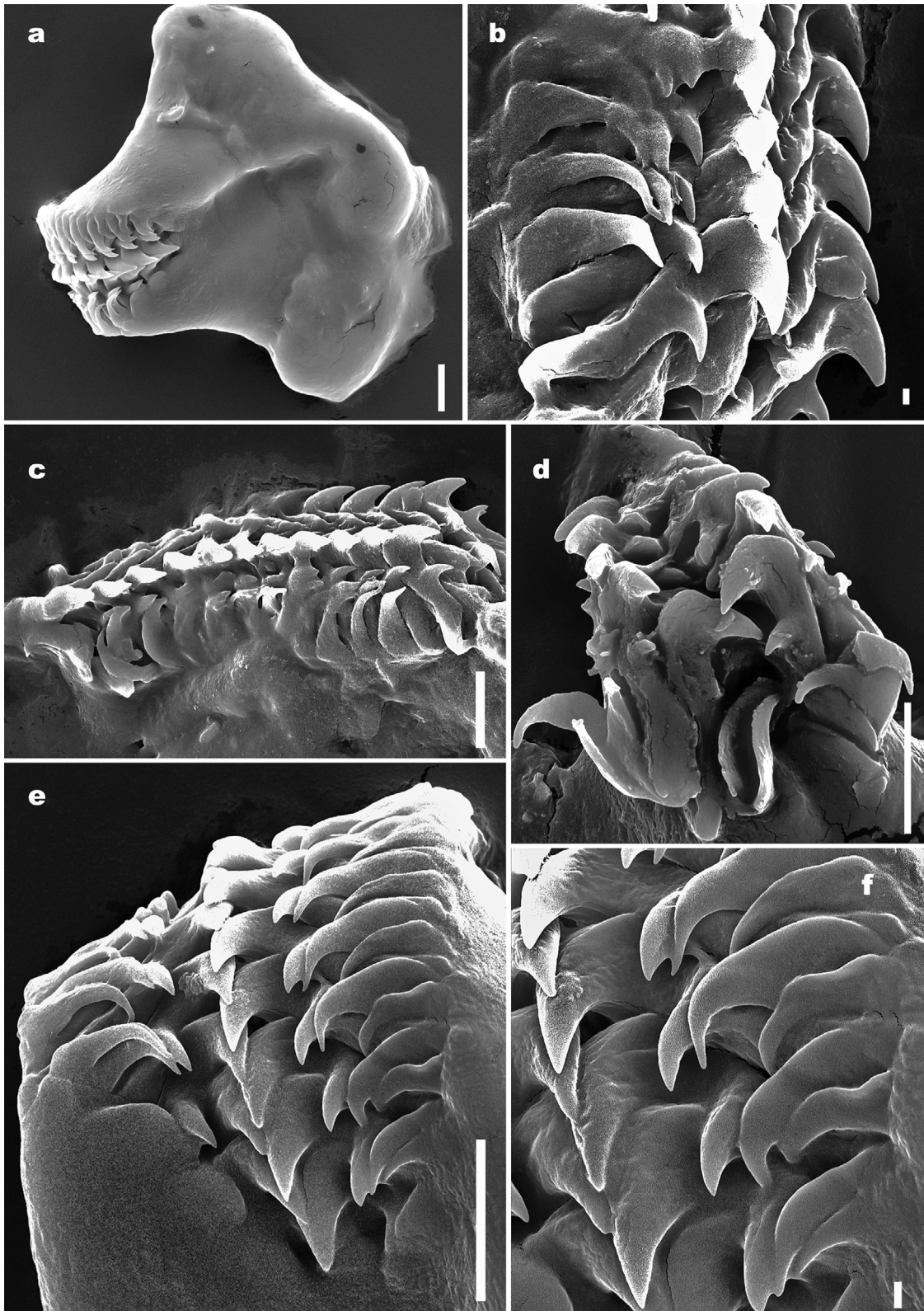
Fig. 3. Scanning electron micrographs of the radula of *Polycerella emertoni*: (a) radular bulb; (b) marginal and lateral teeth; (c) radular membrane and teeth in lateral view; (d) detail of the lateral teeth; (e) detail of the anterior teeth of the radula; (f) marginal teeth. Scale bars = 10 µm, except in (b) and (f): 1 µm
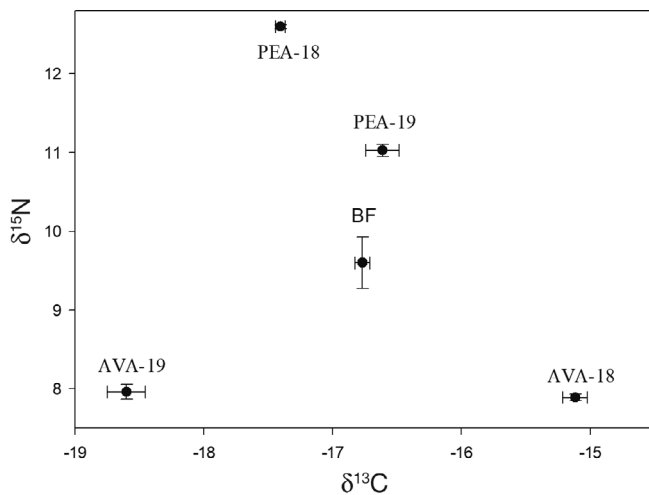
Fig. 4. Stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) of *Polycerella emertoni* from Alfacs Bay in 2018 and 2019 (PEA-18 and PEA-19, respectively), and of possible food items including *Amathia verticillata* (AVA-18 and AVA-19, respectively), and the biofilm of microalgae growing on the bryozoan (BF). Error bars are SE

(9.89 ± 0.32‰) and *A. verticillata* (7.96 ± 0.09‰) (Table 1, Fig. 4). For elemental contents, %C was higher in *P. emertoni* (39.03 ± 0.35%), but was not significantly different between *A. verticillata* and the biofilm (12.20 ± 0.46 and 13.06 ± 0.27%, respectively)

Table 1. One-way ANOVA for differences in stable isotopic signatures and elemental contents among food items (AVA: *Amathia verticillata*; BF: biofilm of microalgae growing on the bryozoan) and the nudibranch (PE: *Polycerella emertoni*) of Alfacs Bay in August 2019, for $\delta^{13}C$, $\delta^{15}N$, %C, and %N. SNK: Student-Newman-Keuls test. Statistically significant results ($\alpha = 0.05$) are indicated in **bold**

ANOVAs	df	MS	*F*	p
δ^{13}C				
Species	2	6.11	87.44	**<0.001**
Error	12	0.07		
SNK	PE = BF > AVA			
δ^{15}N				
Species	2	11.77	58.11	**<0.001**
Error	12	0.20		
SNK	PE > BF > AVA			
%C				
Species	2	6.11	1726	**<0.001**
Error	12	0.67		
SNK	PE > AVA = BF			
%N				
Species	2	134.30	3630	**<0.001**
Error	12	0.04		
SNK	PE > AVA > BF			

(Table 1). Elemental N was significantly different between organisms, with higher values in *P. emertoni* (11.16 ± 0.10%), followed by *A. verticillata* (2.76 ± 0.11%) and the biofilm (1.67 ± 0.03%) (Table 1).

3.3. Stable isotope mixing models

Results for the 50th percentile showed that the main component of the *P. emertoni* diet in Alfacs Bay was the biofilm of microalgae growing on the bryozoan (99.90%), with only a minor contribution from *A. verticillata* (0.10%). Changes of ±1‰ in the value of $\Delta^{15}N$ did not cause any change in the outcome of the results, whereas changes of ±0.5‰ in the value of $\Delta^{13}C$ resulted in a variability of 81–100% contribution (respectively for −0.5 and +0.5 rates) of periphyton to the nudibranch diet.

3.4. Stomach contents and biofilm community analysis

Diatom cells were found in 12 of the 15 individuals of *P. emertoni* whose stomach contents were examined (8 of the 11 examined by light microscopy and 4 out of 4 by SEM). The diatom species and their abundance seemed to vary among individuals (see Table 2, Fig. 5h–n). The 2 digested samples of the biofilm growing on the bryozoan *A. verticillata* showed a rich and highly diverse community of benthic diatoms (Figs. 5a–g & 6), some of the most common being *Nitzschia* cf. *incognita*, *Navicula* cf. *normaloides*, *N.* cf. *salinicola*, *Halamphora coffeaeformis* group, *Hyalosynedra* cf. *hyalina*, *Cocconeis* spp., *Mastogloia* cf. *lanceolata*, *M.* cf. *cuneata*, *Seminavis* spp., *Grammatophora* spp., and *Brachysira* spp.

Determining potential selectivity of *P. emertoni* for particular diatom species or guilds was beyond the scope of this study. However, the approach we used could easily be adapted to look for dietary preferences.

3.5. Seasonal abundance of *A. verticillata* and *P. emertoni*

Seasonal temperatures in Alfacs Bay ranged between 13.7 and 30.8°C, with an average of 20 ± 0.8°C, and this was the most influential variable affecting the growth of *A. verticillata* (Fig. 7). There was significant temporal variability in bryozoan abundance in Alfacs Bay across sampling months and years, with higher occurrence from July to August at water tem-

Table 2. Stomach contents of 11 *Polycerella emertoni* examined under light microscopy (LM) and 4 *P. emertoni* observed with scanning electron microscopy (SEM). Diatom density was assessed visually and in a qualitative way (++++ = very high; +++ = high; ++ = moderate, + = low; 0 = diatom cells not observed). Voucher slides and SEM stubs are held in the Institute of Agrifood Research and Technology (IRTA) center of Sant Carles de la Ràpita

Individual	Voucher slide or stub	Diatom species observed	Diatom density
1	LMA	Many cells of cf. *Stenoneis*; some cells of *Hyalosynedra* cf. *hyalina*; some *Mastogloia* spp.	++++
2	LM1	Some cells of cf. *Stenoneis*, *Navicula* sp. *salinicola*, *Hyalosynedra* cf. *laevis* and *Mastogloia* cf. *inaequalis*	+++
3	LM2	*Mastogloia* sp.	+
4	LM3	Some cells of *Striatella unipunctata*, *Navicula* cf. *salinicola*, cf. *Stenoneis*, round fragilarioid, *Proschkinia* sp.	+++
5	LM4	–	0
6	LM5	Some cells of *Cocconeis* cf. *scutellum*; *Navicula* cf. *normaloides*; *Mastogloia* cf. *lanceolata*	+++
7	LM6	–	0
8	LM7	–	0
9	LM8	Few cells of *Mastogloia* cf. *lanceolata*	++
10	LM9	Some cells of *Tabularia* cf. *fasciculata*; *Brachysira estonarium*, and cf. *Stenoneis*	++
11	LM10	Some cells of *Nitzschia* cf. *incognita*, *Navicula* cf. *salinicola*, and *Halamphora coffeaeformis* group	++
12–15	SEM1–4	Some cells of *Nitzschia* cf. *incognita*, *Navicula* cf. *salinicola*, *N.* cf. *normaloides*, *Halamphora coffeaeformis* group, *Cocconeis* spp., *Mastogloia* cf. *lanceolata*, *M.* cf. *cuneata*, *Hyalosynedra* cf. *hyalina*, *Striatella unipunctata*, *Diploneis* sp.	Not applicable

peratures from 27 to 30.8°C (Table 3, Fig. 8). Spatial variability among study sites was not significant, and neither was the Month × Site interaction (Table 3).

Results from multiple regression analysis showed a significant correlation between *A. verticillata* abundance and water temperature ($r^2 = 0.58$, df = 1, $F = 23.17$, p < 0.05), whereas oxygen, salinity, and pH did not have a significant effect.

Student's *t*-test showed significant differences in the abundance of *P. emertoni* and its egg masses across months and bays (Table 2). Both individuals and egg masses were dominant in Alfacs Bay during the summer period, whereas abundances in Fangar Bay peaked in October (Table 4, Fig. 9).

4. DISCUSSION

This study provides the first record of herbivorous behavior in the order Nudibranchia by evidencing feeding on periphyton by the cryptic nudibranch *Polycerella emertoni*. This evidence was achieved using an array of techniques including video recording, stable isotope ratios, and stomach contents. Our video recording showed an individual of *P. emertoni*

repeatedly sucking a kenozooid of *Amathia verticillata* but without actual loss of bryozoan tissue or damage to it. This suggests that *P. emertoni* feeds on the biofilm covering the external branching structure of the bryozoan. According to the Bayesian stable isotope mixing model used, this biofilm may constitute up to 99% of the diet of *P. emertoni*, although contributions ranging from ca. 80 to 100% are possible depending on variations in the fractionation rates (3.4 ± 1‰ for ^{15}N and 0 ± 0.5‰ for ^{13}C). The δ^{13}C signature of *P. emertoni* (−16.61‰) was very similar to that of the biofilm (−16.76‰), as expected for diet sources under the hypothesis of null ^{13}C fractionation (Post 2002). This biofilm was present in large abundance on the surface of *A. verticillata*, and was mostly composed of a diverse array of diatom species which were also found in the stomach contents of most of the individuals of *P. emertoni* examined (e.g. *Nitzschia* cf. *incognita*, *Navicula* cf. *normaloides*, *N.* cf. *salinicola*, or *Hyalosynedra* cf. *hyalina*). An important next question to answer is whether *P. emertoni* exhibits any selectivity in its feeding across the 3-dimensional structure of the biofilm, including from mainly attached, sometimes chain-forming species to several solitary and mobile ones.
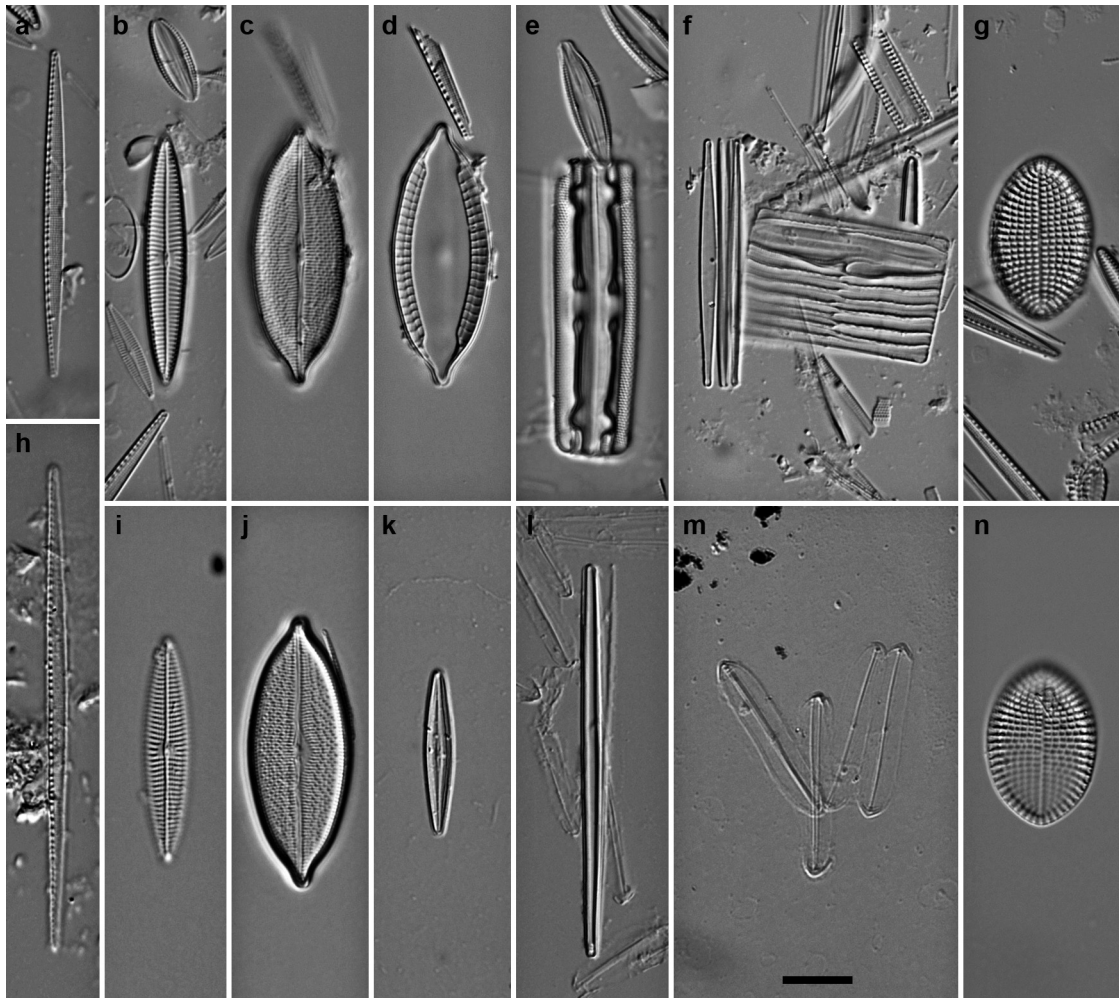
Fig. 5. Light microscope photographs of diatom species: (a–g) from biofilm on *Amathia verticillata*; (h–n) from stomach contents of *Polycerella emertoni* individuals. (a,h) *Nitzschia* cf. *incognita*; (b,i) *Navicula* cf. *normaloides*; (c,d,j) *Mastogloia* cf. *lanceolata*; (e) *Grammatophora* sp. and *Halamphora* sp. (smaller cell at top); (f) *Hyalosynedra* cf. *hyalina* (left) and *Hyalosira* sp. (right); (g,n) *Cocconeis* cf. *scutellum*; (k) *Brachysira* cf. *estoniarum*; (l) *Hyalosynedra* cf. *hyalina*; (m) cf. S*tenoneis*. Scale bar in panel m = 10 µm and applies to all panels

Although this study provides the first and only evidence of herbivorous behavior in nudibranchs, there are some other groups of Heterobranchia whose species are characterized by different feeding behaviors, including herbivory. For instance, species from the order Cephalaspidea show the greatest diversity of feeding strategies, ranging from herbivory to active predation (Kohn 1983). In the superorder Sacoglossa, most species described to date are strict herbivores (Raven et al. 2001), but some in the genera *Olea* and *Calliopaea* are indicated to be specialized egg-predators (Coelho et al. 2006, Filho et al. 2019).

The existence of a possible association between radula morphology and food type was investigated by Nybakken & McDonald (1981) in an array of nudibranch species feeding on different types of prey, such as bryozoans, cnidarians, and tunicates. Unfortunately, their description was limited to the number of teeth per row, and the full radular formula was not provided. The only species known to be similar to *P. emertoni* in their radula morphology are *Triopha catalinae*, *Limacia cockerelli* (Nybakken & McDonald 1981, described as *Laila cockerelli*), and *Antiopella barbarensis*, which supposedly display a food preference for hard bryozoan colonies, which is not consistent with the diet of *P. emertoni*. Nybakken & McDonald (1981) noticed that the teeth of *P. emertoni* have a relatively narrow base compared to other species of nudibranchs with a similar number of teeth per row (such as *T. catalinae*), suggesting that this character might also be important for determining diet type. On the other hand, *P. emertoni* displays a
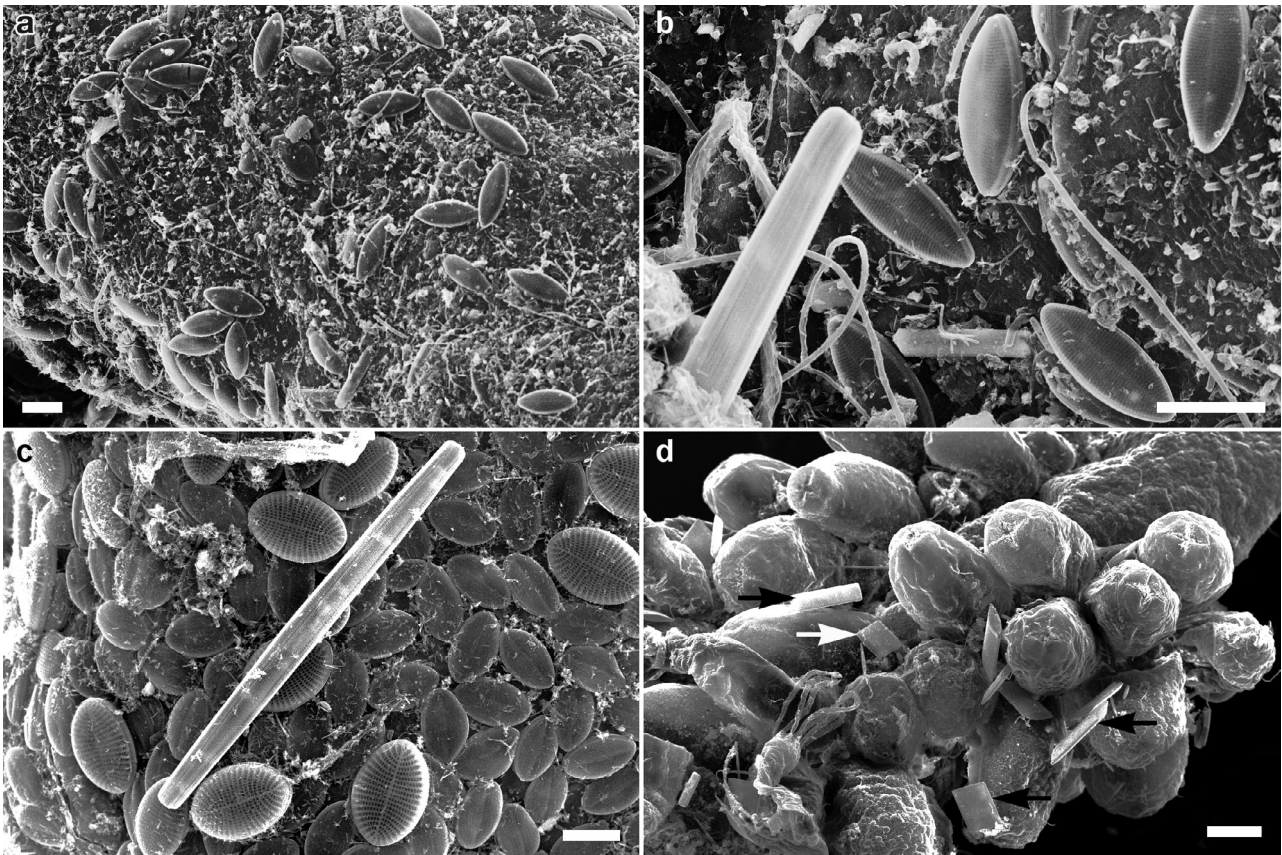
Fig. 6. Scanning electron photographs of the biofilm on *Amathia verticillata*: (a,b) overview and detail of attached diatom cells and organic matrix; (c) high abundance of appressed *Cocconeis* cells with a single *Nitzschia* species (elongated cell in center); (d) slim, rectangular cells of *Hyalosira* (arrows) between polyps on *A. verticillata*. Scale bars = 10 µm, except in panel d: 100 µm

radular morphology and formula (28–30 × 2.1.0.1.2) like those in some species of similar size and behavior in the genus *Okenia* (family Goniodorididae). For instance, *O. zoobotryon*, presumably a specialist consumer of *A. verticillata,* has a very similar radular formula of 25 × 1.1.0.1.1 (Pola 2014). *O. polycerelloides* (29 × 1.1.0.1.1, Sales et al. 2019), *O. problematica* (10–12 × 1.1.0.1.1, Pola et al. 2019), and *O. evelinae* (28 × 1.1.0.1.1, Marcus 1957) are also comparable to *O. zoobotryon*, which could mean that they have a similar diet. The radula of *P. emertoni* and these *Okenia* species is also long and narrow, with few teeth in each row; the lateral teeth have 1–2 cusps and denticulation on their inner face and smaller marginal teeth. Other investigated species in the Polyceridae family, such as *Polycera hedgpethi* (10–11 × 3–4.2.0.2.3–4, Miller 2001) and *P. glandulosa* (28–40 × 3.1–2.0.1–2.3, Behrens & Gosliner 1988), also display radular formulas somewhat different from both *P. emertoni* and the previously mentioned *Okenia* species. It is also worth noting that the

radula of *P. emertoni* also differs from most other radulae since it has no denticles at the end of the teeth, although the functionality of these denticles is unclear. Despite these differences in the number and shape of teeth, the radulae of these small nudibranchs are generally very similar in structure: long, narrow, and with at least 1 well-developed lateral tooth with at least 1 hook-shaped cusp, which may reflect an array of specialized feeding strategies, including the micro-herbivory condition of *P. emertoni*. However, this potential relationship is not straightforward and needs to be investigated further, including the proper determination of dietary items with analytical techniques similar to those used in the present study.

The abundance of *P. emertoni* was strongly seasonal in the study areas of the Ebro Delta. The numbers of both individuals and egg masses peak between July and October, depending on the bay, at water temperatures between 22 and 27°C. Seasonality has already been reported in some nudi-
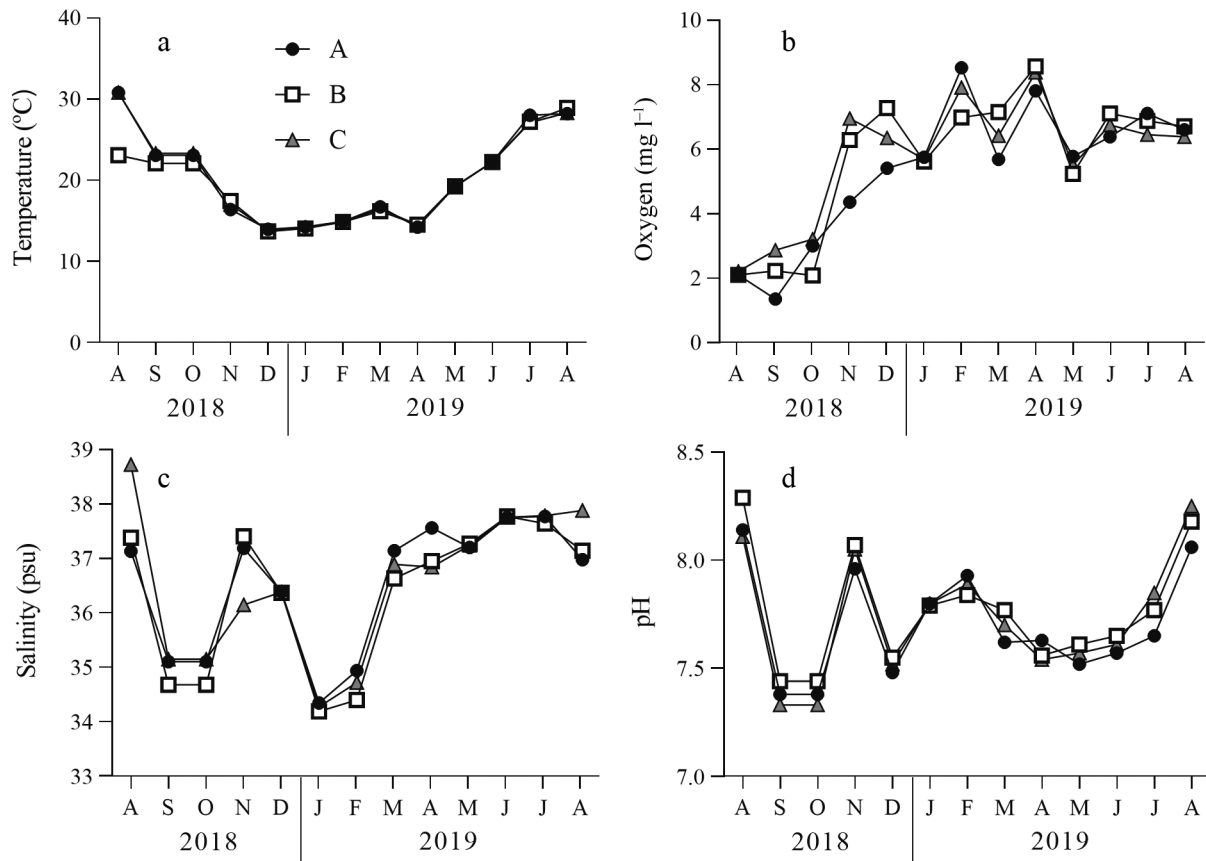
Fig. 7. Time series (August 2018 to August 2019) of environmental data at the 3 study sites (A, B, and C) in Alfacs Bay, Catalonia: (a) temperature (°C); (b) oxygen (mg l^{-1}); (c) salinity (psu); (d) pH. Error bars are SE

Table 3. One-way repeated measures ANOVA results for differences in the abundance of *Amathia verticillata* across 3 sites and between months: 2018 (Aug, Sep, Oct, Nov and Dec) and 2019 (Jan, Feb, Mar, Jun, Jul, Aug). Abundance data were log(x) transformed. Student-Newman-Keuls test. Statistically significant results are indicated in **bold**. [a]α = 0.05, [b]α = 0.01 (see Section 2.7.2 for details)

RM-ANOVA	df	MS	*F*	p
Site	2	11.92	1.45	0.27[a]
Error	12	8.22		
Month	12	34.78	5.78	**<0.001**[a]
Month × Site	24	10.30	1.71	0.02[b]
Error	144	6.01		
SNK (Month)	Aug-18 = Sep-18 = Jul-19 = Aug-19 > other months			

branch species (Chambers 1934), and it is possibly related to their high thermal sensitivity (Clark 1975). For instance, Lambert (1990) reported large summer peaks in the nudibranch community (including *Doto coronata*, *Dendronotus frondosus*, and *Eubranchus exiguus*) associated with colonies of the hydrozoan *Obelia geniculata* in Cape Neddick

(York, ME). Another possible explanation of nudibranch seasonality is high specificity for their dietary resources (Aerts 1994). In the Mediterranean, *P. emertoni* has been consistently observed associated with the pseudo-indigenous bryozoan *A. verticillata* (Camps & Prado 2018), suggesting that the bryozoan provides a unique resource that is not readily available elsewhere. Yet, *P. emertoni* from the Atlantic has been observed in other habitats such as on filamentous algae (Verrill 1881), indicating that *A. verticillata* is not itself the food source but other resources associated with those substrates are. *Okenia zoobotryon*, which is taxonomically close to *P. emertoni*, is also primarily found associated with *A. verticillata*. However, small numbers of adults have also been observed on the macroalga *Gracilaria* spp. and on other bryozoans, such as *Amathia maxima* (Robinson 2004, described as *Bowerbankia maxima*), thus also raising questions concerning the diet of this species. It remains to be determined whether these filamentous algae and bryozoans also possess a diatom epiphyton and whether *Okenia* feeds on it.
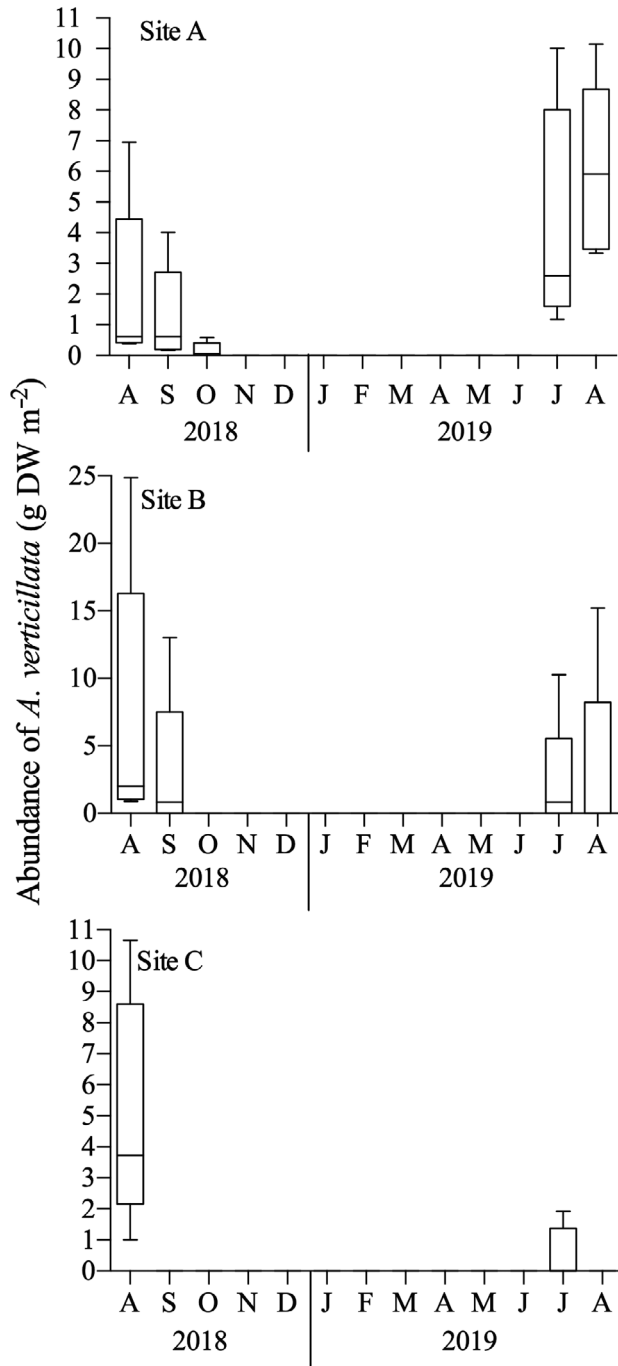
Fig. 8. Abundance of *Amathia verticillata* (g dry weight [DW] m^{-2}) (5th–95th percentile) per month (August 2018 to August 2019) at the 3 study sites (A, B, and C) in Alfacs Bay, Catalonia. Boxplots: central line = median, box = upper and lower quartiles and error bars = SE

Table 4. Student's *t*-test results for differences in the abundance of *Polycerella emertoni* and egg masses of *P. emertoni* between bays (A: Alfacs; F: Fangar) and in different months when presence was observed: 2018 (Jul, Aug, Oct) and 2019 (Jul, Aug, Oct). Statistically significant results (α = 0.05) are indicated in **bold**

Student's *t*-test	df	*t*	p
Individuals of *P. emertoni*			
Oct (A) vs. Oct (F)	58	−4.83	**< 0.001**
Jul (A) vs. Jul (F)	58	11.70	**< 0.001**
Aug-18 (A) vs. Aug-19 (A)	58	5.47	**< 0.001**
Egg masses of *P. emertoni*			
Oct (A) vs. Oct (F)	58	−5.62	**< 0.001**
Jul (A) vs. Jul (F)	58	7.16	**< 0.001**
Aug-18 (A) vs. Aug-19 (A)	58	3.75	**< 0.001**

in relation to predation, anti-predation, warning, or reproduction (Randall 2005). In nudibranchs, the loss of the shell as a defensive mechanism has been compensated for by adaptations to prevent being detected, such as homochromy (Faulkner & Ghiselin 1983), but all currently known cryptic nudibranchs are thought to feed on their habitat. In fact, many marine invertebrates camouflage themselves as their habitat but feed on other species, so they do not necessarily incorporate coloration patterns from their diet. For instance, the pink shrimp *Pontonides unciger*, which lives firmly attached to the coral *Cirripathes* spp., provides a remarkable example of commensal mimicry by adopting the shape and color of the polyps (Tazioli et al. 2007) while feeding on zooplankton (Terrana et al. 2019). Another similar example is the crab *Xenocarcinus tuberculatus*, which displays the same varied colorations of its black coral host (Tazioli et al. 2007), despite its diet being based on detritus and zooplankton (Gosliner et al. 1996). Also, some species of caprellids can change the color of their exoskeleton depending on the substrate where they shelter and may exhibit a significant affinity for particular bryozoans (Keith 1971), although their diet is mainly based on detritus, tiny crustaceans, and diatoms (Guerra-García et al. 2015).

The abundance of the bryozoan *A. verticillata* showed the same marked seasonality as *P. emertoni*, as large mats were developed during the summer and disappeared progressively in September–October (Micael et al. 2018). According to McKinney (1983) and Robinson (2004), *A. verticillata* is capable of establishing new colonies from small fragments of the stolon that are filled with a yolk-like substance and constitute resting structures persisting over the winter period (Jebram 1973). In spring, when temperature and food conditions are favorable, fragments may

An alternative plausible explanation for the high degree of specificity between cryptic nudibranch species and their habitats could be related to their capacity to mimic particular substrates. Mimicry has been widely observed in many marine invertebrates
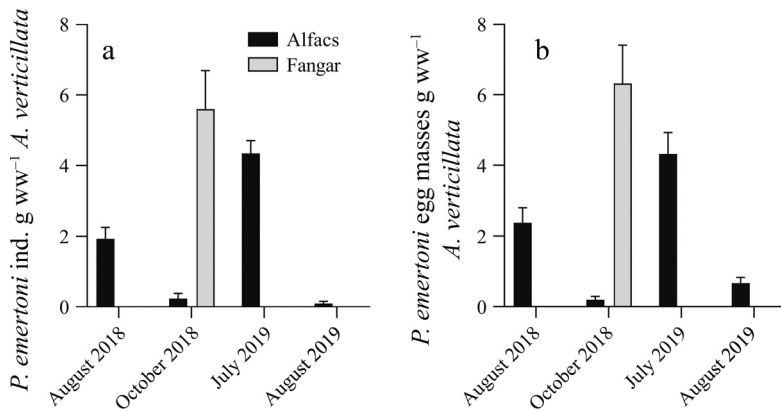
Fig. 9. Abundance of *Polycerella emertoni* (a) individuals and (b) egg masses per g wet weight (WW) of *Amathia verticillata* in Alfacs and Fangar Bays in the months when present. Error bars are SE

settle on a variety of natural and artificial substrates and grow asexually (McKinney 1983, Robinson 2004). Small-sized nudibranchs, taking advantage of the presence of their highly seasonal food, exhibit very rapid growth and can grow, mature sexually, reproduce, and lay eggs from a few days to a few weeks after the larval metamorphosis. These nudibranchs are known as opportunists or r-strategists; they do not usually maintain stable populations, and they suffer large fluctuations in the abundance of individuals over short periods of time. There are usually several generations in a single year. The larvae of benthic invertebrates may remain in the plankton for periods ranging from hours to months before settling and metamorphosing into their juvenile form (Hadfield & Paul 2001). If the larvae of *P. emertoni* are capable of surviving long periods in the plankton (on the order of months), the summer reappearance of *A. verticillata* may provide the specific biological cues required to initiate settlement as indicated for other species of nudibranchs (Hadfield & Paul 2001), but more studies are needed to confirm this idea.

Overall, the findings of our study question the general paradigm that all nudibranchs are carnivores and point to the need for a reassessment of the trophic ecology of the many species in this order, particularly for those that are similar in behavior and size to *P. emertoni*. At least a new trophic category of biofilm-grazer needs to be considered alongside the 4 ecological categories traditionally recognized (Todd 1981). Until more nudibranch species are studied using a similar approach to that applied in the present work, the full implications of our findings for food web/ nutrient transfers will not be clear. Nonetheless, our study provides evidence of a previously neglected route for periphyton carbon and other components

(such as silicon, which is metabolized and accumulated mostly by diatoms) to enter higher trophic levels during the summer period when the species become seasonally abundant. Animals take up silicon in their diet and incorporate it into connective tissues and bones, where it plays a physiological role in the calcification process (Harst & Obreza 2010). Hence, grazing of diatoms by *P. emertoni* may provide a pathway for silicon transfer to secondary consumers throughout the trophic food-web. In the particular case of Heterobranchia, Carefoot & Pennings (2003) also found that heavier silicon-rich food was readily eaten by sea hares, and significantly reduced the time spent swimming, which has possible implications for habitat fidelity in these species. In turn, removal of periphyton may also have positive effects on the settlement of bryozoan larvae (see for instance Dahms et al. 2004) adjacent to conspecific adults, as well as enhance the survival of small detached fragments of *A. verticillata*, thus favoring its dispersal and introduction into new areas.

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