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The use of stable isotopes to measure the ingestion rate of potentially toxic benthic dinoflagellates by harpacticoid copepods

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ABSTRACT

Phycotoxins synthesized by benthic dinoflagellates are known to bioaccumulate in macrofauna and hence represent a risk for human health. However, the presence of toxins synthesized by benthic dinoflagellates in smaller marine organisms than macrofauna has not been considered despite the fact that such small organisms have an important ecological role in the benthic food web. This present study quantified, for the first time, the trophic relationship between benthic dinoflagellates and meiofauna by using stable isotope enriched dinoflagellates during ingestion experiments. Results showed that harpacticoid copepods were not able to discriminate, during ingestion, between the potentially toxic cells of *Ostreopsis* cf. *ovata* and the non-toxic cells of *Amphidinium* cf. *carterae,* even when another food resource, such as diatoms (e.g. *Odontella* sp.), was provided (Kruskal Wallis test, $p > .05$).

1. Introduction

Over the last ten years, the frequency and the geographic extent of harmful algal blooms (HAB) has increased worldwide (Hallegraeff, 1993; Van Dolah, 2000; Cohu et al., 2011). Among the species which are able to generate HAB, around twenty benthic marine species have been identified to produce a wide variety of toxins including the most potent toxins occurring in nature (Accoroni et al., 2016; Chomérat et al., 2019; Hoppenrath et al., 2014; Litaker et al., 2017; Rodríguez et al., 2018; Tubaro et al., 2011; Verma et al., 2016; Yasumoto et al., 1987).

Benthic dinoflagellates synthesizing these toxins are particularly dangerous due to their impact on marine life (Shears and Ross, 2009) and human health (Alcala et al., 1988; Ciminiello et al., 2006; Friedman et al., 2008). Indeed, herbivorous and filter-feeding marine organisms can ingest and accumulate toxins produced by benthic dinoflagellates throughout their life (Chungue et al., 1977; Gleibs and Mebs, 1999; Yasumoto et al., 1976; Yasumoto et al., 1971).Toxins produced by benthic dinoflagellates can transfer between different trophic levels, through predation and bioaccumulation processes thereby reaching high concentrations in top predators which can be consumed by humans (Lewis and Holmes, 1993; Randall, 1958; Vernoux, 1988). Ingestion of seafood, previously contaminated by these toxins, can lead to mass mortalities of marine organisms involving important ecological impacts over large spatial scales (Shears and Ross, 2009) as well as economic impacts affecting the shellfish farming sector (Shumway, 1990). Due to the thermostability of these toxins (Kohli et al., 2015), each benthic toxic genus is responsible for specific human health issues, nevertheless leading in rare cases to death (Bagnis et al., 1979; Alcala et al., 1988; Onuma et al., 1999).

The genera *Gambierdiscus, Fukuyoa, Ostreopsis, Prorocentrum, Coolia,* and *Amphidinium* are frequently involved in benthic dinoflagellate blooms (Hoppenrath et al., 2014; Leung et al., 2018; Smith et al., 2017).

The most serious human poisoning events are related to the occurrence of *Gambierdiscus*, *Fukuyoa* (Bagnis et al., 1979; Chinain et al., 2019, 2014; Friedman et al., 2017), and *Ostreopsis* (Alcala et al., 1988; Randall, 2005) genera which are known to synthesize potent neurotoxins (Alloisio et al., 2016; Dechraoui et al., 1999). The species of *Gambierdiscus* and *Fukuyoa* are known to produce ciguatoxins (Yasumoto et al., 1977; Pearn, 2001; Litaker et al., 2017; Munday et al., 2017) which are potentially lethal for humans, identified as ciguatera fish poisoning (Chinain et al., 2019; Chinain et al., 2014; Yasumoto et al., 1977). Toxic *Ostreopsis* species synthesize ovatoxins and palytoxins (Ciminiello et al., 2008; Patocka et al., 2018; Rossi et al., 2010)

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causing human palytoxicosis and clupeotoxism after the consumption of certain tropical crustaceans and fish species which are able to bioaccumulate these toxins (Alcala et al., 1988; Onuma et al., 1999). In temperate regions, toxins synthesized by *Ostreopsis* spp. are involved in irritations by direct contact (Tichadou et al., 2010) and poisoning by inhalation (Gallitelli et al., 2005; Durando et al., 2007), however no incident has yet been reported by ingestion, even though these toxic compounds were detected in seafood.

Toxins produced by *Prorocentrum, Coolia,* and *Amphidinium* are less dangerous for humans than those synthesized by the genera *Gambierdiscus, Fukuyoa* and *Ostreopsis*. The genus *Prorocentrum* is distributed worldwide (Rodriguez et al., 2010; Richlen and Lobel, 2011) and is able to produce okadaic acid (Valdiglesias et al., 2013). This phycotoxin accumulates in shellfish and crustaceans (Kumagai et al., 1986; Vale and Sampayo, 2002) is responsible for human diarrhetic shellfish poisoning (Tripuraneni et al., 1997). Species of *Coolia* synthesize lipophilic toxins but these organisms have not yet been related to human poisoning events (Ben-Gharbia et al., 2016). Some *Amphidinium* species synthesize amphidinols and amphidinolides (Paul et al., 1997; Kobayashi, 2008). Laboratory scale experiments have shown that toxins produced by the genus *Amphidinium* can affect marine organisms (Pagliara and Caroppo, 2012), but there is still no evidence showing that the bioaccumulation of these toxins throughout trophic levels, including humans, is harmful (Botana, 2014).

The effects of toxins on benthic macrofauna consumed by humans have been intensively studied for several decades, for instance on filter feeders, (Lee et al., 1988; Amzil et al., 2012), macrophyte grazers (Amzil et al., 2012; Biré et al., 2015; Biré et al., 2013; Chungue et al., 1977; Yasumoto et al., 1976) and carnivorous fish (Lewis and Endean, 1984), and this due to the presence of toxic benthic dinoflagellates in coastal areas. However, such effort focusing on macrofaunal organisms has led to overlook the potential transfer of benthic dinoflagellates toxins to higher trophic levels through smaller size organisms representing the meiofauna.

Meiofauna are a prominent component of the benthos and consist of marine metazoans with variable sizes ranging from 40 μm to 500 μm (Giere, 2009). Meiofauna play two important roles in the benthic marine food web. They *i*) actively graze microalgae (Blanchard, 1991; Montagna et al., 1995) and to a certain extent bacteria (Pascal et al., 2009), and *ii*) constitute the predominant food source for a variety of benthic and pelagic predators (Gee, 1989; Coull, 1990). In the pelagic environment, zooplankton copepods can ingest dinoflagellates (Breteler et al., 1999; Breteler et al., 1990) and can consequently transfer their toxins throughout the pelagic food web (Maneiro et al., 2000, Jansen et al. 2006). Harpacticoid copepods associated to macrophytes can dominate the meiofauna (Beckley and McLachlan, 1980; Guidi-Guilvard et al., 2012; Johnson and Scheibling, 1987) and mainly graze on epiphytic algae (De Troch et al., 2007, Pavaux et al., 2019). Benthic copepods are ingested by benthic and pelagic predators (Gee, 1989, Coull, 1990) and could consequently create an important link between benthic toxic dinoflagellates and higher trophic levels. To our knowledge, this trophic link has never been measured.

The aim of the present study is to quantify ingestion rates of harpacticoid copepods fed with two species of benthic toxic dinoflagellates (*Ostreopsis* cf. *ovata* and *Amphidinium* cf. *carterae*), each having a distinct level of toxicity. Feeding experiments were also undertaken with and without a non-toxic diatom, *Odontella* sp., in order to evaluate the impact of another food resource on the ingestion rate of dinoflagellates.

2. Material and method

2.1. Micro-algal culture conditions

The dinoflagellate clonal cultures of *Amphidinium* cf. *carterae* (MCCV092), *Ostreopsis* cf. *ovata* (MCCV070) and the clonal culture of the diatom *Odontella* sp. (MCCV081) were initiated using specimens collected in Bois Jolan (16°14′08.2"N — 61°20′59.8 W, Guadeloupe, Caribbean Sea). These strains are maintained in the Mediterranean Culture Collection of Villefranche, France (MCCV strain number). Non-axenic cultures were grown in L1 medium (Guillard and Hargraves, 1993) using autoclaved, aged 0.2 μm filtered seawater with a salinity of 35. The cultures were maintained at 27 °C with a 12:12 light:dark cycle provided by cool-white fluorescent tubes in a Memmert incubator. Stock cultures were grown in 15 mL culture medium in flat culture flasks in order to optimize the surface culture area for gas exchange and growth of benthic dinoflagellates. During the exponential growth phase, clonal cultures were successively diluted in order to scale up the culture volume from 15 mL (in tissue culture flask of 25 cm^2 surface area, Corning) to 1 L (in fernbach culture flasks of 314 cm^2 surface area, Scott/Duran).

The biovolume of cells sampled from each cultivated strain was evaluated under an inverted microscope (Zeiss Axiovert 40 C) using the approximate geometrical shapes of the dinoflagellates, and the mathematical equation suggested for each genus (Hillebrand et al., 1999). The ellipsoid shape was chosen for *Amphidinium* cf. *carterae*, the cone and half sphere shape was chosen for *Ostreopsis* cf. *ovata* and the cylinder shape for *Odontella* sp. Five cells of each genus were used to determine the biovolume.

2.2. Dinoflagellates identification

A volume of 5 mL of each clonal culture was centrifuged for 6 min at 2000 rpm. The supernatant was removed and cell cultures were re-suspended with 1 mL of sterile water (MilliQ, Millipore). After a second centrifugation step (2 min at 2000 rpm), only the cell pellets were kept and homogenized with 40 μL of sterile water and a fraction of 10 μL of cell pellet was transferred to 0.2 mL polymerase chain reaction (PCR) tubes. Then, PCR tubes were stored at —20 °C until further analysis.

Approximatively 400 base pairs of the internal transcribed spacer region (ITS1–5.8 s-ITS2) ribosomal DNA (rDNA) were amplified by PCR using the primer 329F (5'-GTGAACCTGCRGAAGGATCA-3′) which is the inverse complementary sequence of the universal eukaryote primer 329-R (Moon-van der Staay et al., 2001) and the designed primer D1R-R (5'-TATGCTTAAAATTCAGCAGGT-3′) which is the inverse complementary sequence of the primer DIR-F (Scholin et al., 1994).

Each PCR tube containing the cell pellets was resuspended in 1 μL of each primer at 10 μM, 1 μL of dNTP at 10 mM, 1 μL of the 50× *Taq* Advantage 2 DNA polymerase (Clontech), 5 μL of 10× Advantage 2 PCR Buffer and 31 μL of sterile water in order to perform PCR reactions in a final volume of 50 μL. The PCR was performed using a MasterGradiant thermocycler (Eppendorf) with the following conditions: one initial denaturation at 94 °C for 10 min followed by 35 cycles each consisting of 1 min at 94 °C, 1 min at 53 °C, 1 min at 68 °C and a final elongation for 10 min at 68 °C. The PCR products were purified using QIAGEN MinElute PCR Purification kit according to the recommendations.

After sequencing by Genewiz, sequences were treated using BioEdit software and were compared to the National Center for Biotechnology Information database using BLASTn tool. The rDNA sequences obtained have been deposited on GenBank with the GenBank accession nos. **MK543271** and **MK543258** for the strains MCCV092 and MCCV070 respectively.

2.3. Labeling of micro-algal cultures

The experiments were run using a dinoflagellate culture (*Amphidinium* cf. *carterae* or *Ostreopsis* cf. *ovata*) and a diatom culture (*Odontella* sp.) in the exponential growth phase characterized by a cell density above 1000 cells mL^{-1}. Cultures of *Amphidinium* cf. *carterae* and *Ostreopsis* cf. ovata were labeled with ^{15}N a week before the beginning of feeding experiments.

Before deploying the stable isotope enrichment experiment, cultures

of dinoflagellates (> 1000 cells mL^{-1}) were filtered through a 10 μm mesh and rinsed with autoclaved 0.2 μm filtered aged seawater at a salinity of 35. Dinoflagellates remaining on the mesh were collected with a pipette. The collection, rinsing, and suspension steps were performed on successive small aliquots of culture (35–40 mL) in order to avoid net clogging due to mucus accumulation. Rinsed dinoflagellates (1500 cells mL^{-1}) were placed in 1 L of L1 culture medium with enriched sodium nitrate ($Na^{15}NO_3$, 99%, MERCK) for one week. During this period, the majority of N available for dinoflagellate intake was in the form of ^{15}N. The suspension and rinsing steps were used to start cultures in low nitrogen medium (Jauzein et al., 2017) and to remove the majority of bacteria present in the growth medium (Rausch de Traubenberg and Soyer-Gobillard, 1990). The same method was used to rinse dinoflagellates and diatoms from their culture medium before the beginning of the feeding assays. In order to evaluate the cell abundances for each culture, suspensions at adequate concentrations were done by using a 1 mL Sedgewick Rafter© counting cell under a standard light microscope.

2.4. Harpacticoid copepods culture conditions

Copepods were collected from stands of *Penicillus* sp. (Ulvophyceae) growing in the region of Bois Jolan (Guadeloupe, Caribbean Sea). They were identified as *Canthocamptus* sp. based on morphological characteristics. To obtain a monospecific culture of copepods, a unique female carrying eggs was isolated in 5 mL of GF/F filtered seawater. The volume of the culture was increased gradually until reaching a final volume of 1 L and the cultures were kept at 25–27 °C with a natural day/night cycle. Copepods were fed weekly with a mixture of 2/3 canned spinach and 1/3 dried fish food. Once a month, 2/3 of the culture volume water was removed and replaced by the same volume of 0.22 μm filtered seawater.

2.5. Feeding assay

Three different controls were designed with: a) 150 live copepods in 0.2 μm filtered seawater, b) 150 dead copepods (frozen and then thawed) in contact with dinoflagellates and c) 150 live copepods trapped in a tube closed by a GF/F filter and immersed in the microcosm containing dinoflagellates. Feeding experiments were performed with d) 150 live copepods in contact with ^{15}N enriched dinoflagellates only (*Amphidinium* sp. or *Ostreopsis* sp.), and e) 150 live copepods in contact with a mixture of enriched ^{13}C diatoms and ^{15}N dinoflagellates (*Amphidinium* sp. or *Ostreopsis* sp.), see Fig. 1. Each treatment was performed in triplicate conditions ($n = 3$) where 150 adult harpacticoid copepods were placed in a 100 mL microcosm (64 cm^2).

Concentrations of diatoms and dinoflagellates were determined in order to reach an equal total biovolume of diatoms and dinoflagellates in each experiment (Table 1). Feeding experiments lasted 4 h with a salinity of 35 and a temperature of 27 °C (Memmert incubator). Experiments were stopped by removing all copepods through a sieve (50 μm mesh). Copepods were preserved in a —80 °C freezer for subsequent chemical analysis.

2.6. Isotope analysis and calculations

The $\delta^{15}N$ of prey (*Amphidinium* cf. *carterae* and *Ostreopsis* cf. *ovata*) and predators (copepods) were measured by EA-IRMS (Elemental Analysis – Isotope Ratio Mass Spectrometry). Nitrogen isotope composition is expressed in the delta notation ($\delta^{15}N$) relative to air N_2: $\delta^{15}N = [(({}^{15}N/{}^{14}N)_{sample}/({}^{15}N/{}^{14}N)_{reference}) - 1] \times 1000$. For each experimental condition, copepod sample was composed by 150 specimens representing a total of 109 ± 7 μg (± standard error).

Incorporation of ^{15}N is defined as excess levels which are above the background ^{15}N level (i.e. copepods in control treatment incubated without ^{15}N enriched dinoflagellates) and is expressed in terms of specific uptake (*I*). *I* was calculated as the product of excess ^{15}N (*E*) and biomass of N per grazer. *I* was converted in dinoflagellate carbon grazed using C/N ratio of each dinoflagellate species measured with EA-IRMS. This C/N ratio was obtained from stable isotope measurements (30 samples of 150 specimens). *E* is the difference between the background ($F_{background}$) and the sample (F_{sample}) ^{15}N fraction: $E = F_{sample} - F_{background}$, with $F = {}^{15}N/({}^{15}N + {}^{14}N) = R / (R + 2)$ and R = the nitrogen isotope ratio. For the $F_{background}$, we used control values measured with control grazers. *R* was derived from the measured $\delta^{15}N$ values: $R = [(\delta^{15}N/ 1000) + 1] \times R_{airN2}$. The ingestion of dinoflagellates was calculated as [*I* × (C/N ratio of enriched dinoflagellate)/ ($F_{enriched\ dinoflagellate}$ × incubation time)] (Pascal et al., 2008).

Individual weights were derived from stable isotope samples and used in ingestion rate calculations.

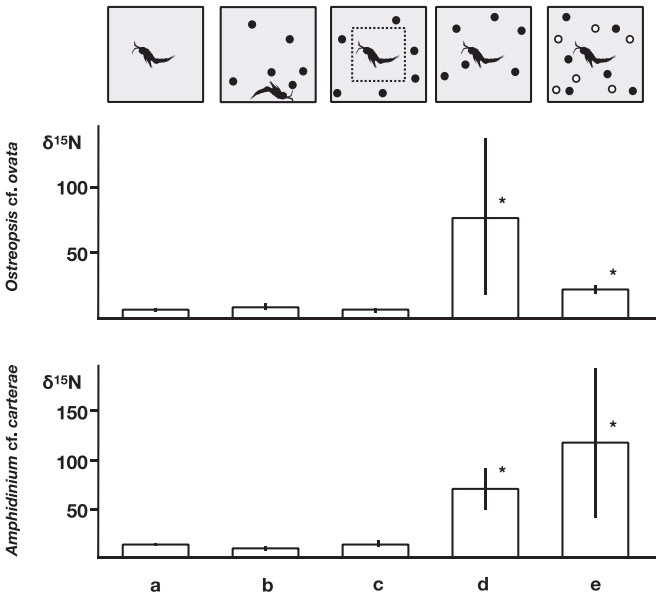

Fig. 1. $\delta^{15}N$ (‰) of copepods (± SE; $n = 3$, 150 specimens per sample) after incubation (4 h).
a) without dinoflagellate.
b) with copepods previously killed before incubation with ^{15}N enriched dinoflagellates ●.
c) caged copepods (GF/F filter) without access to ^{15}N enriched dinoflagellates.
d) with ^{15}N enriched dinoflagellates ●.
e) with diatoms ○ and ^{15}N enriched dinoflagellates ●.
* significant difference between a) and other incubation conditions (Kruskal Wallis tests, $p < .05$).

Table 1
Cell concentrations of labeled diatoms and dinoflagellates during feeding assays.

	Concentration (cells.mL^{-1})	Individual biovolume (μm^3)	Total biovolume (μm^3)
Experiment 1			
Diatom (*Odontella*)	100	3874	387,400
Dinoflagellate (*Amphidinium*)	600	677	406,200
Experiment 2			
Diatom (*Odontella*)	2400	3874	9,297,600
Dinoflagellate (*Ostreopsis*)	500	17,933	8,966,500

2.7. Data analysis

The $\delta^{15}N$ of copepods after all incubations was not normally

distributed which led to the use of non-parametric tests. Kruskal-Wallis tests were used to assess $\delta^{15}N$ and average ingestion rate of copepods. Dunn's test is a multiple pairwise comparison method allowing comparisons of the mean of the rank of each treatment after a Kruskal-Wallis test. Values are presented as means ± standard deviations (SD), except when specified otherwise.

3. Results

The ITS sequence of 411 bp from the strain MCCV092 collected in Guadeloupe matched with a sequence of *Amphidinium* cf. *carterae* (KY697961.1) through GenBank. No data is available on the exact location where the strain was sampled. This match was made with a E-value of 0, an identity percentage of 98.56% and on 100% of query coverage.

The ITS sequence of 370 bp for the strain MCCV070 collected in Guadeloupe matched in GenBank with a sequence of *Ostreopsis* cf. *ovata* (MH844087.1) collected in Ecuador. This match was made with a E-value of 0, a percentage of identity of 98,64% and on 100% of query coverage.

The ^{15}N enriched dinoflagellates *Ostreopsis* cf. *ovata* and *Amphidinium* cf. *carterae* used during ingestion experiments presented a $\delta^{15}N$ of 96,569‰ and 555,151‰ respectively. Their individual ratios of C/N were 18.4 and 11.7, respectively. *Ostreopsis* cf. *ovata* and *Amphidinium cf. carterae* cell biovolumes reached 17,933 ± 7081 μm^3 and 677 ± 64 μm^3 respectively. The clonal culture of *Odontella* sp. used during ingestion experiments presented a biovolume of 3874 ± 508 μm^3. A total of 4500 harpacticoid copepod specimens were isotopically determined from which weight, C and N contents were derived. The individual weight of an adult copepod was estimated at 724 ± 99 ng copepod^{-1} with 30.9% of C and 14.5% of N.

Copepods were incubated in three control conditions a) copepods which were not fed with ^{15}N enriched dinoflagellates and presented a $\delta^{15}N$ of 7.0 ± 0.7‰ (n = 3), b) copepods which were euthanized (frozen and then thawed) before incubation and c) copepods trapped in GF/F filter without having access to ^{15}N enriched dinoflagellates. The $\delta^{15}N$ of copepods from both b) and c) control conditions were not significantly different from control a), Kruskal-Wallis, $p > .05$, and this conclusion was validated with clonal cultures of both *Ostreopsis* cf. *ovata* and *Amphidinium* cf. *carterae* (Fig. 1 and letters associated).

In contrast, copepods incubated with ^{15}N enriched dinoflagellates presented a significantly higher $\delta^{15}N$ compared to the control a). This difference was observed for both dinoflagellate species (Kruskal-Wallis, $p < .05$). The $\delta^{15}N$ of copepods incubated with enriched *Ostreopsis* cf. *ovata* and *Amphidinium* cf. *carterae* was not affected by the addition of diatoms (Kruskal Wallis, $p > .05$).

The average ingestion rate of copepods was not significantly different between *Ostreopsis* cf. *ovata* (372 ± 412 pg C ind^{-1} h^{-1}) and *Amphidinium* cf. *carterae* (49 ± 82 pg C ind^{-1} h^{-1}), Kruskal Wallis test, $p > .05$.

4. Discussion

4.1. Potential toxicity of benthic dinoflagellates in the Caribbean Sea

Investigating the toxicity of benthic dinoflagellates in the Caribbean Sea is recent and studies generally focus only on several genera such as *Gambierdiscus* (Díaz-Asencio et al., 2019; Litaker et al., 2017), *Prorocentrum* (Moreira González, 2013) and *Amphidinium* (Moreira González, 2013).

Despite a great diversity of *Ostreopsis* species observed in the Caribbean Sea (Ballantine et al., 1988; Besada et al., 1982; Bomber et al., 1988; Faust, 2009; Faust, 1995) no studies have yet focused on the actual production of toxins by *Ostreopsis* cf. *ovata* in this area. In the Mediterranean Sea, environmental conditions have been shown to modify the toxicity of *Ostreopsis* cf. *ovata* (Pezzolesi et al., 2012; Scalco et al., 2012). Indeed, high toxicity levels were measured when water temperatures increased above 25° (Pezzolesi et al., 2012; Scalco et al., 2012) and with a salinity of 32 (Pezzolesi et al., 2012). These environmental conditions, which seem to optimize the toxin production of *Ostreopsis* cf. *ovata* in the Mediterranean Sea, were similar to those applied in this present study which suggests that Caribbean strains could be toxic (Boisnoir et al., 2019; Boisnoir et al., 2018). In addition, toxin production by *Ostreopsis* cf. *ovata* strains collected in the Mediterranean Sea increased during cell growth (Guerrini et al., 2010; Pistocchi et al., 2011). This trend was also observed for the Brazilian strains (Nascimento et al., 2012). However, the increase of toxin production during growth was not measured for all the Mediterranean strains of *Ostreopsis* cf. *ovata* (Scalco et al., 2012). The risk of using aged cells, in post-exponential growth phase, is that the toxins produced by *Ostreopsis* cf. *ovata* are released in the medium (Guerrini et al., 2010) which can hence reduce the feeding efficiency of harpacticoid copepods (Pavaux et al., 2019).

The toxicity of the genus *Amphidinium* from the Caribbean and inherent toxin content, was studied on one single species *A. massartii* (Moreira González, 2013). To our knowledge, no study has yet investigated the relationship between the environmental conditions and the toxicity of *Amphidinium* cf. *carterae*. However, strains of *Amphidinium* cf. *carterae* collected off the coasts of Bahamas, in the Mediterranean Sea and in China had hemolytic and antilarval activities (Kong et al., 2016; Meng et al., 2010; Pagliara and Caroppo, 2012).

Moreover, cells of *Amphidinium* cf. *carterae* from Egypt collected during the exponential growth phase were found to be toxic on *Artemia salina,* whilst cells collected in the post-exponential phase had no effect (Ismael et al., 1999). On the other hand strains from the Northern Arabian Sea did not show any significant toxicity on *Artemia salina* (Baig et al., 2006).

4.2. Methodological considerations

Identifying and quantifying copepod ingestion rates of specific microalgae species are relevant practices when considering the context of harmful algal blooms (Haley et al., 2011). Similarly to planktonic organisms, several methods can be used to quantify the ingestion rate of benthic dinoflagellates by epiphytic copepods. However, it remains difficult to undertake *in situ* copepod feeding experiments without depending on laborious, intrusive and potentially biased incubation approaches (Nejstgaard et al., 2008). Methods used to quantify ingestion rates of microalgae all present both strengths and weaknesses. Furthermore, methods which are initially adapted for planktonic organisms can be difficult to implement on benthic organisms.

Relationships between different trophic levels can be evaluated by identifying ingested organisms in the feces or in the gut content and thus by undergoing microscope observations. This method is nevertheless laborious even for a trained observer (Nejstgaard et al., 2008) and a high fraction of food items remains impossible to identify (Gowing and Wishner, 1992). In order to bypass direct microscope identification, another indirect approach based on the measurement of specific pigments of dinoflagellates in the gut content of copepods can be applied (Kleppel et al., 1988; Oechsler-Christensen et al., 2012). Actually, most studies involving copepod feeding rely on this approach although several limitations have been highlighted (Bustillos-Guzmán et al., 2002; Pandolfini et al., 2000) such as *i*) pigment degradation during the digestion process (Pandolfini et al., 2000) and *ii*) limited specificity of pigments for a given microalgal group (Antajan et al., 2004; Irigoien et al., 2004). However, this method remains largely used as it is quick and inexpensive (Nejstgaard et al., 2008). Biomarkers could be an alternative, however there is a lack of specificity for dinoflagellates, particularly regarding amino acid (Guisande et al., 2002) and fatty acid (Desvilettes et al., 1994; Graeve et al., 1994) compositions. A new promising strategy is the use of prey-specific DNA barcodes (Sheppard and Harwood, 2005). This approach was used to

quantify specific phytoplanktonic species present inside gut contents and fecal pellets of zooplankton (Nejstgaard et al., 2008; Nejstgaard et al., 2003). The DNA-based approach may provide a way to rapidly measure ingestion rates, even when the targeted microalgae are present in low abundances within a mixed community (Haley et al., 2011). With this approach, cells of interest which are used as prey must be previously sequenced in order to develop specific primer sets in order to amplify their DNA (Sheppard and Harwood, 2005). Another inconvenience of this method is the possible interference of a large amount of non-target DNA with the primer or the presence of co-purifying material from the host copepod during quantification (Nejstgaard et al., 2008). However, the use of high throughput DNA sequencing combined with better-designed primers and improved databases will undoubtedly generate more studies employing DNA-based approaches (Ho et al., 2017).

Alternatively, grazing experiments offer a straightforward method to estimate prey consumption and do not present drawbacks associated with label specificities. The most current grazing experiment method is based on the disappearance of prey cells over time (Frost, 1972). This method is reliable for planktonic organisms (Campbell et al., 2005; Haley et al., 2011; Turner, 2014) but presents limitations for benthic organisms. When microalgae are present in low concentrations, the accuracy of cell counting decreases (Campbell et al., 2005; Haley et al., 2011) which leads to an overestimation of ingestion rates. Moreover, benthic dinoflagellates can form agglomerates, which may sink and attach to the surface of the cultivation flasks, thereby increasing this bias. Methods used to detach benthic cells can damage cell integrity and introduce a bias in interpretations. Indeed, an empty theca can be considered as a consumed cell with an ingested cytoplasmic content or as a cell which is not grazed.

Grazing experiments can also be performed using pre-labeled prey. In this case, dinoflagellates can be labeled using radioactive isotopes (Lampert and Taylor, 1985; Napp and Long, 1989; White and Roman, 1991) but with the inconvenience to present legal restrictions. Compared to radioactive isotopes, using stable isotopes to enrich dinoflagellates is more appropriate especially for investigators who are limited by radioactive material regulations. The method using pre-labeled dinoflagellates enriched with stable isotopes was consequently chosen in this present study and controls were conducted to assess its efficiency. For instance, passive adhesion of labeled dinoflagellates on the cuticle of copepods (Fig. 1.b) could overestimate the ingestion of dinoflagellates. However, controls using dead copepods revealed a limited bias due to such events. Caged copepods were not able to ingest benthic dinoflagellates but could consume their soluble secretions through cage meshes. Results showed a limited labeling for caged copepods which highlighted that the transfer of dinoflagellate compounds through the dissolved form was limited (Fig. 1.c). Even if 150 specimens were pooled for each sample, measured ingestion rates presented a high variability potentially due to different feeding behaviors between specimens.

4.3. Ingestion rate

Copepod ingestion of toxic planktonic dinoflagellates have been previously measured for genera such as *Alexandrium* (Lasley-Rasher et al., 2016; Sopanen et al., 2011; Teegarden and Cembella, 1996), *Karenia* (Prince et al., 2006; Schultz and Kiørboe, 2009; Walsh and O'Neil, 2014), and *Gymnodinium* (da Costa et al., 2012; Koski et al., 1998; Paffenhöfer, 1971). These studies revealed that different factors can lead to difficulties in interpretation when describing interactions between grazers and cell prey. Furthermore, different predators and various clonal cultures of dinoflagellates which are often used during the feeding assay experiments make the comparison between studies problematic (Teegarden and Cembella, 1996). The interaction between grazers and cell prey can be highly specific (Teegarden, 1999; Teegarden and Cembella, 1996) and even site-specific when a single grazer is considered (Teegarden and Cembella, 1996; Uye and Takamatsu, 1990). Indeed, within a species, trophic interactions can be population-specific and linked with population history (Colin and Dam, 2002). For instance, a same species of grazer, present at two different geographic sites, and fed with the same toxic dinoflagellate species, can present different ingestion rates, which suggests that historical exposure of grazers can impact the ingestion (Colin and Dam, 2003, 2002). There are also ontogenetic considerations, such as different developmental stages (e.g. nauplii *vs*. copepodides vs adult copepods) which certainly display diet differences in the type and amount of preyed items.

Trophic interactions between dinoflagellates and the meiobenthos are less investigated than with the macrobenthos and zooplankton. Harpacticoid copepods can graze on different food items due to the large diversity of microalgal species present in the microphytobenthos (Azovsky et al., 2013) however, they are usually selective in their ingestion (Azovsky et al., 2005; Buffan-Dubau et al., 1996). Indeed, in temperate regions, copepods were found to feed on a broad diversity of diatoms (Cnudde et al., 2011; Decho, 1986; Rzeznik-Orignac and Fichet, 2012; Wyckmans et al., 2007) and bacteria (Cnudde et al., 2013; Cnudde et al., 2011; Pascal et al., 2013; Pascal et al., 2009).

In tropical areas, potentially toxic dinoflagellates can be a major component of the microphytobenthic communities (MacIntyre et al., 1996). To our knowledge, the ingestion rate of potentially toxic dinoflagellates by meiofauna has never been measured in benthic environments.

The present study indicates that harpacticoid copepods can feed on potentially toxic benthic dinoflagellates even when another food source is available. Indeed, ingestion rates of dinoflagellates were not affected when adding diatoms as a food resource, which suggests that dinoflagellates are *i)* part of the regular copepod diet and *ii*) are not neglected when another food resource is available. Similar ingestion rates were found when copepods were fed with *Ostreopsis* sp. and with *Amphidinium* sp. even though *Ostreopsis* cells have a cellulosic theca (Schmidt, 1901) whilst *Amphidinium* cells have none (Fensome et al., 1993). This result suggests that rigid cellulosic walls can be easily broken down by copepods in order to ingest the cytoplasmic content. Moreover, the high $\delta^{15}N$ variability found in the present study when applying feeding conditions d) and e) could be due to fluctuations of the body size explained by *i*) sexual dimorphism in harpacticoid copepods where the females are larger than the males and/or *ii*) different shapes of the mouthpart which determine food ingestion (Giere, 2009). Observations showed that the body size of copepods could influence the ingestion rate of planktonic copepods (Turner and Tester, 1989). Indeed, the body size is an important factor for many physiological processes (Peters, 1985) since in general terms, maximal ingestion rates are inversely correlated to the body size (Moloney and Field, 1989).

4.4. Ingestion role in regulation of blooms

Predation of dinoflagellates by meiofauna have received only little attention and this mainly due to technical constraints associated to the quantification procedure (Danovaro et al., 2007). Blooms of benthic toxic dinoflagellates are currently increasing worldwide (Hallegraeff, 1993; Van Dolah, 2000; Cohu et al., 2011) and the position of the meiofauna within these events needs further clarification mainly regarding the regulation of toxic dinoflagellate abundances through ingestion and transfer of toxins in the food web. In the global context of climate change now experienced by the oceans, quantification of predation rates might be useful to understand the ecological role of benthic toxic dinoflagellates and to forecast how these relationships will evolve.

Algal blooms are possible only if algal growth exceeds the loss by predation and senescence (Buskey et al., 1997). The role of predation must hence be further assessed in order to better describe the regulation of benthic toxic dinoflagellate blooms. Population blooms of benthic dinoflagellates may be controlled by meiobenthic organisms in a

comparable way to those occurring in planktonic environments. Model simulations have already shown that during the first stages of pelagic dinoflagellate blooms, growth can be retarded or inhibited with a low pressure of predation by copepods since each grazed cell is, in proportion, an important loss when considering such a small local population (Haley et al., 2011). However, the impact of low predation was minimal when simulations were carried out with higher micro-algae abundances, above 100 cells L^{-1} (Haley et al., 2011), which highlights the fact that predation pressure has an important role in bloom dynamics, especially in the early development stages. Some dinoflagellates are able produce cysts (Anderson, 1998; Faust, 1992; Tian et al., 2017) which make them sink down to the seabed, thereby being less available for planktonic copepods (Butman et al., 2014; Dale et al., 1978; Mohamed and Al-Shehri, 2011). It has been suggested that a decrease in the abundance of dinoflagellates due to grazing is relatively minor compared to the increase of abundance resulting from a gradual release of germinated cells from the benthic cyst beds (Anderson et al., 2005).

Planktonic dinoflagellates are ingested by copepods even at low concentrations (Haley et al., 2011). Therefore, benthic toxic dinoflagellates, which are found all year round in Guadeloupe and Martinique at viable densities (Boisnoir et al., 2019), could consequently be grazed and assimilated by copepods on a permanent basis. Meiofauna represent a key component of coastal benthos, since over 75% of the total meiofauna production is transferred to higher trophic levels through predation (Danovaro et al., 2007), especially due to macrofaunal and other epibenthic predators (Chardy and Dauvin, 1992). Harpacticoid copepods ingesting toxic benthic cells could bioaccumulate the toxins synthesized by the benthic dinoflagellates and contaminate secondary consumers when they are consumed. Furthermore, the toxicity of dinoflagellate cysts has been shown to be higher in benthic cells than in planktonic cells (Dale et al., 1978) which can expose meiobenthic grazers to a more important toxic risk, able to induce mass mortalities and promote the development of dinoflagellate blooms. Natural marine toxins are a considerable increasing threat when bioaccumulation takes place within the food chain (Ramos and Vasconcelos, 2010). Indeed, the ingestion of benthic toxic dinoflagellates is a way of introducing phycotoxins in the food web, and to our knowledge, this transfer has been rarely considered. The role of the meiofauna in this transfer of toxins has been considered as minor, however, more interest should be given to estimate the bio-magnification potential through specific models since the amplification effect of such toxins is liable to contribute to the emergence of diseases related to toxic benthic dinoflagellates.

Declaration of Competing Interest

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

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