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Domoic acid accumulation in the sardine *Sardina pilchardus* and its relationship to *Pseudo-nitzschia* diatom ingestion

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ABSTRACT: Planktivorous fish are key potential vectors for the phycotoxin domoic acid (DA), produced naturally by diatoms from the genus *Pseudo-nitzschia*. The diet of the Atlantic sardine *Sardina pilchardus* is largely dominated in number by microplanktonic species such as chain-forming diatoms, making the accumulation of this toxin and its transfer to the higher trophic levels likely. DA concentration in sardine tissues and *Pseudo-nitzschia* ingestion were monitored fortnightly during 2002 and 2003 off the NW coast of Portugal, where seasonal upwelling events are responsible for the occurrence of algal blooms. Sardine stomach content analysis showed that *Pseudo-nitzschia* prey reached concentrations as high as 7.8×10^6 cells g^{-1}; in some cases this diatom genus represented more than 99% of the phytoplanktonic prey identified in the stomachs. Four different diatom species were distinguished using scanning electron microscopy (SEM): *P. australis*, *P. pungens*, *P. pseudodelicatissima* and *P. delicatissima*. DA accumulation in sardines was linearly dependent on *P. australis* consumption. Toxin content per individual cell was estimated by comparing DA and *P. australis* concentrations in the stomach contents. DA production by *P. australis* was significantly higher in the summer months than during the spring. In both years, DA in sardine guts was initially detected in May and peaked several times until late summer. Toxin distribution in the different tissues was also determined, with the highest DA levels detected in the intestine. The maximum toxin concentration observed in sardine guts was 128.5 µg DA g^{-1}. No DA was found in the sardine muscle; consequently implications for human health appear minimal.

KEY WORDS: Domoic acid · *Pseudo-nitzschia australis* · *Sardina pilchardus* · Stomach contents

INTRODUCTION

Phytoplankton is the basis of marine food webs, directly supporting filter-feeding organisms. Some species of dinoflagellates and diatoms produce toxins that can cause poisoning in vertebrates. The accumulation and transfer of these toxins through the food chain can cause intoxication of consumers at higher trophic levels. Massive deaths of sea birds and marine mammals due to their predation on planktivorous fish have been reported in California, USA (Work et al. 1993, Sierra Beltrán et al. 1997, Lefebvre et al. 1999, Scholin et al. 2000). It was shown that the northern anchovy *Engraulis mordax*, a planktivorous filter-feeder, was responsible for these events, acting as a domoic acid vector (Fritz et al. 1992, Lefebvre et al. 2001, 2002).

Domoic acid (DA), known as the amnesic shellfish toxin, is produced by some species of the diatom genus *Pseudo-nitzschia* (Subba Rao et al. 1988, Bates et al. 1989, Buck et al. 1992, Garrison et al. 1992). The illness referred to as amnesic shellfish poisoning (ASP) was first recognized in 1987 in Canada, where at least 3 elderly people died and more than 100 became ill, suffering from varying degrees of gastrointestinal and neurological illness, after consumption of contaminated blue mussels (Quilliam & Wright 1989, Todd 1993). The toxin is a water-soluble amino acid that

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binds irreversibly to glutamate receptor sites, causing destructive neuronal depolarization (Debonnel et al. 1989) and permanent short-term memory loss in mammals (Perl et al. 1990, Todd 1993).

Although much attention has been paid to the effects of phycotoxins in higher organisms including marine mammals, sea birds and also humans, the trophic links between these organisms should also be elucidated. In Europe, extensive research has focussed on DA accumulation in bivalves; however, studies of toxin transfer through the pelagic food chain are scarce. Recent studies in California indicate that planktivorous fish are potentially more effective vectors than bivalves (Scholin et al. 2000, Lefebvre et al. 2002).

During a period of shellfish contamination off Portugal in 2000, sardine *Sardina pilchardus* was found to accumulate high levels of DA (Vale & Sampayo 2001). This omnivorous species is the most abundant fish in Portuguese waters with high commercial value. Sardines are primarily filter-feeders, whose diet composition is closely related to plankton availability (Varela et al. 1988, 1990). In terms of biovolume, sardine diet is mostly supported by mesozooplankton organisms, which are assimilated more efficiently than phytoplankton (van der Lingen 1998, Bode et al. 2004). However, it is numerically dominated by organisms smaller than 200 µm, with large quantities of phytoplankton being consumed (Garrido 2003). Diatoms are especially abundant on the Portuguese coast during the spring months (Moita 2001), when they can account for more than 90% of the total number of prey in sardine stomachs (Garrido 2003). This suggests that sardines could act as important vectors of the phycotoxin domoic acid.

The aim of this work was to study the seasonality and transfer of DA from the toxin producers to a direct consumer. To accomplish this, DA levels in sardine tissues were monitored during 2002 and 2003 in the fishing area of Peniche (Portuguese NW coast), a region where seasonal upwelling events give rise to algal blooms, including *Pseudo-nitzschia* spp. Sardine diet was analyzed and microplankton prey in stomach contents were identified and quantified, paying particular attention to toxic phytoplankton species, in order to establish a link between *Pseudo-nitzschia* ingestion and DA occurrence.

MATERIALS AND METHODS

Sample collection. Sardine samples were obtained fortnightly during 2002 and 2003 from purse-seiners fishing off Peniche (NW Portuguese coast) at night, at a depth range of 12 to 70 m and 6 to 25 km from the coast. Sampling was intensified in late spring 2003, when the first *Pseudo-nitzschia* cells were observed in high concentrations in sardine stomach contents (7 samples in May and 4 in June).

Sardine stomach content analysis. Stomach content analysis was performed with sardines collected during 2003. Analysis showed a low coefficient of variation of the mean stomach content weights (CV <25%) between individuals from the same sample date and length class, which led us to assume minimal differences in their diet. In each sample, stomach contents from 10 sardines of 18 cm total length (representative of the most abundant length class in the fishery) were pooled and homogenized prior to the analysis. A portion of the homogenate was used for DA determination (1 g), the remainder was sieved (200 µm) to exclude large prey (>200 µm) and a subsample was taken for identification and quantification of the microplankton under a light microscope (400×).

Scanning electron microscopic (SEM) analysis. When *Pseudo-nitzschia* cells were present in stomachs, a subsample of the stomach contents was taken for their identification and enumeration using SEM. Diatom frustules were cleaned using von Stosch's method (Hasle & Syvertsen 1996). Samples were coated with gold and examined using a JEOL JSM-5200, usually at 20 kV accelerating voltage. The percentage of each *Pseudo-nitzschia* species in a total count of at least 100 cells was determined. To distinguish these species from each other, diverse characters were observed, such as the shape of the valve (which is asymmetrical in the case of the *P. australis*, as opposed to the symmetric shape of the strongly silicified *P. pungens*), the width of the cell, the number and size of poroids, and the presence of a central nodule (Hasle & Syvertsen 1996, Hasle et al. 1996, Skov et al. 1999). *P. pseudodelicatissima* and *P. delicatissima* were identified according to the previous authors, although they are under taxonomic revision (Lundholm et al. 2004).

Toxin extraction and HPLC analysis. Sardines were dissected 5 to 10 h after capture and kept fresh (4 to 7°C), to minimize DA leakage and contamination across tissues. Whole guts (the digestive tract and all internal organs including the gonads) of 5 individuals were removed and homogenized with a Polytron PT 3100. A 5 g aliquot was kept at –20°C for subsequent DA determination. Three naturally contaminated samples, each derived from 5 individuals, were selected to study the DA distribution in the following sardine tissues: (1) intestine, (2) stomach, (3) liver, (4) gonads, (5) brain and (6) muscle.

Extracts for analysis were prepared according to Quilliam et al. (1995) with some modifications (Vale & Sampayo 2001). The extraction was performed with aqueous 50% methanol (ratio 1:4) at 20000 rpm (3360 × *g*) with a homogenizer probe for 1 min, followed by

10 min centrifugation at 4000 rpm (2240 × *g*). The supernatant was filtered (0.22 µm) and the equivalent of 1.0 mg extract (5 µl) was injected into the HPLC column without any further clean-up.

HPLC analysis was performed on a Hewlett-Packard (HP) Model 1100 equipped with an in-line degasser, quaternary pump, autosampler, oven and diode-array detector (DAD). Data collection and treatment of results were performed by the HP Chemstation software. The column used was a Nucleosil 100-5 C-18 (125 × 3 mm, 5 µm), with a guard-column Lichrospher 100 RP-18 (4 × 4 mm, 5 µm). The detection wavelength was set at 242 nm with a 10 nm bandwidth, and the reference wavelength at 450 nm with a 100 nm bandwidth. A confirmatory wavelength at 262 nm was used.

Calibration was performed with a full set of DA standards (0.5, 2, 4 and 10 µg ml^{-1}). A single point calibration, with a working solution of 4 µg ml^{-1} DA in 10% acetonitrile, was performed after 6 consecutive samples. DACS-1D-certified DA standard was purchased from the National Research Council of Canada (NRC). Under these conditions, the detection limit was 0.04 µg ml^{-1}, corresponding to 0.2 µg g^{-1} in tissue.

Statistics. A least squares linear regression analysis was applied to data, using the statistical software R version 1.9.1. (www.r-project.org). DA concentration in each stomach was first considered as a function of the interaction between *Pseudo-nitzschia australis* cell counts and season, and of *P. pseudodelicatissima* concentration (which only occurred during the summer), through a multiple regression analysis. Two seasons were considered, spring (March until May) and summer (June until September), since no DA production was found during the rest of the year. The variables that were not significant to the model were removed through backward stepwise regression analysis (Draper & Smith 1998).

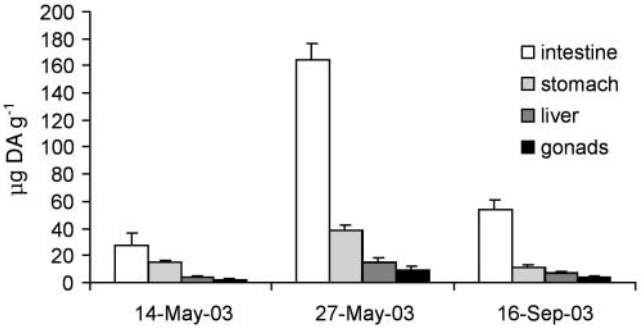

Fig. 2. *Sardina pilchardus*. Domoic acid (DA) concentration (µg g^{-1}, mean + SD) in the different sardine tissues of 3 selected samples (5 individuals each)

RESULTS

Domoic acid in sardines

Domoic acid was found in sardine guts during both 2002 and 2003 (Fig. 1). In 2002, DA was detected from early May until late August, reaching its peak concentration (43 µg g^{-1}) in the first week of July. In 2003, a small amount of DA (1.3 µg g^{-1}) was detected in the sardine guts during March. In the same year, several DA peaks were detected from May to middle September, reaching a maximum concentration of 128.5 µg DA g^{-1} in late June.

Analysis of DA distribution in different sardine tissues (Fig. 2) showed higher concentrations of the toxin in the intestine, followed by the stomach, liver and gonads. The intestine presented levels about 3 times higher than the stomach, and about 9 and 15 times higher than the liver and gonads, respectively. DA was not detected in the brain, nor in the muscle.

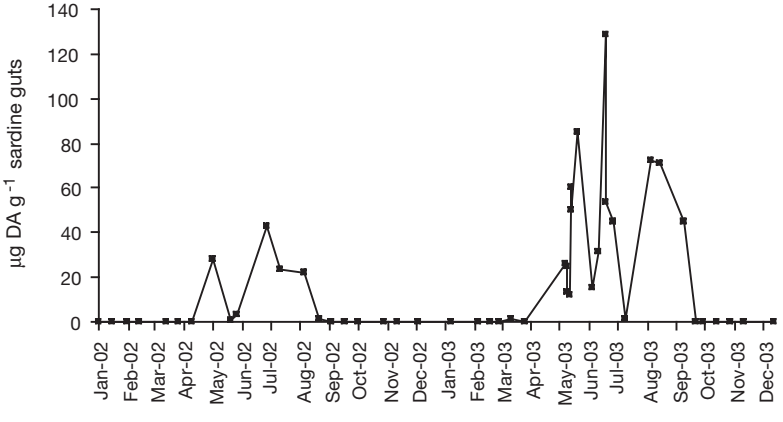

Fig. 1. *Sardina pilchardus*. Domoic acid (DA) concentration (µg g^{-1}) detected fortnightly in sardine guts collected during 2002 and 2003

Sardine stomach content analysis

Pseudo-nitzschia and DA showed similar seasonal patterns of occurrence (Fig. 3). In 2003, *Pseudo-nitzschia* were absent until mid-March, when low concentrations were observed for the first time. During this period, the microplanktonic prey identified in the sardine stomach contents consisted primarily of dinoflagellate species (such as *Scrippsiella* spp.) and the most abundant diatom was the chain-forming benthic *Paralia sulcata*. During the first half of May, there was a sudden rise in the abundance of *Pseudo-nitzschia*, with densities 3 orders of mag-

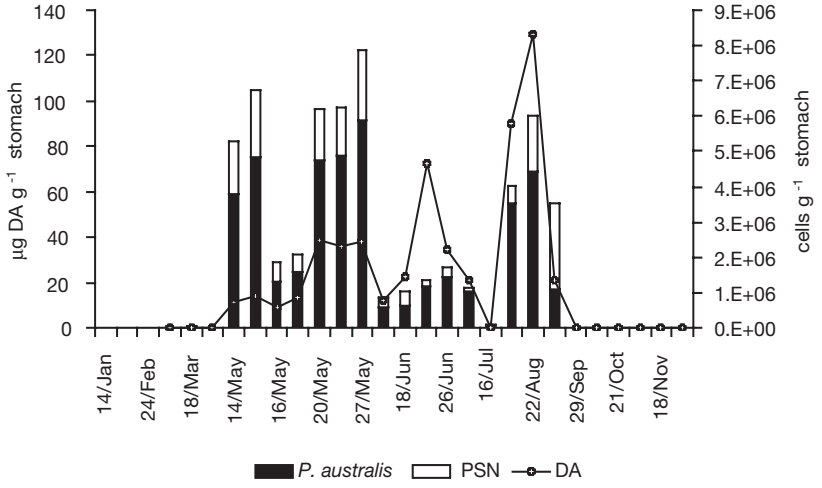

Fig. 3. *Sardina pilchardus*. Concentrations of domoic acid (DA; µg g^{-1}), *Pseudo-nitzschia australis* (cells g^{-1}) and the other *Pseudo-nitzschia* spp. (PSN) observed in stomach contents of sardines collected during 2003

nitude above those registered for all other phytoplankton. High concentrations of these diatoms were observed in sardine stomachs until mid-September. The maximum concentration was observed in late May, when 7.8×10^6 cells g^{-1} of sardine stomach content were recorded. In 75% of the stomach contents containing *Pseudo-nitzschia* spp., this diatom was the most abundant phytoplankton prey, representing up to 99% of the total number of phytoplankton cells found in the stomachs during May and August (Fig. 4).

Four species of the diatom genus *Pseudo-nitzschia* were identified using SEM (Table 1). Whenever *Pseudo-nitzschia* spp. occurred, *P. australis* and *P. pungens* were present, and in 80% of the cases *P. australis* accounted for more than 60% of the *Pseudo-nitzschia* species. *P. pseudodelicatissima* and *P. delicatissima* occurred during the summer, from June until September.

Whenever DA was detected in sardines, *Pseudo-nitzschia* cells were found in their stomachs. Moreover, *P. australis* cells were consistently identified during the toxic periods (Fig. 3). *P. pseudodelicatissima*, which is also considered toxic in some regions (review in Bates 2000), was present in the stomach contents during the summer. These results highlight a link between DA and the number of *P. australis* cells in the stomach content. A linear regression model was used to investigate the relationship between DA content and the interaction between cell counts and season. As the intercept was not significant, regression lines were forced to cross zero. No statistically significant relationship was found between *P. pseudodelicatissima* cell counts and DA concentration in the stomachs. Significant differences were found between seasons ($p < 0.001$), generating 2 different slopes. The final regression model related DA concentration to the interaction between *P. australis* cell counts and season ($R^2 = 0.93$) (Fig. 5). The estimated DA content per *P. australis* cell showed higher values during the summer, ranging from 19 to 34 pg DA $cell^{-1}$ (however, a concentration of 61 pg DA $cell^{-1}$ was detected for a single sample), while spring values ranged from 3 to 9 pg DA $cell^{-1}$. The average DA content per cell estimated from the model was 30 and 6 pg during the summer and spring, respectively.

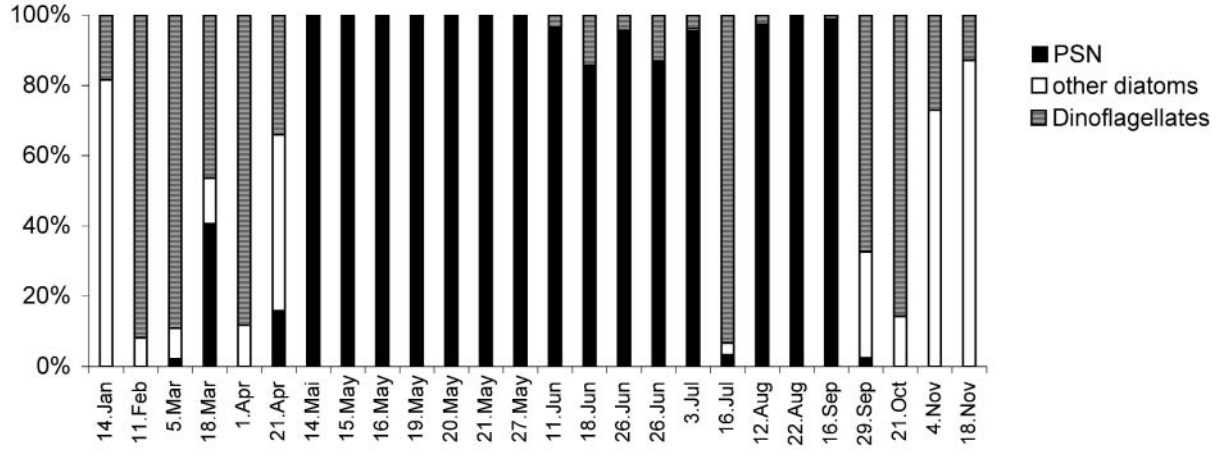

Fig. 4. *Sardina pilchardus*. Relative abundance (%) of *Pseudo-nitzschia* spp. (PSN), other diatoms and dinoflagellates found in sardine stomach contents during 2003

Table 1. *Sardina pilchardus*. Relative abundance (%) of different *Pseudo-nitzschia* spp. in stomach contents during 2003

Date	*P. australis*	*P. pungens*	*P. delicatissima*	*P. pseudodelicatissima*
14 May	72	28	0	0
15 May	72	28	0	0
16 May	72	28	0	0
19 May	76	24	0	0
20 May	77	23	0	0
21 May	78	22	0	0
27 May	75	25	0	0
11 Jun	66	10	0	24
18 Jun	63	9	28	0
26 Jun	83	14	0	3
16 Jul	8	22	49	21
12 Aug	86	6	0	8
22 Aug	74	8	0	18
16 Sep	31	8	3	58

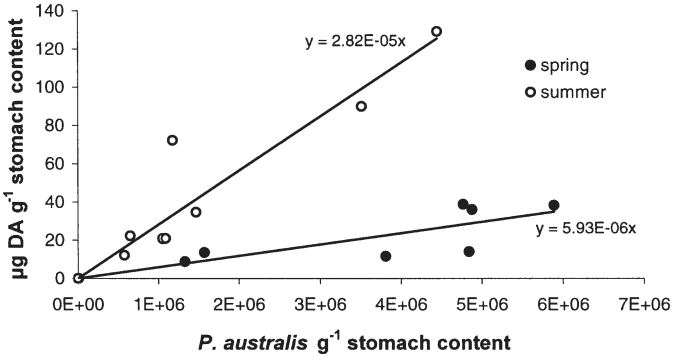

Fig. 5. Linear regression between DA concentration and the number of *Pseudo-nitzschia australis* cells in the stomach content samples during the spring (n = 9) and the summer (n = 10). Equations are of the form $y = ax$, since the intercept was not significant. As DA production was significantly different between seasons, 2 different slopes are presented. R^2 applies for the total regression model

DISCUSSION

The importance of pelagic fish for DA transfer to higher trophic levels has been proposed by several authors (e.g. Lefebvre et al. 1999, 2002, Scholin et al. 2000). Sardines have been proposed to be a particularly relevant group since they are able to filter and digest phytoplankton species efficiently and can accumulate large quantities of DA in their guts. *Sardina pilchardus* is the most abundant planktivorous fish off the Portuguese coast and is the target of a directed purse-seine fishery of economic relevance. Over the years, several aspects of the biology and dynamics of this species have been discussed. However, studies characterizing their feeding habits and diet composition are scarce. Observations have revealed that South African sardine *Sardinops sagax* maximize net energy through prolonged bouts of low-cost filter-feeding, despite being capable of particulate feeding. This results in the ingestion of a large number of phytoplanktonic cells (van der Lingen 1994, 1995). In fact, although zooplankton is the major contributor in terms of biovolume, phytoplankton is numerically dominant in the stomach contents of adult sardines collected off Portugal (Garrido 2003).

In this work, sardine phytoplanktonic prey were characterized by the numerical dominance of diatoms from the genus *Pseudo-nitzschia*, mainly during spring and summer months (99% of the total phytoplankton prey in some cases). Previous studies have confirmed the presence of higher densities of *Pseudo-nitzschia* spp. on the Portuguese coast during the spring and summer months, associated with the occurrence of upwelling events (Abrantes & Moita 1999, Moita 2001). *Pseudo-nitzschia* cells were especially abundant in sardine stomachs during May 2003, with concentrations up to 7.8×10^6 cells g^{-1}, a higher value than the average levels found by McGinness et al. (1995) in stomach contents of Californian northern anchovies.

In this study, 4 species of *Pseudo-nitzschia* were identified and the toxic *P. australis* was found to dominate. In addition, a link was established between the concentration of DA and the number of *P. australis* cells in each stomach content. The observations presented here show that DA production by *P. australis* increased during the summer, reaching values 1 order of magnitude higher than those obtained during the spring. DA production is affected by several environmental conditions, such as irradiance and temperature (Lewis et al. 1993, Bates 1998), pH (Lundholm et al. 2004) and, mainly, by nutrient availability (review in Bates 1998), namely silicate and phosphate, as well as trace metals such as iron. Theories developed from laboratory studies, mainly of *P. multiseries*, have indicated that production of DA is associated with physiological stress caused by silicate and phosphate limitation (Bates et al. 1991, Pan et al. 1996a,b, 1998, Bates 1998). Although field measurements of nutrient availability were not carried out in this work, it has been reported in other surveys that nutrients such as silicate and phosphate decrease sharply in surface waters during the summer, whereas in spring months, there is a strong fluvial nutrient input (Bôto 1945, Moita 2001). Recent studies of the relationship between trace metal availability and DA production by toxigenic *P. multi-*

series and *P. australis* have suggested that production is directly induced by Fe-deficient or Cu-stress conditions (Rue & Bruland 2001, Maldonado et al. 2002). Therefore, changes in trace metal conditions in coastal waters are likely to change intracellular DA concentration and consequently influence the toxic effect of these diatom species.

In some regions of the world, *Pseudo-nitzschia pseudodelicatissima* has been shown to produce DA at low concentrations (7 fg DA cell^{-1}, Martin et al. 1990; 0.12 pg DA cell^{-1}, Rhodes et al. 1998; 36 fg DA cell^{-1}, Pan et al. 2001, up to 4.6 pg DA cell^{-1}, Trainer et al. 2002, reviewed in Bates 2000). In this study, *P. pseudodelicatissima* occurred in sardine stomachs during the summer but was not related to DA production. This is similar to the results of Scholin et al. (2000). Furthermore, no DA was detected in sardine stomachs collected in mid July, when *P. pseudodelicatissima* cells reached a concentration of 1.9×10^4 cells g^{-1}.

This work represents the first effort to estimate DA production by the diatom *Pseudo-nitzschia australis* from cells found in stomach contents. *Pseudo-nitzschia* abundance might be slightly underestimated and, consequently, DA content per individual cell slightly overestimated, due to digestion. Moreover, *Pseudo-nitzschia* consumed by sardine zooplanktonic prey were not counted and might increase the DA values detected by HPLC. Nevertheless, DA levels registered in this estimation (3 to 34 pg DA cell^{-1}) were comparable to concentrations measured in field populations of *P. australis* from the California coast, where most estimates ranged between 7 and 32 pg DA cell^{-1} and the highest cellular levels reached 75 to 78 pg DA cell^{-1} (Scholin et al. 2000, Trainer et al. 2000). Cell cultures of this species have shown a similar DA production (12 to 37 pg cell^{-1}, Garrison et al. 1992; 2 pg cell^{-1}, Rhodes et al. 1996; 26 pg cell^{-1}, Cusack et al. 2002). As suggested by Trainer et al. (2002), laboratory measurements of specific cellular toxicity do not necessarily reflect actual levels in the field, which emphasizes the importance of studies using routine analysis under natural conditions.

The maximum DA concentration (128.5 µg g^{-1} sardine guts) registered in late June 2003 was within the range detected during the toxic events in California that led to severe intoxication in marine mammals (30 to 110 µg g^{-1}, Scholin et al. 2000; 169 to 728 µg g^{-1}, Lefebvre et al. 2002). Furthermore, higher DA concentrations (492.4 µg DA g^{-1}) were recorded during 2000 in the guts of sardines collected off Portugal (Vale & Sampayo 2001). *Sardina pilchardus* is at the base of the marine food web, so it can act as an important domoic acid vector to several predators such as dolphins, which are large consumers of sardines in Portuguese waters (Silva 1999). Human intoxications due to sardine ingestion are unlikely to occur since DA was not detected in the muscle, the edible part. As described for other vertebrates, just small amounts of the toxin cross the gastrointestinal tract or blood–brain barrier (Iverson et al. 1989, Preston & Hynie 1991). Only during acute toxic episodes, with particularly high toxin concentrations in the viscera, have trace levels of DA been detected in sardine and anchovy muscle (>500 µg g^{-1}, Lefebvre et al. 2001, 2002). Consumption of sardine juveniles without evisceration might represent a risk to human health, although juveniles appear to be less effective at retaining phytoplankton than adults (Bode et al. 2003).

DA mainly accumulated in the intestine, followed by the stomach. Some possible reasons might be the higher content:tissue ratio of the intestine (with a thinner wall than the stomach) and the differential rates of prey digestion, assimilation and elimination, as reported by van der Lingen (1998), which are likely to increase phyto:zooplankton proportion within the intestine. No DA was found in the brain tissue and low levels were detected in the gonads. Earlier studies have demonstrated that high concentrations of DA might have a neurological effect in anchovies (Lefebvre et al. 2001), as it does in mammals, but it is not known whether it affects sardine reproduction. However, in this region, sardines mainly spawn during the winter and spring months.

On the Portuguese coast, upwelling events are responsible for the occurrence of algal blooms, including *Pseudo-nitzschia* species, during spring and summer months. Consequently, high concentrations of this diatom are found in sardine stomachs during this period. Several environmental factors, such as the exhaustion of nutrients, are probably responsible for the higher production of DA by *P. australis* observed during the summer in this study. Our results show that sardines accumulate high concentrations of *Pseudo-nitzschia* and DA in their guts, acting as important toxin vectors in the marine food web, mainly during summer conditions.

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