Domoic Acid - A New Toxin in the Croatian Adriatic Shellfish
Toxin Profile

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Abstract: This is the first study that presents concentrations of domoic acid detected in the whole shellfish tissue from breeding and harvesting areas along the Croatian coast of the Adriatic Sea during the period 2006 to 2008. Shellfish sample analyses after SAX cleaning procedures, using a UV-DAD-HPLC system, showed the presence of domoic acid in four species. The most prevalent of those species were the blue mussel (Mytilus galloprovincialis), followed by European flat oyster (Ostrea edulis), Mediterranean scallop (Pecten jacobaeus) and proteus scallop (Flexopecten proteus). Domoic acid, a potentially lethal phycotoxin that causes amnesic shellfish poisoning (ASP), was detected for the first time in January 2006 with the highest value of 6.5486 μg g⁻¹ in whole shellfish tissue. Pseudo-nitzschia spp. bloom events preceded these high domoic acid concentrations. According to this study, retention of domoic acid in the blue mussel M. galloprovincialis is more than 42 days. This investigation indicates the first presence of domoic acid in Croatian shellfish, but in concentrations under the regulatory limit (20 μg g⁻¹), therefore shellfish consumption was not found to endanger human health.

Keywords: Amnesic shellfish poisoning (ASP); retention of domoic acid; mussels; Pseudo-nitzschia; Adriatic Sea
1. Introduction

Occurrence of harmful algal species in the marine environment is a well known cause of human poisoning, mostly by the consumption of contaminated marine products such as shellfish. The seriousness of the threat of amnesic shellfish poisoning (ASP) has been recognized after a case of poisoning in Canada in 1987 [1] when a toxic episode caused the death of at least three people and more than a hundred exhibited neurological problems after consuming blue mussels (*Mytilus edulis*) from Prince Edward Island contaminated with domoic acid (DA) at levels up to 790 µg g⁻¹ [2]. Domoic acid production was linked to the diatom *Pseudo-nitzschia multiseries* and thus this species was identified as a causative organism of the poisoning [3]. This species was also found to have been involved in DA accumulation in French shellfish [4]. After that case, ASP research in the area expanded, with DA subsequently being found in relation to other *Pseudo-nitzschia* species, including *P. australis* [5–7], *P. pseudodelicatissima* [8], *P. pungens* [9], *P. seriata* [10], *P. calliantha* [11], *P. multiseries* [12] and *P. fraudulenta* [5,6,11]. In addition to the above marine diatoms of the genus *Pseudo-nitzschia*, known producers of DA are red algae of the genus *Chondria* [13,14].

Domoic acid is a non-protein, crystalline, water soluble, cyclic amino acid that binds irreversibly to glutamate receptor sites, causing destructive neuronal depolarization and permanent short-term memory loss in mammals [15]. Apart from the neurotoxic properties of DA, genotoxic effects on fish have been established experimentally. Domoic acid induces the formation of micronuclei and DNA strand breaks in peripheral erythrocytes [16]. There are currently ten known isomers of DA, including the isodomoic acids A through H and the domoic acid 5’ diastereomer [17]. Recent research suggests that isodomoic acid A, B and C pose a lower risk to human health than DA itself [18].

Domoic acid accumulates in filter-feeding shellfish by consuming DA producing phytoplankton. In Europe, reports of DA in wild or cultivated shellfish have included the coast of Portugal [19], as well as the Mediterranean regions of France [4], Italy [20] and Greece [21]. James et al. conducted a study, following the first discovery of DA in Ireland in December 1999, and determined the extent of DA contamination of four bivalve species shellfish [22]. DA concentrations in king scallop and variability both within and between different harvesting grounds from around the Isle of Man were reported by Bogan et al. [23]. During 1996, an ASP toxic episode affected Galician (northwest Spain) scallops (*Pecten maximus*) [24].

Trophic transfer of DA is possible by vectors other than shellfish such as copepods, krill [25,26], tunicates [27], cephalopods, cuttlefish [28], planktivorous fish [6,19], and top predators such as sperm whales [7], seabirds [29] and sea lions [30,31]. Fish are very potent vectors because of a lack of acute symptomology during toxic blooms which allows fish to continue feeding and to continue to accumulate and pass on the toxin through the food web to susceptible organisms [32]. Following the Canadian toxic outbreak, DA has been detected in a variety of shellfish throughout the world [4,19,21,33]. Ciminello et al. [20] collected mussels from the Italian western part of the Adriatic Sea during the period 2000 to 2004, analysed the samples by HILIC/MS and recorded the presence of DA as a new toxin that entered the mussel (*M. galloprovincialis*) toxin profile in December 2000 (2.5 µg g⁻¹).

Investigation of DA toxicity in Croatian waters has started to ensure the safety of shellfish for human consumption. According to Regulation (EC) 853/2004 [34] and Croatian legislation, the
maximum permitted level for DA in shellfish edible parts is 20 µg g\(^{-1}\). The purpose of this paper is to establish the temporal and spatial distribution of DA in different species of shellfish of the Adriatic Sea, and the occurrence of DA compared with the level of *Pseudo-nitzschia* spp. abundance in seawater.

2. Results and Discussion

A total number of 1,407 shellfish samples and corresponding seawater samples collected from Croatian breeding and harvesting areas were analyzed from 2006 until 2008. Area of investigation comprise of 22 shellfish breeding and 4 harvesting areas (ZI1, ZI2, ZI3 and SI4) in three zones: North Adriatic (ZI1, ZI2, ZI3, SS1, SV1, LZ1, ME1, UB1 and RZ1), Central Adriatic (SI1 – SI5) and South Adriatic (MZ1 – MZ7 and US1). Means and ranges of temperature measured at each farming area during this three year period are given in Table 1 and the salinity is given in Table 2.

**Table 1.** Surface and bottom temperature at the investigated stations for 2006 – 2008 period.

| Research area of the Adriatic Sea | Mean | Min. | Max. | Std.dev. |
|----------------------------------|------|------|------|----------|
| **Surface**                      |      |      |      |          |
| North                            | 17.3 | 7.6  | 26.7 | 5.8      |
| Central                          | 16.8 | 5.4  | 27.1 | 6.1      |
| South                            | 17.8 | 6.5  | 27.3 | 6.0      |
| **Bottom**                       |      |      |      |          |
| North                            | 16.7 | 8.1  | 24.9 | 5.2      |
| Central                          | 17.4 | 12.0 | 22.1 | 2.9      |
| South                            | 17.6 | 6.7  | 25.4 | 4.5      |

**Table 2.** Surface and bottom salinity at the investigated stations for 2006 – 2008 period.

| Research area of the Adriatic Sea | Mean | Min. | Max. | Std.dev. |
|----------------------------------|------|------|------|----------|
| **Surface**                      |      |      |      |          |
| North                            | 33.5 | 3.7  | 37.7 | 7.1      |
| Central                          | 15.2 | 1.9  | 38.3 | 9.1      |
| South                            | 33.7 | 24.2 | 37.9 | 2.8      |
| **Bottom**                       |      |      |      |          |
| North                            | 36.6 | 28.8 | 37.9 | 1.4      |
| Central                          | 36.5 | 32.4 | 38.2 | 1.3      |
| South                            | 36.2 | 29.8 | 37.9 | 1.2      |

**Figure 1.** Difference between the surface and the bottom (10 m) salinity at central Adriatic Sea stations SI1 – SI5 with mean monthly values; bars indicate min. and max. values for the 3-year investigation period (2006–2008).

The Central Adriatic Sea samples showed a noticeable difference in salinity between the surface and the bottom water layers (Figure 1). The bottom salinity ranged from 32 to 37 psu, while the
surface layer salinity ranged from almost pure fresh water of 1 to 36 psu. Temperature ranged from 5.4 °C to 27.1 °C in February and August, respectively.

During the period of investigation, DA was detected for the first time in this area on the 16th January 2006 in a mussel sample from SI1, and its presence was also evident through February and March. A maximum measured concentration of 6.5486 μg g⁻¹ was detected in February at SI1 and decreased towards SI5 as the influence of fresh water diminished (Figure 2).

**Figure 2.** Domoic acid in shellfish samples (*Mytilus galloprovincialis*) from the central Adriatic Sea in 2006 (see Figure 1).

During March the influence of fresh water became more significant at farms located further downstream, therefore the highest concentrations were in the SI2 samples (Figure 2). All measured concentrations of DA were below the regulatory level of 20 μg g⁻¹. The accumulation of DA in mussels was the result of a *Pseudo-nitzschia* spp. bloom (>10⁶ cells L⁻¹) that occurred in February 2006 after a rainfall event (Figure 3b). Although relatively high sea temperatures (14–17 °C) tend to be associated with increased abundance of *Pseudo-nitzschia* according to Walz *et al.* [35], this study shows that in the central Adriatic Sea, *Pseudo-nitzschia* spp. blooms have occurred in, for this species, extremely cold waters (Figure 4). Namely, in February 2006, the lowest measured temperatures during the eight-year period were found, with a monthly mean of 6 °C.

Furthermore, this occurrence of contamination of mussel cultivated along the shoreline reveals that 94% of domoic acid content was eliminated from the blue mussel tissue within 42 days (period from the 16th of January to the 27th of March), while their detoxication lasted for 55 days (period from the 16th of January until the 9th April, when the toxin was not longer detected). DA was not detected during 2007 and 2008 in the central Adriatic Sea and *Pseudo-nitzschia* spp. abundances were below 1.0 × 10⁵ cells L⁻¹ and 2.0 × 10⁴ cells L⁻¹, respectively (Figure 3b).
Figure 3. Annual variability of *Pseudo-nitschia* spp. number of cells from North Adriatic (a), Central Adriatic (b) and South Adriatic (c). Periods of the DA occurrence are framed.
Figure 4. Mean monthly temperatures with the ranges during 2001 to 2008 period (excluding 2006) and in 2006 at central Adriatic Sea stations SI1-SI3.

The maximum abundances of *Pseudo-nitzschia* spp. in the central Adriatic Sea were already found during mid-winter (February) in 1999, when it contributed up to 85% of the total diatom abundance. Morphological analysis had shown that the dominating *Pseudo-nitzschia* species corresponded to *P. calliantha* [36]. It was also discovered in samples from the Gulf of Trieste (Italian north-west Adriatic Sea) and Kastela Bay in the east-central Adriatic Sea [37]. Caroppo et al. [38] found increasing abundance of *P. calliantha* (up to $1.48 \times 10^5$ cells L$^{-1}$) during winter water conditions in the Italian south-west Adriatic region, while Spatharis et al. [39] counted up to $7 \times 10^6$ cells L$^{-1}$ in the inner part of the Mediterranean Kalloni Gulf (Greece) in February 2005.

Phytoplankton biomass and production are highly variable both in space and time [37] and the phytoplankton dynamics in coastal systems are very complex, particularly in the areas affected by freshwater discharge such as the highly stratified Krka Estuary (SI1-SI5). This study confirmed previous observations on the preference of *Pseudo-nitzschia* species for enclosed water bodies with nutrient loading [39]. *Pseudo-nitzschia* spp. was widely distributed across the Adriatic Sea of both warm and cold climate conditions within the phytoplankton community throughout the investigation period. There are seasonal variations in phytoplankton abundances with increasing numbers in early spring and autumn (Figure 3).

Contaminated samples from the north Adriatic Sea were primarily those of *F. proteus* (ZI1), *P. jacobaeus* (ZI2) and *O. edulis* (ZI3); while the least represented species was the mussel *M. galloprovincialis* (Figure 5). Consequently, although often used as biomarkers, mussels are not necessary good indicator species with respect to ASP contamination. Concentrations of DA were within the range of 0.1117-1.6567 μg g$^{-1}$ (Figure 4). Although DA concentrations were low, a potential risk to human health due to repetitive long-term and low-level exposure calls for continuing investigation of DA [41]; as for the example, Goldstein et al. [42] characterized a chronic DA toxicosis syndrome in sea lions that is distinguishable from acute toxicosis.

Toxin was recorded in the north Adriatic Sea after increasing *Pseudo-nitzschia* spp. abundances up to $1.5 \times 10^6$ cells L$^{-1}$ for 2006, $2.0 \times 10^5$ for 2007 and $1.5 \times 10^5$ for 2008 (Figure 3a). *Pseudo-nitzschia calliantha* was found in seven shellfish areas throughout the northern Adriatic Sea as the dominant diatom species in the summer and autumn of 2007 [37]. *Pseudo-nitzschia* spp. were always present throughout the study period in the south Adriatic Sea, although with abundances mostly lower than at the other parts of the investigated area (Figure 3c), and with no DA detected.
It should be taken into considering that not all *Pseudo-nitzschia* species are toxic and that even toxic ones do not always express toxicity. Nevertheless, according to these extensive data on the *Pseudo-nitzschia* spp. abundances in the study area of the Adriatic Sea, we can assume that if the abundance does not reach $1.0 \times 10^5$ cells L$^{-1}$ then the area can be considered safe with respect to ASP. If the abundance exceeds $1.0 \times 10^6$ cells L$^{-1}$ it might indicate the possible occurrence of ASP contamination. These results add to the previously published observations of domoic acid occurrence in shellfish found in many maritime countries of the European Union.

**Figure 5.** Domoic acid in shellfish samples *Mytilus galloprovincialis* (SS1, RZ1 and ME1), *Flexopecten proteus* (ZI1), *Pecten jacobaeus* (ZI2) and *Ostrea edulis* (ZI3) from the north Adriatic Sea in (a) 2006, (b) 2007 and (c) 2008.

3. Experimental

3.1. Collection of shellfish samples

In 2000, Croatia initiated a monitoring program of its shellfish breeding and harvesting areas in accordance with EU Directive 79/923/EEC [43] and EU Directive 91/492/EEC [44]. It includes analysis of shellfish for the presence of algal toxins (for ASP, DSP, and PSP) and seawater for the presence of toxic phytoplankton species.
Domoic acid was investigated in the Croatian most important cultivated bivalve species, *M. galloprovincialis*, from both breeding farms and wild populations of *Flexopecten proteus* (ZI1), *Pecten jacobaeus* (ZI2), *Ostrea edulis* (ZI3) and *Mytilus galloprovincialis* (SI4) during 2006 until 2008 (Figure 6).

The investigated area comprised of three different farming zones along the eastern Adriatic coast (Figure 6) where the climate is characterized by hot and dry summers (through May to September) and mild and rainy winters. The northern Adriatic breeding farms are located along the coast of the Istrian Peninsula (Figure 6). The central Adriatic breeding and harvesting farms are located in the Šibenik area (Figure 6) influenced by the Krka River Estuary, one of the most productive zones along the Croatian Adriatic coast with fresh water in the surface layer throughout the entire area. This locality is characterized by reduced water exchange with the outer bay area and high nutrient concentrations originating from anthropogenic pollution [45]. The southern Adriatic breeding and harvesting farms are located in the Mali Ston Bay, except for US1 which is located in the aquatorium of Mljet Island (Figure 6). Mali Ston Bay is the most important shellfish farming area in Croatia, with more than one hundred years of tradition.

3.2. Preparation of shellfish samples

The method used for ASP toxins, DA and epi-DAs determination followed the protocol proposed by Quilliam *et al*. [46]. For representative samples, weights of about 100.00 g of soft tissue were used. Homogenized samples (approximately 4.0 g) were extracted with MeOH/H$_2$O (1:1, 16 mL) at 10,000 rpm for 5 min. After 40 min of centrifugation at 4,000 rpm, supernatant (5 mL) was filtered through a 0.45 µm membrane filter (HVHP, Millipore). Subsequent clean up by strong anion exchange (SAX) solid phase extraction (SPE) was necessary to avoid tryptophan interference. Since DA can decompose when frozen, prepared SAX-cleaned extracts were analyzed immediately.

3.3. HPLC analysis

The HPLC system consisted of a Varian ProSTAR 230 Solvent Delivery Module, 310 UV/Vis Detector, 335 Photodiode Array Detector and 410 Autosampler. The column used was a Pinnacle II C18, 250 × 4.6 mm (Restek), with a C18 Guard Cartridge (20 × 4 mm), at the temperature of 40 ºC. Domoic acid was detected by UV absorption at a wavelength of 242 nm, the DA absorption maximum. The mobile phase consisted of 100 mL acetonitrile, 0.2 mL trifluoroacetic acid and up to 1000 mL deionised water. Domoic acid has a retention time of around 12.8 min. Figure 7 shows a typical chromatogram of DA in mussel SAX-cleaned extract.

A standard calibration curve was performed by using an externally DA certified calibration solution for domoic acid (National Research Council of Canada, Halifax, NS, Canada), prepared in seven different concentrations (0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 25.0 µg mL$^{-1}$) and measured in triplicate by HPLC.
**Figure 6.** Area of DA and phytoplankton community investigation along Croatian Adriatic coast comprise of 22 shellfish breeding and 4 harvesting areas (ZI1, ZI2, ZI3 and SI4) in three zones: North Adriatic (a), Central Adriatic (b) and South Adriatic (c). Stations where domoic acid was appeared are framed with dashed line.
Figure 7. HPLC-UV chromatogram of contaminated mussel SAX-cleaned extract contains 6.5486 µg g⁻¹ DA, retention time (DA) = 12.859 min.

Calibration curves were always linear, with correlation coefficients greater than 0.99. To establish an acceptable performance of the extraction procedure used, DA recovery was tested over an expected range of concentrations (Figure 8).

Figure 8. Recovery of DA in mussel tissues spiked at different levels using sax clean-up, n = 5.

Table 3. Domoic acid concentrations in certified referent material ASP-Mus-c, n = 6.

| Certified Concentrations (µg g⁻¹) | Amount found (µg g⁻¹) | Coefficient of variation (%) | Recovery (%) |
|-----------------------------------|----------------------|----------------------------|--------------|
| 41 ± 2                            | 40.76                | 0.13                       | 99.40        |
| 41 ± 2                            | 40.61                | 0.10                       | 99.05        |
| 41 ± 2                            | 40.27                | 0.23                       | 98.23        |
| 41 ± 2                            | 39.42                | 0.74                       | 96.14        |
| 41 ± 2                            | 39.91                | 0.32                       | 97.34        |

The accuracy of the procedure was determined by analysis of certified reference material ASP-Mus-c (National Research Council of Canada, Halifax, NS, Canada) and expressed as a ratio between the concentration found and the accepted reference concentration (Table 3). Limit of detection (LOD) was determined based on a generally accepted 3:1 signal-to-noise ratio. It was performed firstly by comparing the measured signal from samples with known low concentrations of DA to those of blank samples and establishing the minimum concentration at which the DA can be reliably detected. The
limit of detection was found to be 0.1025 µg of DA per g of shellfish tissue. Secondly, it was determined by comparing the measured signal from certified calibration solutions with known low concentrations of DA to those of a blank solution and establishing the minimum concentration at which the DA can be reliably detected; the limit of detection of this method was 0.0220 µg mL⁻¹.

3.4. Phytoplankton, temperature and salinity water sample analysis

Water samples for physical parameters and phytoplankton composition were collected at the same time as samples of shellfish taken at the stations shown in Figure 6. Phytoplankton samples were collected with a PVC sampler at the integrated depths from the surface to 5 m. The PVC line was pulled to close its base with a rubber stopper from the surface until the desired depth or the near-bottom of the water column. The collected water samples were fixed with glutaraldehyde solution (final concentration of 0.5%) and phytoplankton species were identified and counted under inverted microscope (Olympus IX50, Tokyo, Japan) according to Utermöhl [47]. Temperature and salinity of seawater was recorded by a CTD YSI63 probe.

4. Conclusions

The ASP investigation of shellfish from both Croatian breeding and harvesting areas revealed a negligible presence of DA over a wide Croatian Adriatic Sea area during the 2006 – 2008 period and all samples showing the presence of the toxin were found to have concentration levels of less than 20 µg g⁻¹. This is the first detection of domoic acid in shellfish from Croatian waters, specifically from the central Adriatic Sea and estuarine waters (January, 2006). Toxin presence may be related to the identified *Pseudo-nitzschia* spp. blooms. Maximum levels of domoic acid detected in Croatian shellfish were found to be 6.5486 µg g⁻¹, with a detection limit of 0.1025 µg g⁻¹. Retention of DA in mussel, according to this study, is more than 42 days. It should be noted that there have been no DA poisoning outbreaks in Croatia to this date. *Pseudo-nitzschia* spp. bloom formation coincides with the temperature and salinity minima that occur in February. This research contributes to the knowledge of the interactions of *Pseudo-nitzschia* spp. and abiotic parameters at regional and global scales for the prediction and prevention of its harmful effects and potential toxicity. It will also contribute to the understanding of the occurrence of ASP in shellfish, a known member of the marine food web able to accumulate DA.

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References

1. Wright, J.L.C.; Boyd, R.K.; de Freitas, A.S.W.; Falk, M.; Foxall, R.A.; Jamieson, W.D.; Laycock, M.V.; McCulloch, A.W.; McInnes, A.G.; Odense, P.; Pathak, V.; Quilliam, M.A.; Regan M.A.; Sim, P.G.; Thibault, P.; Walter, J.A.; Richard, D.J.A.; Dewar, C. Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. Can. J. Chem. 1989, 67, 481–490.

2. Todd, E.C.D. Domoic acid and amnesic shellfish poisoning—a review. J. Food Protect. 1993, 56, 69–83.

3. Bates, S.S.; Bird, C.J., DeFreitas, A.S.W.; Foxall, R.; Gilgan, M.; Hanic, L.A.; Johnson, G.R.; McCulloch, A.W.; Dodense, P.; Pocklington, R.; Quilliam, M.A.; Sim, P.G.; Smith, J.C.; Rao, D.V.S.; Todd, C.D.; Walter, J.A.; Wrigh, J.L.C. Pennate diatom Nitzschia pungens as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edwards Island, Canada. Can. J. Fish. Aquat. Sci. 1989, 46, 1203–1215.

4. Amzil, Z.; Fresnel, J.; LeGal, D.; Billard, C. Domoic acid accumulation in French shellfish shellfish in relation to toxic species of Pseudo-nitzschia multiseries and P. Pseudodelicatissima. Toxicon 2001, 39, 1245-1251.

5. Cusack, C.K.; Bates, S.S.; Quilliam, M.A.; Patching, J.W.; Raine, R. Confirmation of domoic acid production by Pseudo-nitzschia australis (Bacillariophyceae) isolated from Irish waters. J. Phycol. 2002, 38, 1106-1112.

6. Costa, P.R.; Garrido, S. Domoic acid accumulation in the sardine Sardinia pilchardus and its relationship to Pseudo-nitzschia diatom ingestion. Mar. Ecol. Prog. Ser. 2004, 284, 261-268.

7. Fire, S.E.; Wanga, Z.; Leightfield, T.A.; Morton, S.L.; McFee, W.E.; McLellan, W.A.; Litaker, R.W.; Tester, P.A.; Hohn, A.A.; Lovewell, G.; Harms, C.; Rotstein, D.S.; Barco, S.G.; Costidis, A.; Sheppard, B.; Bossart, G.D.; Stolen, M.; Durden, W.N.; Van Dolah, F.M. Domoic acid exposure in pygmy and dwarf sperm whales (Kogia spp.) from southeastern and mid-Atlantic U.S. waters. Harmful Algae 2009, 8, 658-664.

8. Pan, Y.; Parsons, M.L.; Busman, M.; Moeller, P.D.R.; Dortch, Q.; Powell, C.L.; Doucette, G.J. Pseudo-nitzschia sp. Cf. pseudodelicatissima-a confirmed producer of domoic acid from the northern Gulf of Mexico. Mar. Ecol. Prog. Ser. 2001, 220, 83-92.

9. Trainer, V.L. Harmful Algal Blooms in the PICES Region of the North Pacific; Taylor, F.J., Trainer, V.L., Eds.; PICES: Sidney, Australia, 2002; Scientific Report 23, pp. 89–118.

10. Fehling, J.; Green, D.H.; Davison, K.; Bolch, C.J.; Bates, S.S. Domoic acid production by Pseudo-nitzschia seriata (Bacillariophyceae) in Scottich waters. J. Phycol. 2004, 40, 622-630.

11. Besiktepe, S.; Ryabushko, L.; Ediger, D.; Yilmaz, D.; Zenginer, A.; Ryabushko, V.; Lee, R. Domoic acid production by Pseudo-nitzschia calliantha Lundholm, Moestrup at Hasle (bacillariophyta) isolated from the Black Sea. Harmful Algae 2008, 7, 438-442.

12. Baugh, K.A., Bush, J.M., Bill, B.D., Lefebvre, K.A., Trainer, V.L. Estimates of specific toxicity in several Pseudo-nitzschia species from the Washington coast, based on culture and field studies. Afr. J. Mar. Sci. 2006, 28, 403–407.

13. Bates, S.S. Domoic acid producing diatoms: another genus added! J. Phycol. 2000, 36, 978–985.
14. Kotaki, Y.; Lundholm, N.; Onodera, H.; Kobayashi, K.; Bajarias, F.; Furio, E.; Iwataki, M.; Fukuyo, Y.; Kodama, M. Wide distribution of Nitzschia navis-varingica, a new domoic acid-producing benthic diatom found in Vietnam. *Fisheries Science* **2004**, *70*, 28-32.

15. Debonnel, G.; Weiss, M.; de Montigny, C. Reduced neuroexcitatory effect of domoic acid following mossy fiber denervation of the rat dorsal hippocampus: further evidence that toxicity of domoic acid involves kainate receptor activation. *Can. J. Physiol. Pharmacol.* **1989**, *67*, 904-908.

16. Cavas, T.; Könen, S. *In vivo* genotoxicity testing of the amnesic shellfish poison (domoic acid) in piscine erythrocytes using the micronucleus test and the comet assay. *Aquat. Toxicol.* **2008**, *90*, 154-159.

17. Jeffery, B.; Barlow, T.; Moizer, K.; Paul, S.; Boyle, C. Amnesic shellfish poison. *Food Chem. Toxicol.* **2004**, *42*, 545–557.

18. Munday, R.; Holland, P.T.; McNabb, P.; Selwood, I.A.; Rhodes, L.L. Comparative toxicity to mice of domoic acid and isodomoic acids A, B and C. *Toxicon* **2008**, *52*, 954-956.

19. Vale, P.; Sampayo, M.A. Domoic acid in Portuguese shellfish and fish. *Toxicon* **2001**, *39*, 893-904.

20. Ciminello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Magano, S.G.; Tartaglione, L.; Quilliam, A. M.; Tubaro, A.; Polleti, R. Hydrophilic interaction liquid chromatography/mass spectrometry for determination of domoic acid in Adriatic shellfish. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2030-2038.

21. Kaniou-Grigoriadou, I.; Mouratidou, T.; Katikou, P. Investigation on the presence of the domoic acid in Greek shellfish. *Harmful Algae* **2005**, *4*, 717-723.

22. James, K.J.; Gillman, M.; Amandi, M.F.; López-Rivera, A.; Puente, P.F.; Lehane, M.; Mitrović, S.; Furey, A. Amnesic shellfish poisoning toxin in bivalve molluscs in Ireland. *Toxicon* **2005**, *46*, 852-858.

23. Bogan, Y.M.; Kennedy, D.J.; Harkin, A.L.; Gillespie, J.; Vause, B.J.; Beukers-Stewart, B.D.; Hess, P.; Slater, J.W. Variation in domoic acid concentration in king scallop (Pecten maximus) from fishing grounds around the Isle of Man. *Harmful Algae* **2007**, *6*, 81-92.

24. Leira, F.J.; Vieites, J.M.; Botana, L.M.; Vye ites, M.R. Domoic Acid Levels of Naturally Contaminated Scallops as Affected by Canning. *J. Food Sci.* **1998**, *63*, 1081-1083.

25. Bargu, S.; Powell, C.L.; Coale, S.L.; Busman, M.; Doucette, G.J.; Silver, M.W. Krill: a potential vector for domoic acid in marine foods webs. *Mar. Ecol. Prog. Ser.* **2002**, *237*, 209-216.

26. Bargu, S.; Lefebvre K.; Silver, M.W. Effect of dissolved domoic acid on the grazing rate of krill *Euphausia pacifica*. *Mar. Ecol. Prog. Ser.* **2006**, *312*, 169-176.

27. Lopez-Rivera, A.; Pinto, M.; Insinilla, A.; Isla, B.S.; Uribe, E.; Álvarez, G.; Lehane, M.; Furey, A.; James, K.J. The occurrence of domoic acid linked to a toxic diatom bloom in a new potential vector: The tunicate *Pyura chilensis* (piure). *Toxicon* **2009**, *54*, 754-762.

28. Costa, P.R.; Rosa, R.; Duarte-Silva, A.; Brotas, V.; Sampayo, M.A.M. Accumulation, transformation and tissue distribution of domoic acid, the amnesic shellfish poisoning toxin, in the common cuttlefish, *Sepia officinalis*. *Aquat. Toxicol.* **2005**, *74*, 82-91.

29. Beltran, A.S.; Palafox-Uribe, M.; Garajales-Montiel, J.; Cruz-Villacorta, A.; Ochoa, J.L. Sea bird mortality at Cabo San Lucas Mexico: evidence that toxin diatom blooms are spreading. *Toxicon* **1997**, *35*, 447-453.
30. Scholin, C.A.; Gulland, F.; Doucette, J.G.; Benson, S.; Busman, M.; Chavez, P.F.; Cordaro, J.; DeLong R.; De Vogelaere, A.; Harvey, J.; Haulena, M.; Lefebvre, K.; Lipscomb, T.; Loscutoff, S.; Lowenstein, L.J.; Marin, III R.; Miller, P.E.; McLellan, W.A.; Moeller, P.D.R.; Powell, C.L.; Rowles, T.; Silvagni, P.; Silver, M.; Spraker, T.; Trainer, V.; Van Dolah, F.M. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 2000, 403, 80-84.

31. Bejrano, A.C.; Gulland, F.M.; Goldstein, T.; St Leger, J.; Hunter, M.; Schwacke, L.H.; VanDolah, F.M.; Rowles, TK. Demographic and spatio-temporal signature of the biotoxin domoic acid in California sea lion (*Zalophus californianus*) stranding records. *Mar. Mamm. Sci.* 2008, 24, 899-912.

32. Lefebvre, K.A.; Noren D.P.; Schultz, I.R.; Boga rd, S.M.; Wilson, J.; Eberhart, B.T.L. Uptake, tissue distribution and excretion of domoic acid after oral exposure in coho salmon (*Oncorhynchus kisutch*). *Aquat. Toxicol.* 2007, 81, 266–274.

33. Honer, R.A.; Kussake, M.B.; Moynihan, B.P.; Skinner, R.N.; Wekell, J.C. Retention of domoic acid by Pacific razor clams *Siliqua patula* (Dixon, 1789) – preliminary study. *J. Shellfish Res.* 1993, 12, 451-456.

34. Anonymous. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Off. J. Eur. Commun.* 2002, L 226, 22-82.

35. Walz, P.M.; Garrison, D.L.; Graham, W.M.; Cattey, M.A.; Tjeerdema, R.S.; Silver, M.W. Domoic acid-producing diatom blooms in Monterey Bay, California: 1991-1993. *Nat. Toxins* 1993, 2, 271–279.

36. Burič, Z.; Viličić, D.; Caput Mihalić, K.; Carić, M.; Kralj, K.; Ljubešić, N. Pseudo-nitzschia blooms in the Zrmanja River estuary (eastern Adriatic Sea). *Diatom Res.* 2008, 23, 51-63.

37. Marić, D.; Burić, Z.; Godrijan, J.; Bosak, S.; Djakovac, T.; Peharec, P. The occurrence of potentially toxic Pseudo-nitzschia calliantha in the coastal waters of the Northern Adriatic Sea. In 20th International Diatom Symposium; Jasprica, N., Car, A., Čalić, M., Eds.; University of Dubrovnik: Dubrovnik, Croatia, 2008; p.182.

38. Caroppo, C.; Congestri, R.; Bracchini, L.; Albertano, P. On the presence of *Pseudo-nitzschia calliantha* Lundholm, Moestrup et Hasle and *Pseudo-nitzschia delicatissima* (Cleve) Heiden in the Southern Adriatic Sea (Mediterranean Sea, Italy). *J. Plankton Res.* 2005, 27, 763-774.

39. Spatharis, S.; Danielidis, B.D.; Tsirtsis, G. Recurrent *Pseudo-nitzschia calliantha* (Bacillariophyceae) and *Alexandrium insuetum* (Dinophyceae) winter blooms induced by agricultural runoff. *Harmful Algae* 2007, 6, 811-822.

40. Cabrini, M.; Cok, S.; Pecchiari, I.; Fonda Umani, S.; Andri, M. Carbon partitioning among the first trophic levels in the North Western Adriatic basin. *Chem. Ecol.* 2002, 18, 95-105.

41. Lefebvre, K.A.; Robertson A. Domoic acid and human exposure risk: a review. *Toxicon* 2010, 56, 218-230.

42. Goldstein, T.; Mazet, J.A.; Zabka, T.S.; Langlois, G.; Colegrove, K.M.; Silver, M.; Bargu, S.; Van Dolah, F.; Leighfield, T.; Conrad, P.A.; Barakos, J.; Williams, D.C.; Dennison, S.; Haulena, M.; Gulland, F.M. Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): an increasing risk to marine mammal health. In *Proc. Roy. Soc. Biol. Sci. Ser. B* 2008, 275, 267–276.
43. Anonymous. Council Directive 79/923/EEC of 30 October 1979 on the quality required of shellfish waters. *Off. J. Eur. Commun.* 1979, *L0923*, 1-9.

44. Anonymous. Council Directive 91/492/EEC laying down the health conditions for the production and the placing on the market of live bivalve molluscs. *Off. J. Eur. Commun.* 1991, *L268*, 15-34.

45. Ninčević-Gladan, Ž.; Skejić, S.; Bužančić, M.; Marasović, I.; Arapov, J.; Ujević, I.; Bojanić, N.; Grbec, B.; Kušpilić, G.; Vidjak, O. Seasonal variability in *Dinophysis* spp. Abundance and diarrhetic shellfish poisoning outbreaks along the eastern Adriatic coast. *Bot. Mar.* 2008, 51, 449-463.

46. Quilliam, M.A.; Xie, M.; Hardstaff, W.R. Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood. *J. AOAC Int.* 1995, 78, 543-553.

47. Utermöhl, H. Zur Vervollkommnung der quantitative Phytoplankton Methodik. *Mitt. Int. Ver. Limnol.* 1958, 9, 1-38.

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