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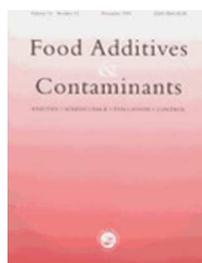
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Effect of ozonation and γ -irradiation on post-harvest decontamination of mussels (*Mytilus galloprovincialis*) containing diarrhetic shellfish toxins

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Additives/Contaminants:	Natural toxicants
Food Types:	Seafood
Abstract:	Contamination of shellfish with diarrhetic shellfish poisoning (DSP) toxins readily occurs during algal blooms. Such phenomena raise important public health concerns and thus comprise a constant challenge to shellfish farmers, the seafood industry and health services, considering the increasing occurrence of toxic episodes around the world. To avoid the detrimental effects of such episodes, research has focused on the use of various detoxification methodologies which should be rapid, efficient, easy to apply, and will not alter quality and sensory properties of shellfish. In the present study, both ozonation (15 mg kg ⁻¹ for 6 h) and γ -irradiation (6 kGy) have been utilized in order to reduce toxin content of contaminated shucked mussels, collected during the DSP episodes of 2007 and 2009 in Greece. DSP toxicity was detected using the mouse bioassay (MBA) while determination of toxin content of the okadaic acid (OA) group (both free and esterified forms) was conducted by LC/MS/MS analysis. Toxin reduction using γ -irradiation was in the range of 12-36%, 8-53% and 10-41% for free OA, OA esters and total OA respectively. Appearance and texture of irradiated mussels deteriorated, pointing to a low potential for commercial use of this method. Ozonation of mussels resulted in toxin reduction in the range of 6-100%, 25-93% and 31-66%

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	<p>for free OA, OA esters and total OA, respectively. Reduction of OA content was substantially higher in homogenized mussel tissue, compared to that of whole shucked mussels. In addition, differences detected with regard to quality parameters (TBA, sensory attributes) between ozonated and control mussels were not considerable. Eventhough varying % reductions in OA and its derivatives were achieved using ozonation under specific experimental conditions tested, it is postulated that upon optimization, ozonation may have the potential for post-harvest commercial DSP detoxification of shucked mussels.</p>

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1 **Effect of ozonation and γ -irradiation on post-harvest**
2 **decontamination of mussels (*Mytilus galloprovincialis*) containing**
3 **diarrhetic shellfish toxins**

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24 **ABSTRACT**

25 Contamination of shellfish with diarrhetic shellfish poisoning (DSP) toxins readily
26 occurs during algal blooms. Such phenomena raise important public health concerns
27 and thus comprise a constant challenge to shellfish farmers, the seafood industry and
28 health services, considering the increasing occurrence of toxic episodes around the
29 world. To avoid the detrimental effects of such episodes, research has focused on the
30 use of various detoxification methodologies which should be rapid, efficient, easy to
31 apply, and will not alter quality and sensory properties of shellfish. In the present
32 study, both ozonation (15 mg kg⁻¹ for 6 h) and γ -irradiation (6 kGy) have been
33 utilized in order to reduce toxin content of contaminated shucked mussels, collected
34 during the DSP episodes of 2007 and 2009 in Greece. DSP toxicity was monitored
35 using the mouse bioassay (MBA) whilst the determination of toxin content of the
36 okadaic acid (OA) group (both free and esterified forms) was carried out by
37 LC/MS/MS analysis. Toxin reduction using γ -irradiation was in the range of 12-36%,
38 8-53% and 10-41% for free OA, OA esters and total OA respectively. Appearance
39 and texture of irradiated mussels deteriorated, pointing to a low potential for
40 commercial use of this method. Ozonation of mussels resulted in toxin reduction in
41 the range of 6-100%, 25-83% and 21-66% for free OA, OA esters and total OA,
42 respectively. Reduction of OA content was substantially higher in homogenized
43 mussel tissue, compared to that of whole shucked mussels. In addition, differences
44 detected with regard to quality parameters (TBA, sensory attributes) between
45 ozonated and control mussels were not considerable. Even though varying %
46 reductions in OA and its derivatives were achieved using ozonation under specific
47 experimental conditions tested, it is postulated that upon optimization, ozonation may

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3 48 have the potential for post-harvest commercial DSP detoxification of shucked
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5 49 mussels.

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10 51 **Keywords:** DSP toxins; decontamination; mussels; ozonation; γ -irradiation; okadaic
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54 Introduction

55 Consumption of shellfish contaminated with toxic algae such as *Dinophysis* spp.
56 can lead to Diarrhetic Shellfish Poisoning (DSP). DSP toxins include okadaic acid
57 (OA) and its derivatives (dinophysistoxins DTX1 and DTX3) and belong to the
58 lipophilic toxins' group, which includes several other different classes of lipophilic
59 compounds: pectenotoxins (PTXs), yessotoxins (YTXs) and several groups of
60 spiroimine-containing toxins, such as azaspiracids (AZAs) and spirolides (SPX)
61 (Yasumoto et al. 1985; Yasumoto 1990; Villar-Gonzalez et al. 2005). DSP symptoms
62 include diarrhea, nausea, vomiting and abdominal pain which appear 30 min to a few
63 hours after ingestion, with complete recovery occurring after several days (Yasumoto
64 et al. 1978). The minimum doses of OA and DTX-1 necessary to induce above
65 symptoms in adults have been estimated to be 40 and 36 mg respectively (Hamano et
66 al. 1986).

67 According to relevant data of the National Reference Laboratory on Marine
68 Biotoxins of Greece (Thessaloniki, Greece), toxins of the OA group have been
69 identified as being responsible for the majority of lipophilic toxin outbreaks during
70 the past 10 years in Greece (Mouratidou et al. 2004; Prassopoulou et al. 2009;
71 Louppis et al. 2010).

72 Given the fact, that so far, the only means of managing marine biotoxin toxic
73 episodes is prevention by means of monitoring programmes, it is evident that the
74 development of shellfish detoxification methodologies from DSP toxins would be
75 desirable. Such methods should be rapid, efficient, easy-to-apply and should not alter
76 the quality and sensory properties of shellfish. Methods investigated so far for this
77 purpose include thermal processing, freezing, evisceration, supercritical CO₂ with
78 acetic acid, irradiation and ozonation (Gonzalez et al. 2002; Reboreda et al. 2010).

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3 79 Food irradiation has been recognized and regulated as an effective food processing
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5 80 technology in many countries, being able to destroy or reduce ubiquitous pests and
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7 81 pathogens that contaminate raw foods (WHO 1988; Olson 1998; Rose & Engeljohn
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9 82 2000). However data in the literature on the use of irradiation for the purpose of
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11 83 detoxification of mussel tissue are scarce (McCarron, et al. 2007). According to EU
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13 84 authorizations in some member states, the highest acceptable radiation dose for fish
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15 85 and shellfish is 3 kGy while especially for frozen peeled and/or decapitated shrimp
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17 86 the respective limit is 5 kGy (EU Parliament, 2009). Furthermore the European Food
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19 87 Safety Authority (EFSA, 2008) has recently proposed a limit of 5kGy for the
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21 88 irradiation of seafood (fresh or frozen).
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25 89 Ozone has been primarily applied for the treatment of drinking water (Bryant et
26
27 90 al. 1992) as well as municipal and industrial waste water (Stover & Jarnis 1981).
28
29 91 Ozone treatment for marine biotoxin reduction in shellfish and/or seawater has so far
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31 92 given quite contradictory results, depending on specific toxin involved and/or matrix,
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33 93 with most studies involving paralytic and neurotoxic toxins. Preliminary studies by
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35 94 Gacutan et al. (1984, 1985) on the use of ozone as a means of shellfish detoxification
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37 95 demonstrated that ozone gas may effectively inactivate PSP toxins from *Perna viridis*
38
39 96 contaminated by *Pyrodinium bahamense*. However, results of a subsequent study by
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41 97 White et al. (1985) were totally contradictory to previous studies, concluding that no
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43 98 detoxification occurred in *Mya arenaria* exposed to ozone treatments. Schneider and
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45 99 Rodrick (1995) studied potential reduction of toxins associated with Florida's red tide
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47 100 organism, *Gymnodinium breve*. Results showed a three log reduction in the total
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49 101 amount of toxin recovered after 10 min (135 mg kg⁻¹) of ozone exposure. Rositano et
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51 102 al. (1998) studied destruction of cyanobacterial toxins by ozone and concluded that
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3 103 free peptide hepatotoxins were rapidly destroyed by ozone in alkaline pH
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5 104 environments.

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7 105 A rather limited number of studies have dealt with ozonation effects on
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10 106 lipophilic toxins and more precisely DSP toxins (Croci et al. 1994; Reboreda et al.
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12 107 2010). These applications, however, involved treatment of the seawater where
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14 108 shellfish grow, rather than the end product itself, so may only apply prior to shellfish
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16 109 harvesting while mussels are still alive. In several countries, including Greece, it is
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18 110 quite common for mussels to be marketed either fresh or processed with usual
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20 111 processing methods including smoking, canning and marinating (Manousaridis et al.
21
22 112 2005). Fresh mussels are sold either as whole in-shell unpackaged products in ice or
23
24 113 as shucked products packaged in polyethylene pouches or glass jars containing liquid
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26 114 (water or brine) and kept under refrigeration. In a relevant survey, it was found that
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28 115 43% of Greek consumers preferred buying sucked mussels in brine rather than live
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30 116 mussels in shell (Batzios et al. 2004).

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34 117 Given the fact that ozone has been declared by the United States Food and Drug
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36 118 Administration (USFDA) in 2001 as a Generally Recognized As Safe (GRAS) food
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38 119 preservative, approved for direct contact with foods (Khadre et al. 2001), the
39
40 120 objective of the present study was to investigate the potential reduction of DSP toxins
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42 121 using ozone gas or irradiation in mussels, post-harvest. MBA as well as LC-MS/MS
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44 122 were used to detect and/or quantify OA, DTX-1 and their acyl derivatives in shucked
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46 123 mussels before and after treatment.

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51 52 125 **Materials and Methods**

53 54 126 *Samples*

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3 127 Mussels (*Mytillus galloprovincialis*) contaminated with DSP toxins were
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5 128 collected during the DSP episodes of 2007 and 2009 from several sampling stations
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7 129 belonging to three different production areas of Greece: Thermaikos Gulf in northern
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9 130 Greece (regions of Thessaloniki, Pieria and Imathia), Maliakos Gulf in central Greece
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11 131 (region of Fthiotida), and Saronikos Gulf in southern Greece (regions of Piraeus and
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13 132 Megara), as well as from the market during the same time periods. A total of 21
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15 133 samples (ca. 250 g each), derived from the Greek National Monitoring Program for
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17 134 Marine Biotoxins (National Reference Laboratory on Marine Biotoxins of Greece,
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19 135 NRLMB), were tested upon arrival to the laboratory by the Yasumoto (1978) mouse
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21 136 bioassay (MBA) method, using the hepatopancreas tissue. Samples, both positive and
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23 137 negative according to the MBA, were stored at -70°C until chemical analyses by LC-
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25 138 MS/MS in order to characterize toxin content and profile before further processing.
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27 139 Analysis was carried out either on whole shucked mussels, which were mildly
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29 140 steamed using hot tap water in order to keep the body in one piece, (for both
30
31 141 irradiation and ozone treatment) or on homogenized whole tissue (for ozone
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33 142 treatment). Mussel tissue quantities of 150 g were required for processing in order to
34
35 143 perform both MBA and LC-MS/MS analyses after treatment. Allocation of samples
36
37 144 was conducted in such a way so as to assure that both DSP negative and positive
38
39 145 samples were included for each treatment. After processing, residual toxicity of all
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41 146 samples was tested by MBA, whereas LC-MS/MS analysis was also carried out in
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43 147 order to quantify the effect of each treatment on toxin content and profile.
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51 **Reagents**
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3 150 Solvents used for both MBA protocols were Tween 60 “for synthesis” grade
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5 151 (Sigma, Sigma-Aldrich, St. Louis, MO, USA) and analytical grade acetone and
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7 152 diethyl ether (Merck, Darmstadt, Germany).

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10 153 Solvents used for the LC-MS/MS analysis were LC grade methanol,
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12 154 chloroform, water, acetonitrile, n-hexane (Merck, Darmstadt, Germany). Okadaic acid
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14 155 (OA) standard solution (24.1 $\mu\text{g ml}^{-1}$) (NRC CRM-OA-b, Institute for Marine
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16 156 Biosciences, National Research Council of Canada, Halifax, Canada) was used for the
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18 157 preparation of the calibration curve used in the LC-MS/MS methodology. A certified
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20 158 Reference Material Mussel Tissue [10.1 $\mu\text{g OA g}^{-1}$ and 1.3 $\mu\text{g DTX1 g}^{-1}$] (NRC
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22 159 CRM-DSP-Mus-b)] was used for recovery determination of the LC-MS/MS method.
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25 160 All standards and reference materials were stored at -20°C .

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29 30 162 ***Mouse bioassay (MBA) protocols***

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32 163 MBA for the determination of DSP toxicity in samples, upon arrival to the
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34 164 NRLMB, was performed as described by Yasumoto et al. (1978) using the
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36 165 hepatopancreas (HP) of mussels. This protocol is routinely used for monitoring
37
38 166 toxicity of incoming samples and is based on testing of the HP of mussels.

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40 167 Sample toxicity after treatment either by γ -irradiation or ozone gas treatment
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42 168 was determined using the EU-harmonized protocol (Version 4.0, CRL-MB, 2007)
43
44 169 (Louppis et al., 2010). This protocol is based on the analysis of whole mussel tissue.
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46 170 Both methods are used with equivalent results according quality control procedures of
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48 171 the laboratory depending on availability of specific tissue (HP or whole flesh).

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50 172 In both protocols, the criterion of toxicity established by the EU Regulation
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52 173 2074/2005 (2005) was employed, which is the death of two out of three mice within
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54 174 24 h of injection with an extract equivalent to 5 g of HP or 25 g of whole flesh tissue.
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3 175 This constitutes a positive result for the presence of the lipophilic toxins mentioned in
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5 176 the above Regulation, including OA, DTX-1, -2 and -3. The mice were allowed
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7 177 laboratory feed and water *ad libitum* throughout the observation period. All animal
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9 178 manipulations were performed in accordance with the EU Directive 86/609/EEC
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11 179 (1986) and the EU Recommendation 2007/526/EC (2007), under official license from
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13 180 the Prefectural Veterinary Service of Thessaloniki, Greece.
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18 182 ***LC-MS/MS analysis***

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21 183 Toxin extraction, subsequent hydrolysis of esterified DSP toxins for LC-
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23 184 MS/MS analysis and the analysis procedure itself were performed according to the
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25 185 method described by Louppis et al. (2010), using whole tissue as sample. Aliquots of
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27 186 20 µl were injected into the LC-MS/MS unit. LC-MS was performed on an Agilent
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29 187 LC 1100 series liquid chromatograph coupled to an Agilent MSD Trap SL (Stuttgart,
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31 188 Germany) equipped with an atmospheric pressure electrospray ionization (ESI)
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33 189 interface. The chromatographic conditions and quality control data for the analytical
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35 190 method (calibration curve range, linearity, retention times etc.) have already been
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37 191 reported by Louppis et al. (2010). Recovery of the method, determined by use of the
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39 192 Certified Reference Material mussel tissue (NRC CRM-DSP-Mus-b) was found to be
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41 193 96% for OA and 92% for DTX-1.
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47 195 ***γ-Irradiation treatment.***

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50 196 Irradiation treatment was conducted at room temperature (20±1°C) in a γ-
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52 197 irradiation sterilization plant (ELVIONY S.A, Mandra Attikis, Greece). Prior to
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54 198 treatment, mussels were packaged in low-density polyethylene (LDPE) pouches, heat
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56 199 sealed using a Boss No48 sealer (Bad Homburg, Germany). Samples were irradiated
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3 200 with a 150kCi ^{60}Co source at 6.0 kGy with actual dose being within $\pm 10\%$ of the
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5 201 target dose. To minimize variations in radiation-dose absorption, pouches were
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7 202 rotated 180° halfway during the irradiation process. Irradiation doses were measured
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9 203 using Harwell Perspex Polymethylmethacrylate Amber 3042 dosimeters. The
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11 204 absorbance signal was measured using a Camspec M 201 UV spectrometer at 640 nm.
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14 205 The average dose rate was 1 kGy/h for all irradiated samples.
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19 207 ***Ozone treatment***

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21 208 Ozone was generated by exposure of pure oxygen gas supplied by a 10m^3
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23 209 cylinder to high voltage electrical discharge. The ozone generator C-Lasky series (C-
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25 210 L010-DTI/C-L010-DSI, AirTree Ozone Technology Co., Baunatal, Germany) was
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27 211 used for the generation of ozone. Mussel samples were ozonated inside a refrigerator
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29 212 (4°C) at a dose of ozone gas of 15 mg kg^{-1} (concentration of ozone gas in the
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31 213 refrigerator cabinet) and exposure time of 6 h. Ozone gas was fed into the refrigerator
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33 214 by means of plastic (PVC) tubing, whereas during treatment, samples (either whole
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35 215 shucked or homogenized) were placed in glass trays in order to achieve the largest
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37 216 possible exposure of the samples to ozone gas. Ozone concentration inside the
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39 217 refrigerator was measured using a model OS-4 ECO-Sensors ozone meter (Santa Fe,
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41 218 USA).
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45 219 In order to further investigate the effect of ozone on DSP toxins, OA standard
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47 220 solution and Certified Reference Material (CRM) Mussel Tissue were also ozonated.
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49 221 During treatment with ozone gas, 1 ml of the standard solution and 3.5 g of CRM
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51 222 tissue were kept in petri dishes in the refrigerator and were exposed to the same ozone
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53 223 dose concentration/time treatment. The final concentrations, as well as the percent
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3 224 reduction of OA and DTX-1 after irradiation and ozone treatment, were measured by
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5 225 the LC-MS/MS method.
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10 227 ***Determination of TBA values and sensory assessment.***

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12 228 Determination of thiobarbituric acid (TBA) values and sensory assessment
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14 229 were conducted only in the case of ozone-treated mussels. TBA values were
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16 230 determined according to the method of Kirk and Sawyer (1991) and results were
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18 231 expressed as mg malondialdehyde (MDA) kg⁻¹ mussel tissue.
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21 232 The sensory attributes of raw (non-toxic) mussels before and after ozonation
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23 233 were evaluated by a panel of seven experienced judges. Each panelist evaluated
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25 234 approximately 20 g of mussel sample. Sensory evaluation was conducted in
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27 235 individual booths under controlled conditions of light, temperature and humidity.
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29 236 Panelists were asked to score odor and texture of mussels using a 0-10 acceptability
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31 237 scale (Manousaridis et al., 2007). Throughout the experiment, the panelists were also
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33 238 presented with a freshly thawed mussel sample, stored at -30°C which served as the
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35 239 control sample.
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41 241 ***Statistical analysis***

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43 242 All determinations were conducted in duplicate. Sensory evaluation was
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45 243 carried out once with 7 panelists (n=7).
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50 245 **Results and Discussion**

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52 246 ***Treatment with γ -radiation.***

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54 247 Results of decontamination trials by γ -irradiation are shown in Table 1. Before
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56 248 treatment, toxin content of positive samples, for free OA, OA esters and total OA
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3 249 concentration (free OA plus OA esters) was in the range of 0.18 to 6.50 $\mu\text{g g}^{-1}$, non-
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5 250 detectable to 7.04 $\mu\text{g g}^{-1}$ and 0.18 to 13.54 $\mu\text{g g}^{-1}$ mussel tissue, respectively. In the
6
7 251 case of negative samples, OA or OA esters were not detected at all or concentrations
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10 252 were below the detection limit of the method (0.045 $\mu\text{g g}^{-1}$). DTX-1 was not detected
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12 253 in any of the samples included in the study.

14 254 Toxin content of all initially DSP/MBA-positive mussel samples was reduced,
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16 255 to various extents after γ -irradiation treatment (Table 1). Toxin reduction was in the
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18 256 range of 12% (from 6.50 $\mu\text{g g}^{-1}$ to 5.70 $\mu\text{g g}^{-1}$) -36% (from 0.19 $\mu\text{g g}^{-1}$ to 0.12 $\mu\text{g g}^{-1}$);
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20 257 8% (from 7.04 $\mu\text{g g}^{-1}$ to 6.50 $\mu\text{g g}^{-1}$) – 53% (from 1.76 $\mu\text{g g}^{-1}$ to 0.82 $\mu\text{g g}^{-1}$) and 10%
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22 258 (from 13.54 $\mu\text{g g}^{-1}$ to 12.20 $\mu\text{g g}^{-1}$) – 41% (from 2.74 $\mu\text{g g}^{-1}$ to 1.60 $\mu\text{g g}^{-1}$) for free
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24 259 OA, OA esters and total OA, respectively. Two of the initially MBA positive
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26 260 samples, whose toxin content was slightly above the regulatory limit of 0.16 $\mu\text{g OA}$
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28 261 equivalents g^{-1} mussel tissue, turned to MBA negative following the γ -irradiation
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30 262 treatment, with total toxin content being lower than 0.16 $\mu\text{g/g}$. It should be noted that
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32 263 OA esters were not detected in these samples. On the other hand, negative (non toxic)
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34 264 samples, which served as negative controls for the treatment, following the
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36 265 experimental decontamination procedure remained MBA-negative with OA
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38 266 concentrations being still non-detectable, which indicates that γ -irradiation did not
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40 267 generate compounds acutely toxic to mice, at least in the case of the samples tested.
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42 268 This, however, cannot exclude the possibility that compounds with long term toxicity
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44 269 or carcinogenic effects can be generated. For instance, compounds such as 2-
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46 270 alkylcyclobutanones, radiolytic derivatives of triglycerides found exclusively in
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48 271 irradiated food, may promote experimental colon carcinogenesis in animals (Raul et
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53 272 al. 2002).

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3 273 To the best of our knowledge, there are no reports in the literature with regard
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5 274 to the effect of γ -irradiation on toxin content of DSP-contaminated shellfish for
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7 275 comparison purposes. The sole relevant work is that of McCarron et al. (2007), who
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9 276 studied the effect of γ -irradiation on concentrations of lipophilic and hydrophilic
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11 277 toxins in methanolic solution and mussel tissue, aiming however to the stabilization
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13 278 of toxins for the preparation of standards and reference materials. The toxins
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15 279 investigated included the lipophilic toxins AZA1, OA, PTX2, YTX and the
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17 280 hydrophilic toxin Domoic Acid (DA). Samples were treated at doses of 6 kGy, 12
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19 281 kGy and 24 kGy. Although all toxin concentrations in solution were reduced to some
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21 282 extent, OA was one of the least affected toxins, with its reduction in mussel tissue
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23 283 being always lower than that in methanolic solutions. In comparison to the controls,
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25 284 there was an approximately 30% reduction in OA concentration for the samples in
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27 285 solution treated with the highest dose. In mussel tissue, on the other hand, there was a
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29 286 gradual reduction in OA concentration with increasing dose, but differences between
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31 287 the samples subjected to progressively increasing doses were not statistically
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33 288 significant. In comparison to the controls, however, the 20% mean reduction of OA in
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35 289 the samples treated with 24 kGy, was statistically significant. Taking into account the
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37 290 irradiation doses applied, this reduction can be considered as lower, compared to the
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39 291 results of the present study.

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45 292 The effectiveness of γ -irradiation for the reduction OA content in mussels
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47 293 proved to be limited. Furthermore, appearance and texture of the product after the
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49 294 decontamination procedure had markedly deteriorated indicating a low potential for
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51 295 commercial utilization of this method. At present, European legislation regarding
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53 296 food irradiation does not include the use of irradiation for the elimination of toxins
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55 297 from seafood (EU Directive 1999/2/EC), whereas ionizing radiation of fish and
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3 298 shellfish is only allowed in some countries at doses not exceeding an overall average
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5 299 absorbed radiation dose of 3 kGy, with the exception of 5 kGy for frozen peeled
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7 300 and/or decapitated shrimp (EU Parliament 2009). It is therefore evident that
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9 301 application of this process for commercial decontamination of shellfish, would
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11 302 require amendment of the current legislation.

14 303 *Treatment with ozone*

16 304 Effect on OA content of standard solution, CRM tissue and samples

18 305 Reduction of OA content by ozone treatment in the methanolic standard
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20 306 solution was 100% (initial concentration: 24.1 mg kg⁻¹, final concentration: ND),
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22 307 whereas in the case of the CRM mussel tissue the reduction observed was 14.2 % for
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24 308 OA (initial concentration: 9.7 mg kg⁻¹, final concentration: 8.3 mg kg⁻¹) and 20.8 %
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26 309 for DTX-1 (initial concentration: 1.2 mg kg⁻¹, final concentration: 0.95 mg kg⁻¹).
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29 310 Results of decontamination trials by ozone gas treatment in mussel samples are
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31 311 presented in Table 2. Before treatment, toxin content of positive samples, for free OA,
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33 312 OA esters and total OA concentration (free OA plus OA esters) were in the range of
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35 313 0.16 to 5.96 µg g⁻¹, ND to 6.84 µg g⁻¹ and 0.98 to 12.80 µg g⁻¹ mussel tissue,
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37 314 respectively, whereas in negative samples OA or OA esters were again non-
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39 315 detectable.
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42 316 Despite the initial very high toxin content (OA and derivatives) of the samples
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44 317 included in the study, which explains the positive MBA test after ozone gas treatment
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46 318 in most of them, a reduction of the toxin content of mussel tissue was observed in all
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48 319 samples, irrespective of mussel sample type (whole shucked or homogenized tissue),
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50 320 after ozone gas treatment (Table 2). This reduction was in the range of 6% (from 2.26
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52 321 µg/g to 2.12 µg/g) – 100% (from 0.16 µg/g to not detected); 25% (from 6.84 µg/g to
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54 322 5.58 µg/g) – 83% (from 0.66 µg/g to 0.11 µg/g) and 21% (from 12.80 µg/g to 10.08
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3 323 $\mu\text{g/g}$) – 66% (from 1.42 $\mu\text{g/g}$ to 0.48 $\mu\text{g/g}$) for free OA, OA esters and total OA,
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5 324 respectively. Practically, this indicates that in the worst case scenario (total OA
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7 325 reduction of 21%), non-marketable mussels with total OA contents of 193 $\mu\text{g OA eq}$
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9 326 kg^{-1} , may be probably marketable after treatment with ozone gas, as toxin content
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11 327 may be decreased to levels lower than the current regulatory limit (160 $\mu\text{g OA eq kg}^{-1}$
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13 328 shellfish tissue). It is interesting to point out that reduction of OA content was
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15 329 somewhat lower in the CRM mussel tissue compared to the mussel tissue samples
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17 330 treated. This could be partly attributed to the fact that the CRM mussel tissue consists
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19 331 of hepatopancreas, which has a much higher lipid content (about 10%) compared to
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21 332 whole mussel tissue (around 3%) (Lubet et al. 1985). This difference in lipid content,
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23 333 may have an impact on the ability of ozone to react with the toxins, taking into
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25 334 account that OA is a lipophilic toxin.

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30 335 In the same context, Croci et al. (1994) studied the depuration of live toxic
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32 336 mussels in ozonated artificial marine water (ozone generator providing 360 mg $\text{O}_3 /$
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34 337 h). A clear reduction of toxicity levels and OA was observed after 3 days of
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36 338 treatment, but some of the samples were still toxic after 6 days of depuration. On the
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38 339 other hand, Reboreda et al. (2010) kept live mussels in ozonated seawater (Redox >
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40 340 450 mV) for 24 h and reported that mussels were not significantly detoxified by this
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42 341 process.

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45 342 An important trend was observed with regard to reduction of toxin content in
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47 343 treated samples, when mussel sample type was considered (Table 2), with percent
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49 344 reduction of OA content in homogenized tissue being much higher in comparison to
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51 345 that observed in whole shucked mussels. Toxin content in homogenized tissue
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53 346 decreased after treatment by 25% (from 0.32 $\mu\text{g/g}$ to 0.24 $\mu\text{g/g}$) – 100% (from 0.16
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55 347 $\mu\text{g/g}$ to not detected); 39% (from 3.26 $\mu\text{g/g}$ to 2.00 $\mu\text{g/g}$) – 83% (from 0.66 $\mu\text{g/g}$ to

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3 348 0.11 $\mu\text{g/g}$) and 51% (from 2.96 $\mu\text{g/g}$ to 1.46 $\mu\text{g/g}$) – 66% (from 1.06 $\mu\text{g/g}$ to 0.36
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5 349 $\mu\text{g/g}$) for free OA, OA esters and total OA, respectively, whereas in the case of whole
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7 350 shucked mussels these values were in the range of 6% (from 2.26 $\mu\text{g/g}$ to 2.12 $\mu\text{g/g}$) –
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9 351 21% (from 1.90 $\mu\text{g/g}$ to 1.51 $\mu\text{g/g}$); 25% (from 6.84 $\mu\text{g/g}$ to 5.58 $\mu\text{g/g}$) – 57% (from
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11 352 0.70 $\mu\text{g/g}$ to 0.30 $\mu\text{g/g}$) and 21% (from 12.80 $\mu\text{g/g}$ to 10.08 $\mu\text{g/g}$) – 33% (from 4.94
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13 353 $\mu\text{g/g}$ to 3.40 $\mu\text{g/g}$), respectively. This observed effect could be possibly attributed to
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15 354 the greater penetration of ozone gas in mussel tissues leading to a higher interaction
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17 355 with OA molecules due to the larger surface area of the homogenized sample.
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21 356 It is also interesting to point out that in the present study percent reduction of
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23 357 OA esters after ozone treatment was in most cases higher than the respective value for
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25 358 free OA in both whole shucked mussels and homogenized tissue (with the exception
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27 359 of samples 13, 17, 18). This is in partial agreement with the findings of Reboreda et
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29 360 al. (2010), who reported an increase in OA and a decrease of DTX3 (OA esters) after
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31 361 ozone treatment.
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35 362 Two of the negative samples, which were used as control samples for the
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37 363 treatment, remained MBA-negative following the ozone decontamination procedure
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39 364 with OA concentrations being still non-detectable, whereas one negative MBA
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41 365 sample (with one mouse dead in the MBA test) provided a positive MBA test after
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43 366 treatment (sample no. 19). This somewhat odd result could be attributed to one or
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45 367 both of the following factors: a) inhomogeneity of the initial sample due to sample
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47 368 origin, as (i) market samples normally consist of a mixture of mussels coming from
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49 369 different sampling locations, (ii) toxic mussels collected from a variety of sampling
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51 370 sites may contain different amounts of OA and its derivatives and b) a possible toxin
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53 371 transformation, which has been also implied by Reboreda et al. (2010). Although in
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55 372 their study ozone did not substantially detoxify mussels, there was a modification in
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3 373 the ratio OA/Total toxin, whereas an increase in OA levels parallel to a decrease in
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5 374 DTX3 (OA esters) levels was also observed in ozonated mussels, suggesting that the
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7 375 oxidative power of ozone could have been responsible for changing the mussels'
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9 376 toxin profile.

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11 377 A possible explanation for the effect of ozone treatment on OA content of
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13 378 mussel tissue may be an interaction of ozone gas with the double bonds present in the
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15 379 OA molecule (Soriano et al. 2003), either in its free or esterified form. Ozone is
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17 380 known to attack double bonds of organic compounds (Smith 2004). Okadaic acid
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19 381 (both free and in esterified form) contains several double bonds which could be a
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21 382 potential target for ozone. Alteration of the free OA structure by ozone treatment may
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23 383 result in a reduction of its concentration because an altered molecule will probably be
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25 384 non-identifiable as OA in LC-MS/MS analysis and furthermore could be less toxic in
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27 385 the MBA. In this context, ozone treatment may also have contributed to a
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29 386 transformation of OA esters back to OA free form, which could account for the lower
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31 387 percent reduction of free OA observed in the present study, as some of the remaining
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33 388 free OA after processing may be derived from the esterified form. Further research is
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35 389 required to elucidate the actual mechanism of action of ozone on OA molecule.

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43 391 TBA values

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45 392 TBA values of mussel tissue samples before and after ozonation were 0.10 and
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47 393 0.21 MDA kg⁻¹. Although the difference in TBA values is almost double, which is
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49 394 expectable since ozone treatment has been reported to have an effect on unsaturated
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51 395 bonds of organic compounds (Gottschalk et al. 2010) and subsequently on fatty acid
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53 396 oxidation, in both cases values are much lower than the proposed TBA limit value of
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55 397 2 mg MDA kg⁻¹ (Connell, 1995). This is indicative of a negligible detrimental effect
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3 398 of ozonation on lipid oxidation of mussel tissue. In partial agreement with our data,
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5 399 Manousaridis et al. (2005) observed no significant differences in the degree of lipid
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7 400 oxidation (TBA) between control and ozonated mussels.
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402 ***Sensory assessment***

403 No apparent deterioration of appearance or texture was noticed visually after
404 ozone treatment. Mean sensory texture scores of mussels before and after ozonation
405 were similar (10.0 vs. 9.8, respectively). On the other hand, odor scores decreased
406 after ozonation compared to the controls (8.1 vs.10.0, respectively), but remained
407 sufficiently high given the lower limit of acceptability (score of 7) below which an
408 apparent off-taste developed (Chang et al., 1998).

409 In agreement with our observation, in a comparative study of ozone treatment
410 effects on shucked mussels' shelf-life, no significant differences were reported in
411 sensory attributes (odor, taste and texture) between control and ozonated mussels
412 (Manousaridis et al. 2005).

413

414 **Conclusion**

415 This study indicates the potential for use of ozonation to decontaminate shucked
416 mussels from DSP toxins. Even though varying % reductions in OA and its
417 derivatives were achieved under specific experimental conditions tested it is
418 postulated that upon further optimization may be potentially exploited for commercial
419 use in the future. At the same time the need for the collection of a larger numbers of
420 contaminated samples is stressed. A thorough statistical treatment of such a data set
421 may verify present preliminary results. Taking into account that mussels are probably
422 one of the best indicator species for evaluating the contamination of shellfish by

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3 423 toxins of the OA group, this processing method could be also applied to other
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5 424 contaminated shellfish species provided that they are marketed as shelled products.

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7 425 Further research is required to establish whether there is an optimal processing
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9 426 time/ozone dose combination in order to achieve even better decontamination effects.

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17
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Table 1. Toxicity of samples (7 of 21) determined by MBA and concentrations of okadaic acid and acyl derivatives by LC-MS/MS of mussels' tissue before and after decontamination by γ -irradiation at 6kGy.

Sample No	Sampling Region	Sampling Year	Mussel sample form	Before decontamination			After decontamination			Free OA reduction (%)	OA esters reduction (%)	Total OA reduction (%)		
				MBA (mice deaths)	LC-MS/MS			MBA (mice deaths)	LC-MS/MS					
					Free OA ($\mu\text{g g}^{-1}$)	OA esters ($\mu\text{g g}^{-1}$)	Total OA ($\mu\text{g g}^{-1}$)		Free OA ($\mu\text{g g}^{-1}$)				OA esters ($\mu\text{g g}^{-1}$)	Total OA ($\mu\text{g g}^{-1}$)
1	Thessaloniki	2009	Whole shucked	Negative (- - -)	ND	ND	ND	Negative (- - -)	ND	ND	ND	-	-	-
2	Fthiotida	2009	Whole shucked	Negative (- - -)	ND	ND	ND	Negative (- - -)	ND	ND	ND	-	-	-
3	Megara	2009	Whole shucked	Positive (+++)	0.18	ND	0.18	Negative (- - -)	0.13	ND	0.13	27	-	27
4	Megara	2009	Whole shucked	Positive (+++)	0.19	ND	0.19	Negative (- - +)	0.12	ND	0.12	36	-	36
5	Megara	2009	Whole shucked	Positive (+++)	6.50	7.04	13.54	Positive (+++)	5.70	6.50	12.20	12	8	10
6	Megara	2009	Whole shucked	Positive (+++)	3.54	3.08	6.62	Positive (+++)	3.06	1.88	4.94	14	39	25
7	Piraeus	2009	Whole shucked	Positive (+++)	0.98	1.76	2.74	Positive (+++)	0.78	0.82	1.60	20	53	41

ND: not detected

Table 2. Toxicity of samples (14 of 21) determined by MBA and concentrations of okadaic acid and acyl derivatives by LC-MS/MS of mussels' tissue before and after decontamination by ozone treatment at 15 mg kg⁻¹ for 6 h.

Sample no	Sampling Region	Sampling Year	Mussel sample form	Before decontamination				After decontamination				Free OA reduction (%)	OA esters reduction (%)	Total OA reduction (%)
				MBA (mice deaths)	Free OA (µg g ⁻¹)	OA esters (µg g ⁻¹)	Total OA (µg g ⁻¹)	MBA (mice deaths)	Free OA (µg g ⁻¹)	OA esters (µg g ⁻¹)	Total OA (µg g ⁻¹)			
2	Thessaloniki	2009	Whole shucked	Negative (- - -)	ND	ND	ND	Negative (- - -)	ND	ND	ND	-	-	-
4	Fthiotida	2009	Whole shucked	Negative (- - -)	ND	ND	ND	Negative (- - -)	ND	ND	ND	-	-	-
5	Piraeus	2009	Whole shucked	Positive (+++)	5.96	6.84	12.80	Positive (+++)	4.50	5.58	10.08	18	25	21
6	Megara	2009	Whole shucked	Positive (+++)	2.26	2.68	4.94	Positive (+++)	2.12	1.28	3.40	6	52	31
12	Thessaloniki	2007	Homogenized tissue	Positive (+++)	1.08	2.24	2.32	Positive (+++)	0.78	0.82	1.60	28	63	52
13	Pieria	2007	Homogenized tissue	Positive (+++)	0.84	0.58	1.42	Positive (+++)	0.24	0.24	0.48	71	59	66
14	Pieria	2007	Homogenized tissue	Positive (+++)	1.42	1.70	3.12	Positive (+++)	1.03	0.32	1.35	27	81	57
15	Pieria	2007	Homogenized tissue	Positive (+++)	0.32	0.66	0.98	Positive (+++)	0.24	0.11	0.35	25	83	64
16	Imathia	2007	Homogenized tissue	Positive (+++)	1.26	1.70	2.96	Positive (+++)	0.84	0.62	1.46	33	64	51
17	Imathia	2007	Homogenized tissue	Positive (+++)	0.16	0.90	1.06	Positive (+++)	ND	0.36	0.36	100	60	66
18	Megara	2007	Homogenized tissue	Positive (+++)	2.10	3.26	5.36	Positive (+++)	0.24	2.00	2.24	89	39	58
19	Market	2007	Whole shucked	Negative (+ - -)	ND	ND	ND	Positive (+++)	0.32	0.38	0.70	-	-	-
20	Market	2007	Whole shucked	Positive (+++)	1.90	0.70	2.60	Positive (+++)	1.51	0.30	1.81	21	57	30
21	Thessaloniki	2009	Whole shucked	Positive (+++)	1.40	0.65	2.05	Positive (+++)	1.13	0.30	1.43	19	54	30

ND: not detected