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Effect of the antioxidant N-acetylcysteine on the depuration of the amnesic shellfish poisoning toxin, domoic acid, in the digestive gland of the king scallop *Pecten maximus*

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Abstract – Domoic acid (DA) is a potent neurotoxin produced by worldwide distributed diatoms of the genus *Pseudo-nitzchia* (PSN) and is responsible for Amnesic Shellfish Poisoning (ASP) in humans. King scallop *Pecten maximus,* a bivalve species of high commercial interest, is regularly subjected to blooms of *Pseudo-nitzschia* sp., thus accumulating and retaining high levels of DA for extended periods, leading to prolonged fisheries and aquaculture closures and important economic losses following increasingly recurrent toxic PSN blooms. The underlying mechanisms behind this accumulation and long toxin retention remain poorly understood so far. Fishermen and the aquaculture industry ask for methods to accelerate DA depuration in contaminated scallops, which has led to investigate the effect of some substances such as the antioxidant N-Acetylcysteine (NAC), which was previously found to improve up to four-fold DA depuration in *P. maximus* adductor muscle. Our study investigated the potential of NAC to accelerate DA depuration in all scallop tissues, including the digestive gland (DG), where most of the toxin is accumulated. Twenty-four contaminated adult scallops were collected following a toxic *P. australis* bloom in the Bay of Brest (France) and half were treated with the antioxidant NAC (250 mg L^{-1}) for 6 days. HPLC toxin quantification analyses did not revealed any significant differences in the DA burdens in the DG between treated scallops and the control group. DA amounts in the adductor muscle and gonads were below the HPLC detection limit in both groups. Our results revealed that NAC does not thus appear as a commercially suitable solution for fisheries and aquaculture industries as DA depuration enhancer in the tested conditions.

Keywords: Domoic acid / amnesic shellfish poisoning / *Pecten maximus* / *Pseudo-nitzschia* N-acetylcysteine / depuration

1 Introduction

Harmful algal blooms (HAB) have considerable impacts on human health and human activities, such as aquaculture, fisheries, and tourism. HABs represents a growing concern around the world, as a consequence of its increase in frequency and intensity (Glibert et al., 2005), highlighted by an increased awareness and monitoring (Lelong et al., 2012; Hallegraeff et al., 2021).

One of the five most commonly recognized HAB-related illnesses is amnesic shellfish poisoning (ASP) caused by domoic acid (DA), a neuroexcitatory water-soluble cyclic amino acid produced by various strains of diatoms of the genus *Pseudo-nitzschia* (PSN) (Bates et al., 1989; Grattan et al., 2016). To this day, 28 toxigenic *Pseudo-nitzschia* species have been discovered worldwide giving DA toxic outbreaks an extended distribution (Lundholm et al., 2009; Trainer et al., 2012; Bates et al., 2018). Neurotoxicity is the critical toxicological effect of DA. In humans, the symptoms of ASP include nausea, gastroenteritis, and vomiting, followed by neurological signs such as confusion, lethargy, disorientation, paresthesia, short-term memory loss, and, in extreme cases, death (Pulido, 2008; La Barre et al., 2014). Those effects are mediated through its high-affinity binding and agonist activity on some forms of glutamate receptors in certain regions of the brain (e.g. hippocampus), leading to cell death (Perl et al., 1990; Lerma et al., 1993).

As filter-feeding organisms grazing on microalgae, bivalves are prone to contamination and rapid accumulation of high quantities of HAB-derived neurotoxins like DA.

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Hence, the consumption of shellfish and fish is the primary vector for HAB-related human health concerns, such as ASP (Lelong et al., 2012; Grattan et al., 2016). Consequently, in Europe, regulations made shellfish marketing banned when DA was found above the limit of 20 mg kg^{-1} of flesh on the whole or individual parts (McKenzie and Bavington, 2002; Wekell et al., 2004).

Most bivalve species, such as the mussels *Mytilus edulis* (Novaczek et al., 1992; Wohlgeschaffen et al., 1992), *M. californianus* (Whyte et al., 1995), and *M. galloprovincialis* (Blanco et al., 2002), the oyster *Crassostrea virginica* (Mafra et al., 2010), or the surf clam *Mesodesma donacium* (Álvarez et al., 2015), depurate DA in a matter of hours or days following a toxic *Pseudo-nitzschia* sp. bloom, or in a matter of weeks, such as the deep-sea scallop *Placopecten magellanicus* (Wohlgeschaffen et al., 1992; Douglas et al., 1997), having thus relatively low impact on their harvest and commercialization.

However, the contamination by DA of the commercially important king scallop, *Pecten maximus,* is problematic as it accumulates high levels of this toxin and retains it in tissues for long periods, up to years in extreme cases (Blanco et al., 2002). This slow depuration, in the case of consecutive toxic *Pseudo-nitzschia* blooms, can lead to reintoxication of the scallops before having depurated from the first bloom (Blanco et al., 2006b; Mauriz and Blanco, 2010). Over the last thirty years, prolonged closures of king scallop harvesting have occurred in Ireland (Bogan et al., 2007a,b), Scotland (Campbell et al., 2001), Spain (Arévalo et al., 1998), Portugal (Vale and Sampayo 2001), and France (Nézan et al., 2006), leading to consequent negative economic impacts on the exploitation of wild and aquacultured king scallops (Blanco et al., 2006a,b; Husson et al., 2016; Trainer et al., 2012). Simultaneously, monitoring in France revealed that toxic *Pseudo-nitzschia* blooms have become more frequent since the year 2000 (Husson et al., 2016). Likewise, data on DA occurrence and DA related toxic events in the UK showed an increase in frequency since the year 2008 (Rowland-Pilgrim et al., 2019).

The duration of the harvesting closures is mostly modulated by the depuration rate of the organisms (Blanco et al., 2002). Fishermen and scallop hatcheries have thus been asking for methods to accelerate the depuration of DA in *P. maximus*, but, to this day, only a few studies have been conducted to acquire knowledge on depuration kinetics of *P. maximus*, and potential acceleration strategies. Blanco et al. (2006a) showed that DA depuration rate in *P. maximus* increased with rising temperature and decreasing salinity, as well as by relocating scallops from the bottom (natural bed) to the surface (attached to a raft). Nevertheless, Bogan et al. (2006) also tested the relocation hypothesis and found no difference between those two conditions. The divergence of those results stresses the complexity of the kinetics of DA accumulation and depuration in king scallops, which seems to be linked to several physical and biological factors.

Nevertheless, one study (Peña-Llopis et al., 2014) proposed a method that enhances the elimination of DA in *P. maximus*, through the exposure to an antioxidant, and that could open new avenues in king scallop depuration. Peña-Llopis et al. (2014) suggested that the use of the antioxidant N-Acetylcysteine (NAC) increased the depuration rate of DA in the adductor muscle of *P. maximus* up to fourfold. Indeed, NAC is a glutathione (GSH) pro-drug that could enhance the GSH pathway, which is involved in the detoxification of many pollutants including biotoxins from HAB, by facilitating excretion through conjugation with GSH.

The aim of our study was to test the use of NAC as a feasible solution for fishermen to accelerate DA depuration rate of contaminated *P. maximus* in order to cope with the harvesting closure of wild and cultured king scallops. In other words, the idea behind is to reduce the impact of the bans by adding a new step after harvesting, which would consist in placing the contaminated scallops in NAC concentrated reservoirs over the time required for the toxic burden to reach levels below the regulatory limit, making the scallops proper for consumption. In order to be economically sustainable, the gain in depuration rate should be important enough to allow this new step to be quick. Thus, to be considered interesting and prone to deeper study, this method should lead to an important decrease of the toxic burdens (i.e. various tens of mg DA kg^{-1}) over the course of 6 days. Otherwise, due to the large extent of DA outbreaks, the scale of production of king scallop in Europe, which can reach up to 60,000 t per year in France only (FAO, 2020), and the large size of the animals, this process would require enormous infrastructures. In addition, if the animals are held in such reservoirs for longer than 6 days, high mortality is expected as well as high additional costs for the production or acquisition of scallop's food. Furthermore, Peña-Llopis et al. (2014) already identified drastic effects of NAC after only two days of treatment.

Hence, we designed a specific experiment that aimed at testing the efficiency of NAC as an enhancer of the depuration of DA in different *P. maximus* tissues and consequently evaluating its potential for fishermen and the aquaculture industry as an implementable solution.

2 Materials and methods

2.1 Source of scallops

Twenty-four contaminated adult scallops *P. maximus* (10.7 ± 0.7 cm; 231 ± 46 g) were collected from a natural bed by dredging at Roscanvel (48° 18' 3.408 " N, 4° 32' 23.108 " O) at the entrance to the Bay of Brest, France, on the 8th of April 2021, during a bloom of *Pseudo-nitzchia australis* (1×10^5 cells L^{-1}) according to the REPHY (REseau de surveillance du PHYtoplancton et des phycotoxines, https://envlit-alerte.ifremer.fr/). After harvesting, scallops were transported to the laboratory and acclimatized for 23 days prior to the experiment and placed in a flow-through aerated 100 L-tank supplied with natural seawater and fed daily with *Tisochrysis lutea* (clone *T.iso*) (Bendif et al., 2013) at a concentration of 5×10^8 cells $scallop^{-1}$ day^{-1}.

2.2 Microalgal cultures

Algal culture of *T. iso*, a species commonly used as feed-in bivalve hatcheries, was produced as the principal diet. *T-iso* was cultured in batch culture, with Conway medium (Tompkins et al., 1995) prepared with 1-μm-filtered seawater. The culture was performed in 6-L glass bottles before being inoculated into 300-L acrylic cylinders maintained at 18 °C under a 12 h–12 h photoperiod. To optimize growth conditions, cylinders were aerated.

2.3 Antioxidant

The antioxidant N-Acetyl-L-cysteine (NAC) stock solution was prepared by dissolving 100 g of NAC solid powder (Sigma-Aldrich®, Darmstadt, Germany) into 1 L of Milli-Q water and stored at 5 °C at a final concentration of 100 g L^{-1}.

2.4 Experimental design

After acclimatization, the 24 scallops were distributed to six 20 L plastic tanks ($n = 4$ scallops $tank^{-1}$) filled with running filtered (4 μm) and aerated seawater, and two experimental conditions were set up: control scallops (3 tanks) and treated with NAC (3 tanks) (12 scallops per condition). Control scallops were all fed continuously (flow rate of 14 mL min^{-1}) for 6 days with *Tiso* at the same concentration of 10^5 cells mL^{-1} using a peristaltic pump, thus reaching 5×10^8 cells $scallop^{-1}$ day^{-1}. Treated scallops were all fed similarly, i.e. continuously (flow rate of 14 mL min^{-1}) for 6 days with *Tiso* at the same concentration of 10^5 cells mL^{-1} with an addition of NAC at 250 mg L^{-1} in their ratio using a peristaltic pump, thus reaching respectively 5×10^8 cells $scallop^{-1}$ day^{-1} and 1.25 g NAC $scallop^{-1}$ day^{-1}. NAC treated scallops were thus supplied with NAC, incorporated in their algal ration, at a concentration of 250 mg L^{-1}, the concentration at which Peña-Llopis et al. (2014) demonstrated significant decrease on DA concentration in scallop muscle tissues.

2.5 Scallop sampling

After 6 days of the experiment, the 24 scallops were sampled. The meat was excised from the shells, and the digestive gland (DG) was carefully dissected from the rest of the tissues (RT: gonad, adductor muscle, gills, and mantle) to avoid a potential transfer of DA between tissues during dissections (García-Corona et al., 2022). The DG, the adductor muscle, and the gonad were stored at −20 °C until toxin quantification by HPLC.

2.6 HPLC analysis and DA quantification

For each scallop, 200 ± 5 mg of each tissue (digestive gland, adductor muscle, and gonad) were sampled for DA quantification. Each frozen sample (–20 °C) was then added to a tube containing 1 mL of a MeOH: water (50:50,v:v) mixture and 250–300 mg of 100–250 μm diameter glass beads and ground using a MM 400 ball mill system (Retsch, Fisher Scientific, Illkirch-Graffenstaden, FR) for 6 min at 30 Hz. The extracts were clarified by centrifugation at 15,000 *g* for 10 min at 4 °C (Eppendorf 5427 R, Thermo Scientific, West Sussex, UK) and the supernatant was isolated. Finally, 200 μL of this extract were filtered using 0.2 μm nylon centrifugal-filtering tubes (VWR International, Radnor, PA, USA) at 10,000 *g* for 5 min at 4 °C and the filtrate was stored in amber-glass autosampler vials (Thermo Scientific, Rockwood, TN, USA) at −20 °C until quantification. DA was quantified in the DG, adductor muscle, and gonad extracts using an HPLC-UV protocol adapted from the LNRBM-ASP 01 (IFREMER) method. The column used was a C18 Jupiter HPLC (Phenomenex 250 × 4.6 mm, 15 μm) with a gradient of the mobile phase ranging from 5% to 25% of acetonitrile and 0.1% of trifluoroacetic acid. The injection volume was 20 μL and the run time was set at 20 min with a flow rate of 1 mL min^{-1}. The column temperature was maintained at 40 °C. The detection wavelength was set at 242 nm. A certified DA standard was purchased from the National Research Council of Canada (NRC) and a six-point calibration curve was obtained by serial dilutions in MeOH: water (50:50, *v:v*) to obtain concentrations of 0.2, 0.5, 1.0, 2.0, 4.0 and 8 μg DA mL^{-1}. The LODs of this HPLC-UV method ranged from 0.2 to 1 mg DA kg^{-1} tissue.

2.7 Statistical analysis

The toxin burdens in the different tissues of the scallops were statistically analyzed using the R package Stats (R v. 4.0.2, R Core Team, 2017). Prior to analysis, Lilliefors (Kolmogorov-Smirnov), and Fligner-Killeen tests, were used to assess normality and homoscedasticity assumptions, respectively (Hector, 2015). The means of DA burdens measured by HPLC were analyzed using a two-sample *t*-test, where the two experimental conditions (control and treated scallops) were used as independent variables. All values are expressed as mean ± standard error (SE). Differences were considered statistically significant at $\alpha = 0.05$ for all analyses (Zar, 2010).

3 Results

No significant differences were found between DA burdens measured in the DGs of scallops treated for 6 days with NAC (57.85 ± 11.3 mg DA kg^{-1}) compared to those of the control group (63.03 ± 18.20 mg DA kg^{-1}) without antioxidant treatment (Fig. 1). The coefficient of variation (CV) was 29% for the control group, and 20% for the treated group. CVs higher than 15% express a significant inter-individual variability in DA concentration in the DG between individuals of the same group. DA concentrations in the adductor muscle and gonads were below the limit of detection of the HPLC-UV method (ranging from 0.2 to 1 mg DA kg^{-1} tissue) in both control and treated scallops.

4 Discussion

The goal of this study was to test the use of a 6 days-exposure of domonic acid (DA) contaminated scallops to N-acetylcysteine (NAC) as a solution for fishermen to accelerate DA depuration in order to cope with the repetitive closures faced by king scallop fishery and aquaculture.

In our experiment, the field-sampled scallops were fairly highly contaminated. Even at the end of the experiment, all the scallops were still exhibiting a DA burden in the DG that was three-fold higher than the European legal limit allowing their marketability as whole animals (20 mg DA kg^{-1}). Our results confirmed the well-accepted statement that in *P. maximus*, DA is mainly accumulated in the DG (Arévalo et al., 1998; Blanco et al., 2002; Bogan et al., 2007a, 2007b; James et al., 2005). Furthermore, DA burdens in adductor muscle and gonads were below the detection limit of the HPLC method (0.2 mg DA kg^{-1}) and all below the current European legal limit (2002/226/EC) allowing the marketability of those

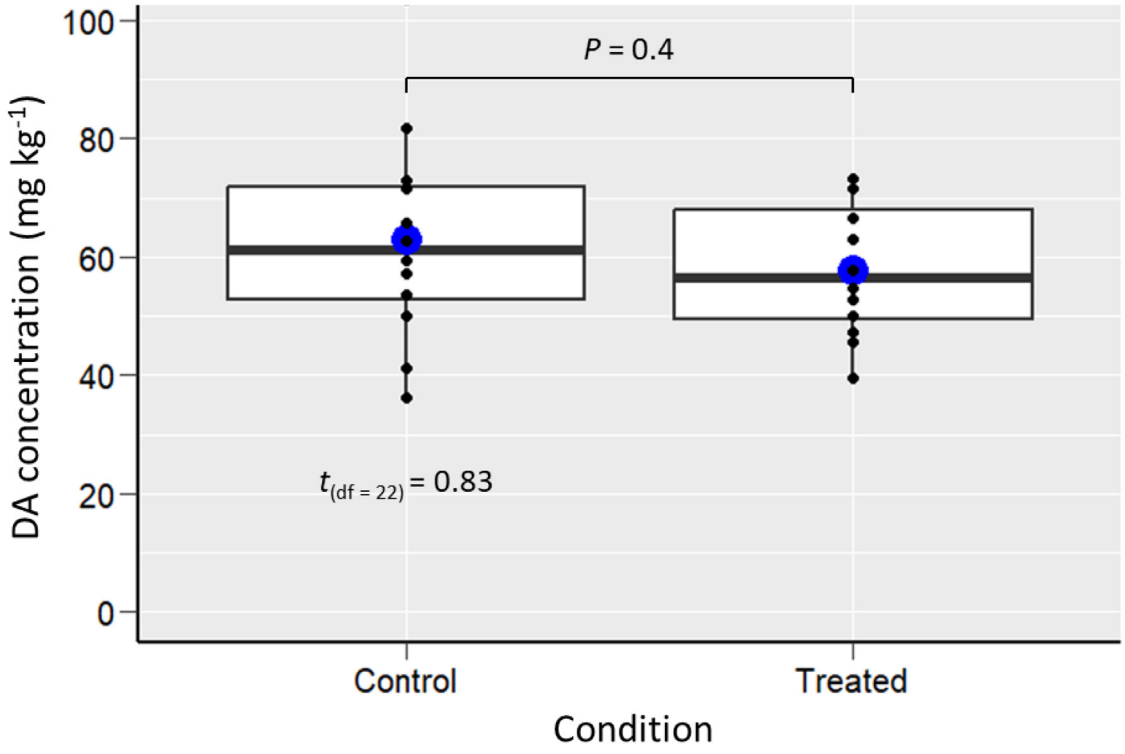

Fig. 1. DA concentration in the digestive gland of scallops *P. maximus* after 6 days of depuration in seawater (control) or seawater supplemented with N-Acetylcysteine (250 mg L^{-1}) (treated). The upper and lower limits of the boxes are the quartiles, the middle horizontal line is the median, the extremes of the vertical lines are the upper and lower limits of the observations, the dots are the individual observations, and the blue dots are the means. Data were analyzed using the experimental condition (two levels) as independent variable in a two-sample *t*-test. The *t*-test statistic and degrees of freedom (df) are reported. The level of statistical significance was set at $\alpha = 0.05$, $n = 12$ scallops per condition.

individual parts after shucking (4.6 mg DA kg^{-1}). In our experiment, no significant acceleration of DA depuration rate was observed in the DG within the 6 day-exposure to NAC. Hence, even though the toxic burdens in adductor muscle and gonads were not sufficient to be quantified by HPLC, it is reasonable to assume that there was no significant effect of the antioxidant on the DA depuration rate in any organ of the scallops.

In Peña-Llopis et al. (2014), scallops with DA levels of 7.6 mg kg^{-1} in the DG and 1 mg kg^{-1} in the adductor muscle which is already below the regulatory limits, and very low compared to levels of contamination in other studies (Blanco et al., 2002, 2006a,b; Bogan et al., 2006a 2007a,b,c) – were exposed during 32 days to several NAC concentrations (0, 50 and 250 mg L^{-1}). Depuration was only enhanced with the highest concentration of NAC tested, 250 mg L^{-1}. After 2 days, the estimated DA concentration in the adductor muscle was twice as low (0.327 mg kg^{-1}) as in the control group (0.771 mg kg^{-1}) or the 50 mg L^{-1} group (0.789 mg kg^{-1}). After 7 days, DA concentration in the adductor muscle was twenty times lower (0.02 mg kg^{-1}) than in the control group (0.402 mg kg^{-1}) or in the 50 mg L^{-1} group (0.436 mg kg^{-1}). Unfortunately, Peña-Llopis et al. (2014) focused only on the adductor muscle – the edible part with the highest market value – with an already low level of DA. Nevertheless, the study of other organs, i.e. DG, gonad, and the rest of tissues seems necessary to conclude on the potential of NAC as a depuration enhancer.

The experiment in this work was designed with a replication sufficient to find, with a probability of ~90%, a toxin depuration of at least 30% of the total DA burdens, as found by Peña-Llopis et al. (2014) in tissues of contaminated scallops. Nevertheless, the reduction of the amounts of DA in the DG using NAC was not significant, thus inferring that toxin depuration was even smaller than the reported by Peña-Llopis et al. (2014) in the adductor muscle of *P. maximus*. Hence, our results do not confirm Peña-Llopis et al. (2014) positive conclusions about NAC as a potential depuration accelerator. For further investigations, it would be interesting to quantify DA in adductor muscle, gonad, mantle, gills, and kidney with a more sensitive quantification method such as HPLC/LC-MS or by working with more contaminated scallops, if possible above the current legal limit for the marketability of individual parts after shucking (4.6 mg DA kg^{-1}) to assess whether NAC could indeed boost DA depuration. Meanwhile, results from this study did not allow to identify any significant difference in digestive gland DA content between experimental and control groups, despite replication, thus indicating that even if NAC could increase DA depuration rate of scallops, it does not appear as a commercially suitable solution for fisheries and aquaculture industries.

Therefore, to achieve acceleration of DA depuration in the king scallop, it seems more pertinent to investigate other methods, such as the combined effect of other antioxidants, like vitamin E, or other amino acids, and increasing

temperature and food availability. As mentioned by Peña-Llopis et al. (2014), glutathione metabolism could be potentially involved in the excretion of DA. Nonetheless, more investigations are needed since the mechanisms of DA accumulation and retention in the king scallop *P. maximus* remain poorly understood. Another lead for investigation is the inoculation of probiotics from other bivalves, such as mussels, which quickly depurate DA (Donovan et al., 2009; Vasama et al., 2014).

5 Conclusions

The results of this work provide evidence that exposure to antioxidants, such as NAC does not appear as a sustainable alternative for the scallop fishing industry to accelerate the decontamination rates of DA in this resource. Moreover, a 6-day exposure to high concentrations of NAC did not allow identifying any significant acceleration of the depuration of DA in the digestive gland of *P. maximus*. Other alternatives have to be investigated to induce faster depuration of this scallop species, which despite being a slow DA depurator, represents a very high commercial interest.

Conflict of interest

All authors approved the final version of this manuscript and declared no conflict of interest.

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Data availability statement

The evidence and data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statements

The adult scallops (*Pecten maximus*) were transported and handled according to the International Standards for the Care and Use of Laboratory Animals. The number of sampled organisms contemplated the rule of maximizing information published and minimizing unnecessary studies. In this sense, 24 scallops were considered as the minimum number of organisms needed for this work.

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