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Margot Deléglise

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# THESE DE DOCTORAT DE

L'UNIVERSITE  
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Spécialité : Biologie Marine

Par

**Margot DELÉGLISE**

**Suivi de la contamination des coquilles Saint-Jacques  
(*Pecten maximus*) par l'acide domoïque et exploration du rôle du  
microbiote dans sa décontamination.**

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## Liste des abréviations

<b>AD / DA</b>	: Acide domoïque / Domoic acid
<b>ASP</b>	: Amnesic Shellfish Poisoning
<b>AZP</b>	: Azaspiracid Shellfish Poisoning
<b>CP</b>	: Ciguatera poisoning
<b>CRPMEM</b>	: Comité Régional des Pêches Maritimes et des Élevages Marins
<b>DSP</b>	: Diarrheic Shellfish Poisoning
<b>DO / OD</b>	: Densité optique / Optical density
<b>HAB</b>	: Harmful Algal Bloom
<b>Hz</b>	: Hertz
<b>HPLC-UV</b>	: High-Performance Liquid Chromatography-Ultraviolet (Chromatographie en phase liquide couplée à un détecteur ultraviolet)
<b>IFREMER</b>	: Institut Français de Recherche pour l'Exploitation de la Mer
<b>MaSCoET</b>	: Maintien du Stock de Coquillages en lien avec la problématique des Efflorescences Toxiques
<b>NAC</b>	: Antioxyant N-acétylcystéine
<b>NSP</b>	: Neurologic Shellfish Poisoning
<b>PSP</b>	: Paralytic Shellfish Poisoning
<b>PST</b>	: Paralytic Shellfish Toxin
<b>REPHY</b>	: Réseau d'observation et de surveillance du phytoplancton et de l'hydrologie dans les eaux littorales : le phytoplancton dans l'eau
<b>REPHYTOX</b>	: Réseau de surveillance des phycotoxines dans les organismes marins : les toxines produites par le phytoplancton dans les coquillages
<b>SOMLIT</b>	: Service d'Observation en Milieu Littoral
<b>TTX</b>	: Tétrodotoxine

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# **INTRODUCTION GÉNÉRALE**

## **CONTEXTE ET OBJECTIFS DE LA THÈSE**

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## Problématique des HAB et de leurs toxines dans le monde

Les efflorescences algales ou blooms algaux sont des phénomènes naturels lors desquels une croissance importante d'une espèce donnée de microalgue est constatée. Ils sont communément appelés marées vertes ou rouges en raison de la couleur donnée à l'eau par la forte concentration de ces microalgues (Dierssen *et al.*, 2006). Avec le réchauffement climatique ces événements sont de plus en plus nombreux et plus fréquents pour les sites déjà touchés (Anderson *et al.*, 2021; Tewari, 2022; Wells *et al.*, 2020) mais on assiste également, depuis une dizaine d'années, à une apparition de ces blooms dans des régions qui jusqu'à présent n'étaient pas concernées comme c'est le cas en Asie ou bien encore dans le Golf du Maine ou la côte Est de la Floride aux Etats-Unis (Bates *et al.*, 2018; Clark *et al.*, 2022; Schreiber *et al.*, 2023). La plupart des événements recensés sont dus à des organismes de la classe des dinoflagellés ou de l'embranchement des diatomées (Lassus, 2016; Turner *et al.*, 2021). Ces proliférations d'algues, parfois nuisibles du fait de leur grande concentration cellulaire, sont recensées pour pas moins de 300 espèces de microalgues sur les presque 5000 espèces connues à ce jour (Turner *et al.*, 2021). Leurs efflorescences peuvent également être toxiques, c'est à dire avec production de toxine et sont dénommées sous le terme anglais de « Harmful algal blooms » (HAB). Les environnements côtiers sont les premiers touchés par ces HAB. Lorsqu'ils se produisent dans ces environnements, les HAB ont un impact direct sur la faune présente, notamment dans l'eau. L'apparition d'une biomasse aussi importante de microalgues peut entraîner une hypoxie du milieu (Milbrandt *et al.*, 2021; Pitcher and Probyn, 2016; Stanisiera *et al.*, 2013). En effet, les bactéries présentes dans le milieu, et dégradant les microalgues qui meurent, appauvrissent le milieu en oxygène. Cela peut également provoquer la mort des animaux par asphyxie induite directement par le colmatage des branchies par les cellules algales. Cela peut atteindre, entre autres, les poissons et les bivalves tels que les huîtres ou les coquilles Saint-Jacques (Lorrain *et al.*, 2000). Ces premières conséquences sont souvent observables par les populations présentes dans les régions affectées et peuvent impressionner par le nombre d'organismes, bien souvent des poissons, remontant à la surface après leur mort. Il existe également une autre problématique posée par ces HAB : celle des toxines, qui peuvent être libérées dans l'environnement ou être intracellulaires et être libérées après digestion des microalgues. En effet, certaines espèces de microalgues sont capables, sous certaines conditions, de produire des toxines, appelées phycotoxines. Les risques liés à ces composés toxiques sont multiples puisque leur rayon d'action ne se cantonne pas aux organismes à proximité immédiate des HAB. La toxine une fois ingérée va se concentrer dans les organes et

tissus des consommateurs primaires qui vont ensuite transmettre la toxine aux consommateurs secondaires (De La Riva *et al.*, 2009; Neves *et al.*, 2021; Scholin *et al.*, 2000). Toute la chaîne trophique est alors contaminée (Bates *et al.*, 1998) par des transferts impliquant les mécanismes de bioamplification<sup>1</sup> (**Fig. 1**). L'une des manifestations de ce genre de contamination a été rapportée par le *Santa Cruz Sentinel* le 18 Août 1961 et est sans doute la plus connue puisqu'elle inspira certainement « Les Oiseaux » à Alfred Hitchcock (Bargu *et al.*, 2012).

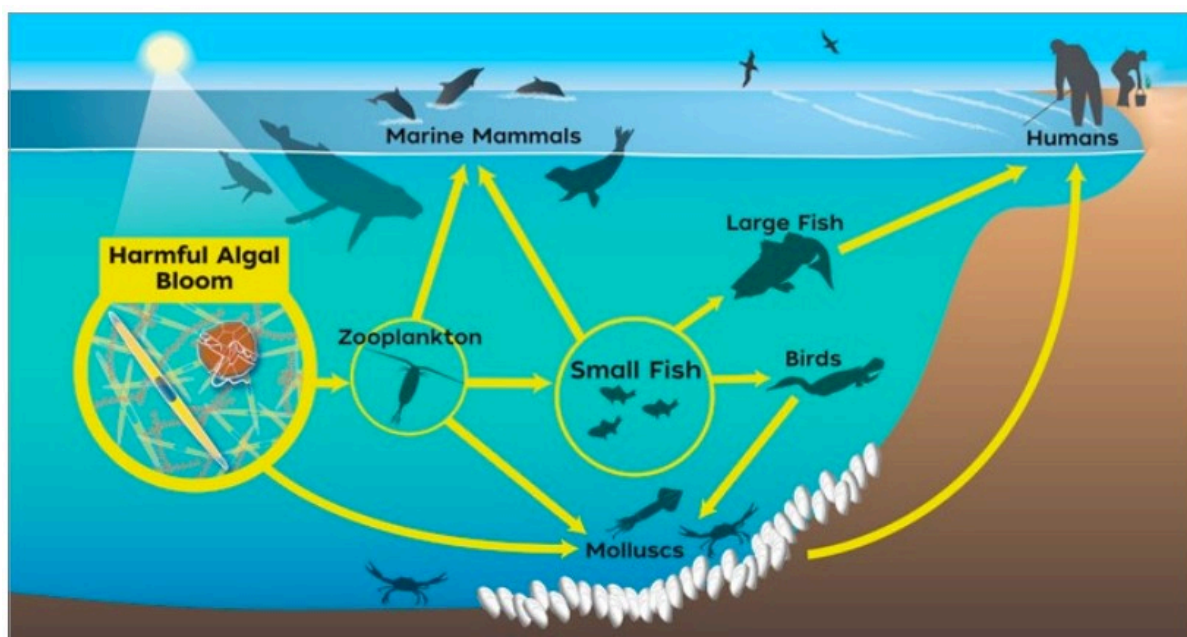


Figure 1 : Représentation des toxines d'algues qui s'accumulent dans le réseau trophique marin. Illustration de Nathalie Renier dans le rapport spécial 2022 de la “Woods Hole Oceanographic Institution”

L'Homme fait également partie des organismes pouvant être affectés par les toxines algales. Le vecteur de référence pour les contaminations humaines est la consommation de coquillages. Il se pose donc un problème de sécurité sanitaire puisque ces toxines peuvent être mortelles (Grattan *et al.*, 2016). Un classement de ces différentes phycotoxines, leurs effets sur l'humain ainsi que le taux de chacune d'entre elles pouvant entraîner des effets délétères sur l'organisme ont été recensés par les autorités sanitaires. Cinq catégories principales de toxines, provoquant toutes des syndromes différents, sont recensées. L'intoxication la plus répandue est celle causée

<sup>1</sup> La bioamplification est le processus selon lequel la concentration d'une substance présente dans un biotope augmente tout au long d'une chaîne alimentaire (Ministère de la culture)

par les toxines diarrhéiques, responsables du syndrome diarrhéique ou “Diarrheic Shellfish Poisoning syndrome (DSP)”. Il y a également les toxines paralysantes, responsables du syndrome paralysant ou “Paralytic Shellfish Poisoning (PSP)”, les toxines neurotoxiques avec le syndrome “Neurologic Shellfish Poisoning (NSP)”. A cela viennent s’ajouter les intoxications par l’azaspiracide (AZP) et la ciguatera (CP). Enfin, les contaminations à l’acide domoïque (AD) une toxine responsable du syndrome amnésiant “Amnesic Shellfish Poisoning: (ASP)” (Tableau 1).

Tableau 1 : Épisodes confirmés d’intoxications humaines dues à des toxines présentes dans des mollusques de 1901 à 2008 (James *et al.*, 2010)

Toxic syndrome	Location of outbreak (year)	Shellfish species	Number of poisonings	Ref.
PSP	USA – California (1927–1936)	Mussels	> 100 (6 deaths)	[16]
	USA – Alaska (1973–1992)		117	[14]
	USA (1998–2002)		43	[123]
	Canada (1880–1970)		187	[17]
	Spain	Mussels	120	[20]
	UK (1968)	Mussels	78	[22]
	Norway (1901–1992)		32 (2 deaths)	[124]
	Portugal (1994)		9	[21, 23]
	Chile (1991–2002)	Mussels, oysters	21 deaths	[24, 26]
	Philippines (1988–1998)		877 (44 deaths)	[25]
DSP	Japan (1976–1984)	Mussels, scallops	> 1000	[39, 40]
	France (1980–1987)	Mussels	7600	[41]
	Denmark (1990–2002)	Mussels	800–900	[43, 47]
	Norway (1984–1985)	Mussels	> 400	[125]
	Spain (1978–1981)	Mussels	> 5000	[43]
	Portugal (2002)	Mussels	58	[126]
	UK (1997)	Mussels	49	[45]
	Ireland (1984–1994)	Mussels	?	[46]
	Canada (1990)	Clams, mussels	16	[127]
	Chile (1970–1991)	Mussels, cholgas	> 100	[128]
	Argentina (2000)	Mussels	40	[129]
	New Zealand		13	[59]
	NSP	USA – North Carolina (1987)	Oysters	48
USA – Florida (1996–2006)		Whelks, clams	23	[56, 61]
New Zealand (1993)		Green mussels, cockles, oysters	186	[58, 132]
ASP	Canada (1987)	Mussels	107 (3 deaths)	[66, 67]
	USA – Washington State (1991)	Razor clams	24	[73, 77]
AZP	The Netherlands (1995)	Mussels	8	[85, 133]
	Ireland – Arranmore Island (1997)	Mussels	20–24	[86]
	Italy (1998)	Mussels	10	[89]
	France (1998)	Mussels	20–30	[89, 94]
	UK (2000)	Mussels	16	[93]
	France (2008)	Mussels	200	[134]





Différentes études ont démontré qu'il existe à ce jour, au moins 29 espèces de cette microalgue capables de produire de l'acide domoïque (Bates *et al.*, 2018; Lundholm *et al.*, 2009). Plus, ou moins toxiques, ces souches sont regroupées sous deux différents groupes, les fines et les larges, afin de permettre leur identification par microscopie photonique dans les prélèvements effectués par les réseaux de surveillance dans le monde. A l'automne 1987, la population de l'île du Prince Edouard au Canada ayant consommé des moules de culture (*Mytilus edulis*) a fait face à 105 cas d'intoxication à l'acide domoïque. Ce premier événement de contamination ASP répertorié a entraîné la mort de 3 personnes (Bates *et al.*, 1989). D'autres cas de contamination de la population ont par la suite été rapportés et c'est pour cela que les instances sanitaires ont décidé de mettre en place un suivi ainsi qu'un seuil de sécurité sanitaire, à la fin des années 80 (Wekell *et al.*, 2004), de 20 mg d'acide domoïque par kilogramme de chair fraîche de coquillage, au-dessus duquel il n'est plus possible de consommer les coquillages.

En France, le réseau de surveillance dédié aux phycotoxines est géré par l'IFREMER dans le cadre du dispositif REPHY-REPHYTOX. Le réseau d'observation analyse les prélèvements d'eau mais aussi de coquillages pour vérifier, un mois puis quinze jours avant l'ouverture de campagnes de pêche, que le taux de toxine ne dépasse pas le seuil autorisé une fois que la présence de cellules algales toxiques est décelée. En cas de dépassement du seuil sanitaire avéré, la décision de clôturer ou d'annuler les campagnes de pêche est notifiée aux pêcheurs qui restent à quai. Si les bivalves sont les premiers surveillés, c'est du fait de leur importante consommation en France mais surtout par leur propension à se contaminer. Ce sont des organismes filtreurs se nourrissant de microalgues, c'est pourquoi ils sont facilement et fortement contaminés. Un fait important à souligner est que tous ces organismes n'accumulent pas l'acide domoïque avec la même intensité et surtout ne présentent pas les mêmes vitesses de dépuración (Blanco *et al.*, 2021). Les observations menées jusqu'à présent ont permis de répertorier les bivalves en deux groupes : les dépurateurs "rapides" et les dépurateurs "lents" (**Tableau 2**).

Tableau 2 : Liste non exhaustive des espèces de mollusques bivalves connus à ce jour pour dépurier rapidement ou lentement l'acide domoïque.

Bivalves à dépuración rapide		Espèces de bivalves à dépuración lente	
espèce	référence	espèce	référence
<i>Agropecten purpuratus</i>	Álvarez <i>et al.</i> , 2020	<i>Pecten maximus</i>	Blanco <i>et al.</i> , 2006
<i>Mytilus galloprovincialis</i>	Blanco, 2002	<i>Siliqua patula</i>	Dusek Jennings <i>et al.</i> , 2020
<i>Mytilus edulis</i>	Mafra <i>et al.</i> , 2010b Mafra <i>et al.</i> , 2010a	<i>Spondylus cruentus</i>	Dao <i>et al.</i> , 2006
<i>Mya arenaria</i>	Dusek Jennings <i>et al.</i> , 2020 Stewart <i>et al.</i> , 1998		
<i>Crassostrea gigas</i>	Jones <i>et al.</i> , 1995		
<i>Crassostrea virginica</i>	Mafra <i>et al.</i> , 2010a		
<i>Mesodesma donacium</i>	Álvarez <i>et al.</i> , 2015		
<i>Placopecten magellanicus</i>	Douglas <i>et al.</i> , 1997		
<i>Ruditapes philippinarium</i>	Dusek Jennings <i>et al.</i> , 2020		

Les bivalves parvenant à dépurier efficacement l'acide domoïque, c'est-à-dire sous seulement quelques heures, jours ou quelques semaines après un bloom, tels que les moules ou les huîtres sont également des espèces commerciales importantes. Les arrêtés pour la fermeture de leur exploitation ont un impact moindre sur l'activité des professionnels du secteur de la pêche ou de l'aquaculture. A contrario, la coquille Saint-Jacques (*Pecten maximus*), ressource économique importante, fait partie des quelques espèces dont la période de rétention de l'acide domoïque peut atteindre des années (Blanco *et al.*, 2006). Pour faciliter la compréhension de cette dépuración à double vitesse, des modèles ont été proposés. Le premier, composé de deux compartiments représente les bivalves avec une cinétique de dépuración rapide, la toxine étant plus facilement transférée d'un de la glande digestive aux autres organes pour être excrétée et un second modèle à un seul compartiment pour les bivalves dépurant lentement (Álvarez *et al.*, 2020).

## Spécificité de la Coquille Saint-Jacques face aux contaminations ASP

La coquille Saint-Jacques fait donc partie des bivalves à dépuración lente de l'acide domoïque. Il existe une très grande variabilité inter-individuelle concernant les taux de toxine présents et ce, dans les différents tissus dosés (Bogan *et al.*, 2007b; Campbell *et al.*, 2001). Une autre spécificité connue de la coquille Saint-Jacques est qu'elle peut accumuler de fortes quantités d'acide domoïque, jusqu'à 2900  $\mu\text{g}\cdot\text{g}^{-1}$  d'acide domoïque (Ramsdell, 2007) dans sa glande digestive et il a été observé que la glande digestive de l'animal accumule à elle seule plus de 80% de la charge totale d'acide domoïque (Mauriz and Blanco, 2010) et peut mettre parfois jusqu'à plusieurs années pour redescendre sous le seuil de sécurité sanitaire. Cette longue contamination par l'acide domoïque entraîne des fermetures prolongées de l'activité de pêche et par conséquent des pertes de revenus pour les professionnels. Par ailleurs, pour assurer la sécurité des consommateurs, la Commission Européenne du 15 Mars 2002 a pris la décision de mettre en place un contrôle sanitaire de pêcher les coquilles Saint-Jacques et de commercialiser les parties comestibles (noix et corail) sous réserve que celles-ci soient obtenues après éviscération par une entreprise agréée (<https://eur-lex.europa.eu/eli/dec/2002/226/oj>) et sous réserve aussi d'analyses d'acide domoïque confirmant un taux d'acide domoïque  $<4.6\text{mg/kg}$  dans l'ensemble de ces parties. Cependant cette autorisation de commercialisation induit des coûts supplémentaires pour les pêcheurs, puisqu'elle fait appel à un intermédiaire et la forte demande pendant les périodes de contamination sature régulièrement les infrastructures, qui ne peuvent répondre favorablement à toutes les sollicitations.

La coquille Saint-Jacques fait également régulièrement face à des épisodes de recontamination. Cette recontamination pourrait être le fait de fines couches de *Pseudo-nitzschia* sp. vivant aux côtés de *Chaetoceros socialis* trouvées plus profondément dans la colonne d'eau et qui pourraient être une source de toxicité en entraînant des épisodes de bloom (Suárez *et al.*, 2008). Ces derniers sont causés par une augmentation dans le milieu de la présence de cellules de *Pseudo-nitzschia* sp. toxique et par l'augmentation du taux d'acide domoïque dans l'eau. Or la coquille Saint-Jacques étant lente à dépurar, elle gardera un niveau élevé d'acide domoïque après un premier épisode toxique et accumulera à nouveau la toxine, faisant ainsi remonter son taux dans les tissus. Plusieurs hypothèses ont été étudiées afin de comprendre quels pouvaient être les mécanismes mis en jeu lors de la contamination et décontamination de la coquille Saint-Jacques et qui pourraient expliquer cette spécificité de l'espèce (Blanco *et al.*, 2006, 2002). Blanco et collaborateurs ont également pu constater que 90% de la toxine était libre dans le

cytosol. À la suite de ces résultats, l'hypothèse d'un manque de transporteurs permettant l'excrétion de la toxine chez la coquille Saint-Jacques pourrait expliquer cette longue rétention. Cependant cette hypothèse n'a pas encore pu être confirmée. Les études sur les cinétiques de contamination des coquilles sont par ailleurs peu répandues car la contamination artificielle en conditions expérimentales est difficile à mettre en place. En effet, l'ajout d'acide domoïque dilué dans l'eau de mer ne garantit pas une contamination aussi importante qu'espérée des animaux et ces expériences sont très coûteuses. De plus, les cultures de *Pseudo-nitzschia* sp. toxiques en grands volumes, nécessaires à la contamination d'un nombre élevé d'individus, sont difficiles à maintenir et ne permettent pas une répétabilité fiable. C'est pourquoi, à ce jour, il existe peu d'informations sur les mécanismes de contamination et de décontamination chez *Pecten maximus*.

Il en va de même pour les études sur la réponse de la coquille Saint-Jacques face aux blooms toxiques de *Pseudo-nitzschia* sp. En effet, bien que les taux d'acide domoïque enregistrés dans la coquille Saint-Jacques soient souvent très importants, la toxine ne semble pas avoir d'effet sur l'intégrité des tissus comme peuvent le provoquer l'exposition à d'autres toxines. Tel est le cas par exemple des composés toxiques produits par la microalgue *Alexandrium* sp. Une fois ingérée par les bivalves cette microalgue peut entraîner, entre autres, des lésions du tube digestif (Haberhorn *et al.*, 2010; Medhioub *et al.*, 2012) pouvant entraîner un dérèglement des fonctions digestives (Fernandez-Reiriz *et al.*, 2008; Li *et al.*, 2002). Ces modifications de l'aspect des tissus est un exemple d'altération visible des tissus contaminés, or aucune observation de ce genre a été faite chez *P. maximus*. L'exposition aux toxines PST produites par *Alexandrium* sp. semble également provoquer des changements dans les taux de filtration de certains bivalves comme les huîtres *Crassostrea gigas* (Pousse *et al.*, 2018). L'étude de Pousse et collaborateurs a mis en évidence que les huîtres qui se contaminent le moins étaient celles dont le taux de filtration était le plus bas. Une exposition à *Alexandrium minutum* entraîne également une réduction temporaire des taux de filtration et de respiration chez les juvéniles de *P. maximus* (Even *et al.*, 2022). Ce comportement adaptatif de filtration contribue à réduire l'ingestion de cellules d'algues et, par conséquent, à abaisser les niveaux de contamination (Mafra *et al.*, 2009; Mafra *et al.*, 2010). Les huîtres juvéniles, comme *Crassostrea gigas*, présentent également des schémas de filtration modifiés lorsqu'elles sont exposées à des cellules de *Pseudo-nitzschia australis* et à l'acide domoïque, ce qui souligne la nature spécifique à l'espèce de ces réponses (Sauvey *et al.*, 2021). En revanche, *Pecten maximus*, semble suivre un modèle

distinct. Contrairement aux huîtres, les juvéniles de *P. maximus* ne présentent aucun changement significatif dans le comportement alimentaire lorsqu'ils sont exposés à de l'acide domoïque dissous ou à des algues toxiques, comme l'a démontré l'étude de Sauvey et collaborateurs. (2021).

La relation complexe entre les réponses physiologiques et la contamination est évidente dans les études de (Morono *et al.*, 2001), où des facteurs tels que la concentration de nourriture, la température et la taille influencent les taux de filtration. La taille, en particulier, joue un rôle multiple, (Thompson and Bayne, 1974) et (Riisgard and Mohlenberg, 1979) soulignent son impact positif sur le taux de filtration, tandis que (Sukhotin *et al.*, 2003) mettent l'accent sur son rôle dans la détermination du taux de respiration. L'influence de la taille sur les niveaux de contamination est également apparente dans les études de Mafra *et al.* (2010), où les petites huîtres présentent une contamination plus élevée par l'acide domoïque en raison de taux d'absorption de toxines plus élevés. De plus, les huîtres de petite taille présentent un taux de dépuración plus élevé que celui des individus de plus grande taille (Mafra *et al.*, 2010 ; (Novaczek *et al.*, 1992). Il est intéressant de noter que les études de terrain sur *P. maximus* présentent des résultats contradictoires concernant la corrélation entre la taille et la contamination par l'acide domoïque, certaines ne montrant aucune association (Arévalo *et al.*, 1998; Campbell *et al.*, 2001) et d'autres révélant une cinétique de contamination et une dépuración plus rapide chez les coquilles Saint-Jacques de petite taille (Bogan *et al.*, 2007a). Les résultats de cette étude montrent une corrélation négative entre la concentration en acide domoïque dans la glande digestive et la taille de la coquille. L'explication possible avancée pour cette différence de contamination est double. En effet, il peut s'agir de la conséquence d'un taux de filtration plus élevé chez les individus de petite taille (Jones *et al.*, 1992) ou d'un effet de dilution par la taille. La dilution par la taille s'explique en période de croissance, les organismes peuvent se développer et augmenter leur masse et donc leur capacité à stocker. Lorsque le taux de croissance ou de stockage est supérieur à l'absorption de la toxine, les concentrations dans les organismes peuvent sembler diminuer lors des analyses. L'étude de Bogan et collaborateurs met en lumière un taux de dépuración plus important chez les coquilles Saint-Jacques de petite taille.

Malgré des recherches approfondies sur diverses espèces de bivalves, il existe une lacune notable dans la compréhension de la réponse physiologique de *P. maximus* pendant la décontamination par l'acide domoïque, ce qui justifie des recherches plus approfondies dans ce domaine.

### **Études menées sur de potentielles voies d'accélération de la dépuración chez la coquille Saint-Jacques**

Quelques études ont été menées afin d'étudier si certains facteurs pouvaient accélérer le processus de dépuración chez la coquille. Tout particulièrement, celles de (Blanco *et al.*, 2006) et (Peña-Llopis *et al.*, 2014). La première étude a été menée en Galice sur des coquilles Saint-Jacques naturellement contaminées lors d'un bloom de *P. australis*. Le protocole consistait à suspendre des coquilles sous des radeaux d'élevage de moules à trois profondeurs différentes, à 2 m, 6 m et 10 m. Des coquilles laissées sur le sédiment ont servi de témoins. La cinétique d'élimination de l'acide domoïque a été suivie dans la glande digestive ainsi que dans deux groupes de tissus : le muscle adducteur, la gonade, le rein, le pied d'un côté et les branchies et le manteau de l'autre. L'expérience s'est déroulée sur 154 jours et les résultats montrent que les glandes digestives des coquilles présentes sous le radeau ont dépuré plus rapidement la toxine que celle des coquilles contrôles. L'accélération de la cinétique est présentée dans l'article comme la possibilité pour des coquilles contaminées à 1000  $\mu\text{g}$  d'acide domoïque par gramme de tissus pendant un bloom de mettre cinq mois à atteindre 250  $\mu\text{g}\cdot\text{g}^{-1}$ , soit le seuil autorisé pour décortiquer le muscle et le vendre, au lieu de huit mois pour les coquilles témoins. Cela représente donc un taux de réduction du temps de rétention de la toxine s'élevant à 30%. Si l'accélération du processus est observée, elle présente toutefois des limites. En effet, Blanco et collaborateurs expliquent que cela ne prend pas en compte les possibles recontaminations des animaux, provoquées dans le milieu naturel par une nouvelle augmentation de la toxine en surface ou au fond de la colonne d'eau. Ils notent également le fait que lors de cette expérience, il n'a pas été possible de déterminer quelle variable environnementale a contribué à cette accélération. Il peut aussi bien être question d'un effet d'un apport accru en lumière, d'une nourriture plus abondante à cette hauteur dans la colonne d'eau ou bien d'une température plus élevée. Il n'est pas non plus à exclure que ces variables agissent en synergie pour obtenir cette dépuración facilitée. Ils notent également que bien que la température ait été considérée comme un facteur permettant une dépuración accélérée des toxines, étant donné son impact sur le métabolisme des bivalves (Bayne and Newell, 1983), il existe une étude de Blanco en 2002

démontrant que la température n'avait pas d'impact clair sur la dépuraison d'acide domoïque des moules *M. edulis* (Blanco, 2002). La deuxième expérience s'est attachée à regarder la cinétique de dépuraison du muscle adducteur lorsque ce dernier est traité avec l'ajout de N-acétylcystéine (NAC). Le NAC est un antioxydant et un précurseur du glutathion (Zafarullah *et al.*, 2003) jouant un rôle dans la détoxification. Les auteurs ont donc exposé des coquilles Saint-Jacques contaminées à hauteur de  $1\mu\text{g.g}^{-1}$  dans le muscle adducteur et  $7.6\mu\text{g.g}^{-1}$  dans la glande digestive par un bloom attribué à *Pseudo-nitzschia sp.*, à des doses de 0,50 ou 250  $\text{mg.l}^{-1}$  de NAC. L'expérience a duré 15 jours et les résultats obtenus montrent une diminution par quatre du temps de dépuraison. Ces résultats, très encourageants sur la partie comestible de l'animal ont amené les professionnels de la pêche à demander une étude de cet antioxydant sur la glande digestive qui retient environ 80% de la charge totale de l'acide domoïque. Cette expérience a été menée, dans le cadre d'un stage que j'ai co-encadré et a fait l'objet d'une publication (Vanmaldergem *et al.*, 2023) ajoutée en annexe. Les résultats sur des glandes digestives de coquilles plus fortement contaminées ( $63\mu\text{g.g}^{-1}$ ) après un bloom de *P. australis* n'ont montré aucune différence significative après 6 jours de traitement à 250  $\text{mg.l}^{-1}$  de NAC renouvelé toutes les 24 h. Le NAC ne semble donc pas être une solution efficace pour accélérer la décontamination de la glande digestive des coquilles Saint-Jacques contaminées au-delà du seuil de sécurité sanitaire.

Ces travaux, bien que très pertinents pour la compréhension des mécanismes pouvant jouer un rôle dans l'accélération de la dépuraison de l'acide domoïque chez la coquille Saint-Jacques, ne semblent pas prendre en compte la faisabilité de ces méthodes pour les professionnels. En effet, l'accélération de la dépuraison doit être avant tout pensée comme une avancée permettant aux pêcheurs de vendre le fruit de leur activité. Or ces deux méthodes apparaissent difficiles à mettre en place. Exposer les centaines de coquilles pêchées et débarquées chaque jour en période de campagne coquillière à des doses élevées de NAC serait trop coûteux et demanderait une logistique et un temps trop important pour que cela soit réalisable et rentable pour les professionnels impliqués. Il en est de même pour le processus mis en avant dans l'étude de Blanco (Blanco *et al.*, 2006). Les stocks de coquilles devraient alors être déplacés dans une zone cumulant les différentes variables exposées ci-dessus (lumière, température et nourriture élevées) puisqu'il serait impossible de les garder en hauteur dans la colonne d'eau. Nous nous sommes donc demandé quelle autre piste pourrait être envisagée pour accélérer la décontamination de la coquille Saint-Jacques à décontaminer.

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## Quelles autres pistes pour accélérer le processus de dépuraction chez la coquille Saint-Jacques ?

Après avoir testé l'action du NAC, et inspirée par ce qui a été publié sur le sujet, j'ai décidé d'étudier la piste de l'utilisation de bactéries pour accélérer la décontamination de l'acide domoïque par les coquilles Saint-Jacques. En effet, l'acide domoïque semble séquestré dans la glande digestive de *P. maximus*. Or aujourd'hui l'importance que revêt le microbiote digestif et son rôle clef dans différents mécanismes est reconnu (Aron-Wisnewsky *et al.*, 2012; Clemente *et al.*, 2012). Aussi une modification importante de ce dernier, appelée dysbiose, peut entraîner des conséquences parfois graves pour l'organisme tout entier (Levy *et al.*, 2017).

Deux études ont particulièrement retenu mon attention. La première, parue en 1998 par Stewart et collaborateurs, a démontré que certains genres bactériens étaient capables de croître en présence d'acide domoïque grâce à la métabolisation de la toxine, et donc de diminuer sa concentration dans le milieu. Les auteurs ont conclu que trois genres de bactéries étaient capables d'utiliser l'acide domoïque pour croître et donc faire diminuer son taux dans le milieu. Il s'agit des genres *Moraxella* sp., *Alteromonas* sp. et *Pseudoalteromonas* sp. Un total de sept souches spécifiques a pu être identifié sans que le nom d'espèce soit divulgué. Après avoir pris contact avec les différents auteurs, leur laboratoire et l'université à laquelle ils appartenaient au moment de l'étude aucune réponse n'a pu être obtenue concernant les espèces précises de ces souches. Néanmoins, ils ont confirmé avec cette étude que les bactéries les plus efficaces pour diminuer le taux d'acide domoïque résidaient dans le tractus digestif de certains mollusques. Parmi les quatre espèces de bivalves suivantes *Modiolus modiolus*, *Placopecten magellanicus*, *Mytilus edulis* et *Mya arenaria* seuls ces deux derniers seraient des hôtes fiables pour ces bactéries. Ces deux espèces de bivalves sont également considérées comme des dépurateurs rapides de l'acide domoïque (Dusek Jennings *et al.*, 2020; Mafra *et al.*, 2010b), il apparaît donc plausible que les bivalves dépurant rapidement cette toxine partagent des espèces bactériennes dont la capacité de dégradation de l'acide domoïque permet une dépuraction accélérée. Une deuxième étude est venue renforcer l'intérêt de tester les bactéries en probiotiques chez la coquille Saint-Jacques, il s'agit de celle de Hagström *et al.*, 2007. Deux expériences ont été menées en parallèle afin de tester, d'une part, les facteurs pouvant influencer la libération de l'acide domoïque à partir d'une culture de cellules algales en décomposition et, d'autre part, la dégradation de l'acide domoïque en solution. Dans ce papier, les auteurs ont démontré que la présence de pseudo-fèces de moules (*M. edulis*), de sédiments provenant d'un site où avait eu



lieu un bloom de *Pseudo-nitzschia* sp., ainsi qu'un consortium bactérien provenant de la microalgue *Pseudo-nitzschia multiseriis*, permettaient de réduire rapidement la teneur en acide domoïque dans le milieu. Il existerait donc des genres bactériens associés aux blooms de *Pseudo-nitzschia* sp. qui utiliseraient également la toxine. Cette étude n'a pas identifié les genres bactériens présents dans les échantillons testés.

A la lecture de ces papiers, il est intéressant de se demander si les coquilles Saint-Jacques sont exemptes de ces bactéries, leur présence au sein de la glande digestive de ce bivalve peut-elle être confirmée ou invalidée ? Par ailleurs, si l'absence de ces bactéries devait se confirmer, alors la question que nous pouvons naturellement poser est la suivante : existe-t-il une possibilité de transférer ces bactéries, capables de métaboliser l'acide domoïque, d'un organisme dépurant rapidement à la coquille Saint-Jacques, un dépurateur lent ?

Identifier ces espèces bactériennes permettrait d'aborder la dépuration des coquilles Saint-Jacques sous un nouvel angle, notamment en rade de Brest où les contaminations récurrentes entraînent des conséquences majeures sur le système socio-économique développé autour de la pêche à la Coquille Saint-Jacques.

### **Cas de la contamination en acide domoïque dans la rade de Brest**

La coquille Saint-Jacques représente de grandes parts de marché et est même le premier bivalve pêché en France avec 60 035 tonnes pêchées en 2018 (Chiffres-clés des filières pêche et aquaculture en France en 2018 – FranceAgriMer). La Bretagne est une région reconnue pour son activité de pêche importante (**Fig. 3**) et la coquille Saint-Jacques est l'une des ressources exploitées puis conditionnée entière et directement par les pêcheurs. Les saisons de campagnes coquillières sont fixées chaque année par décision du Comité Régional des Pêches Maritimes et des Élevages Marins (CRPMEM) de Bretagne. Cette décision est conditionnée par les résultats des analyses effectuées par le REPHY. L'apparition des fortes contaminations à ASP en rade de Brest depuis 2004 a entraîné les premières fermetures des campagnes de pêche. L'effondrement de l'exploitation des coquilles Saint-Jacques s'est produit en 2004, 2007, 2014, 2017 (**Fig. 4**). Pour les professionnels, cela se traduit par un arrêt de leur activité et donc un manque de revenus. Le nombre de licences accordées pour cette activité est également en baisse puisque passé de 60 bateaux en 2004 à environ 32 en 2023.

## Pêche fraîche débarquée par région en 2021 <sup>(1)</sup>

Ventes

Valeur (millions d'euros) / Quantité (tonnes) <sup>(2)</sup>

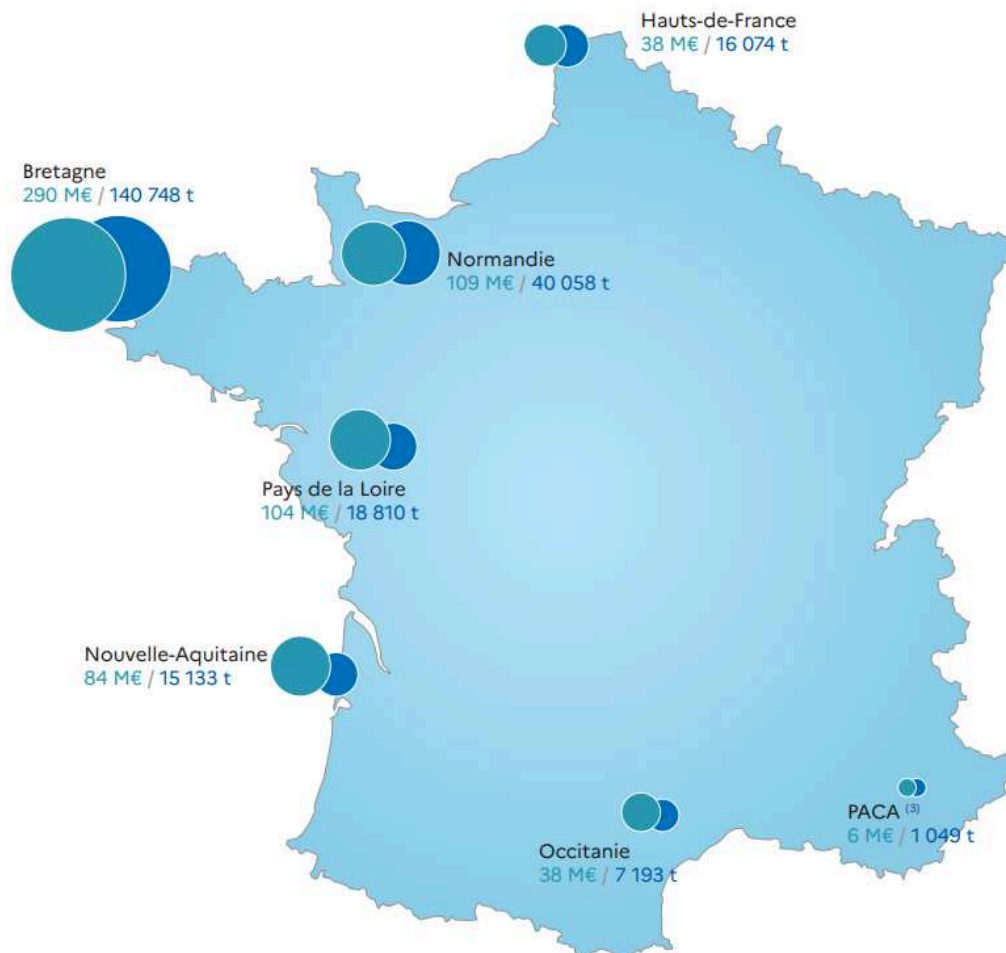


Figure 3 : Carte des ventes en tonnes (en bleu foncé) et des montants générés en millions d'euros (en bleu clair) par la pêche totale débarquée par région pour 2021 - FranceAgriMer

Bien que le suivi des contaminations des coquilles Saint-Jacques et autres bivalves par le réseau de surveillance national permette de détecter les événements toxiques avant les campagnes de pêche, permettant ainsi de mettre à l'abris les consommateurs, il existe des divergences entre la présence de l'acide domoïque et celle des cellules de *Pseudo-nitzschia* sp. En effet, il arrive que des efflorescences de microalgues soient localisées en surface sans que cela n'induisse de forte contamination dans l'eau de surface, traduisant d'un bloom d'espèce non ou peu toxique. A l'inverse, il se peut, comme évoqué précédemment dans cette introduction, que les bivalves comme les coquilles Saint-Jacques présentent une recontamination par l'acide domoïque de

leurs tissus sans qu'un bloom n'ait été détecté en surface (Rowland-Pilgrim *et al.*, 2019). Comment alors, expliquer cette contamination des bivalves au fond de la colonne d'eau ? Quelle est la relation entre l'animal et la contamination dans le milieu naturel mais également quelles sont les sources de cette dernière ? A ce jour, aucune étude n'a été menée sur l'ensemble de ces données pour répondre à ces questions qui permettraient de mieux comprendre le phénomène de contamination de la coquille Saint-Jacques.

Le suivi de la présence d'espèces de microalgues problématiques telle que *Pseudo-nitzschia* est déjà en place en France et de nombreuses informations sont connues.

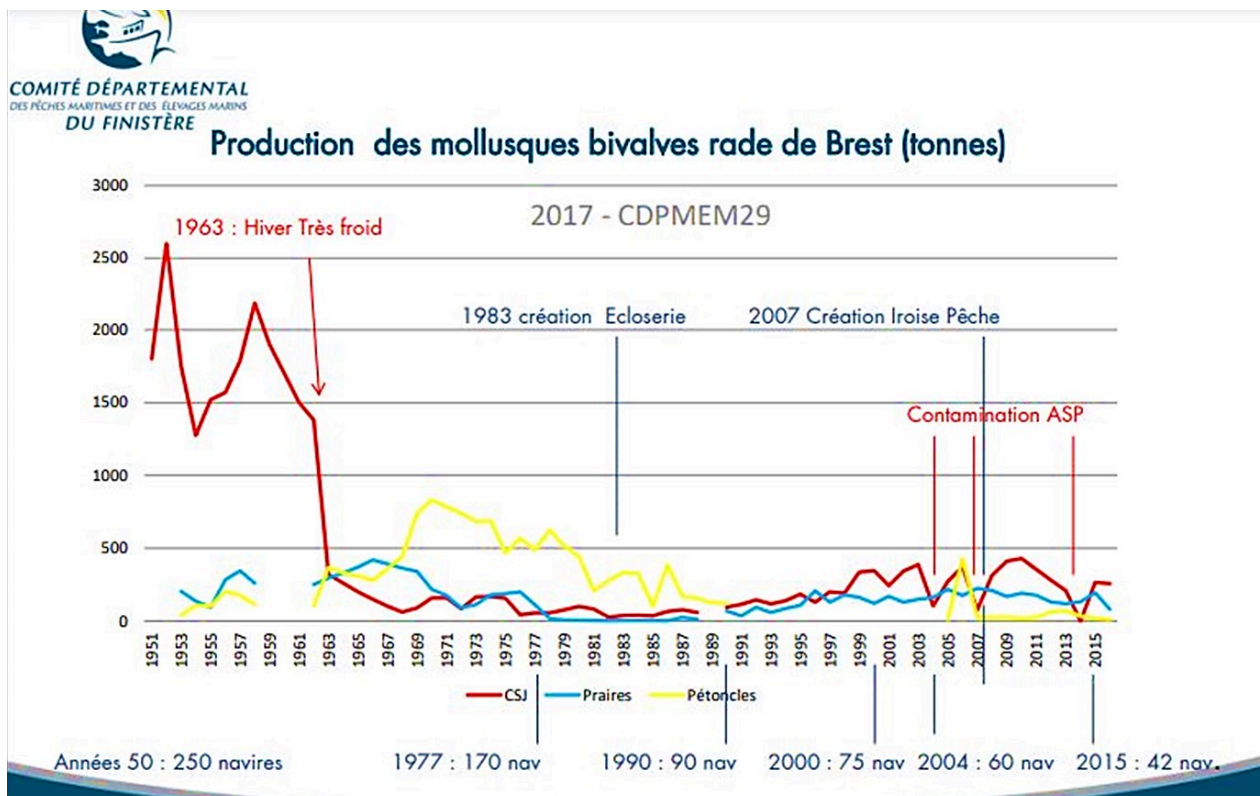


Figure 4 : Tonnage pêché des mollusques bivalves (coquille Saint-Jacques en rouge ; praire en bleu ; pétoncles en jaune) de 1951 à 2015. Données du Comité départemental des pêches maritimes et des élevages marins (<https://www.comitedespeches-finistere.fr/>) du Finistère présentée à l'atelier Zabri par Solenne Le Guennec le 9 mars 2017.

Sur la façade Atlantique du pays par exemple, le genre *Pseudo-nitzschia* est généralement observé de mars à novembre (Husson *et al.*, 2016). Dans la rade de Brest plus spécifiquement, les blooms sont généralement observés en mai et parfois en septembre et les concentrations en cellules restent élevées de mai à juin (Husson *et al.*, 2016) (**Fig. 5**). Entre 2004 et 2009, ce sont *P. multiseriata* et *P. australis* qui seraient à l'origine des blooms toxiques conduisant aux fermetures. Dernièrement, en avril 2021, un bloom de *P. australis* a été observé en rade de Brest atteignant  $1.1 \times 10^5$  cell.L<sup>-1</sup> d'après le relevé du REPHY à la date du 30 mars 2021 (<https://envlit-alerte.ifremer.fr/>). Ceci s'est traduit par un épisode de contamination des coquilles Saint-Jacques par l'acide domoïque et par conséquent la fermeture de la pêche pendant 8 mois. Le tonnage pour cette année est d'ailleurs tombé à moins de 20 000 tonnes captées. En rade de Brest, les efflorescences de *Pseudo-nitzschia* sp. les plus problématiques et entraînant des fermetures de pêches les plus longues semblent dues à l'espèce *P. australis* (Belin *et al.*, 2021; Husson *et al.*, 2016).

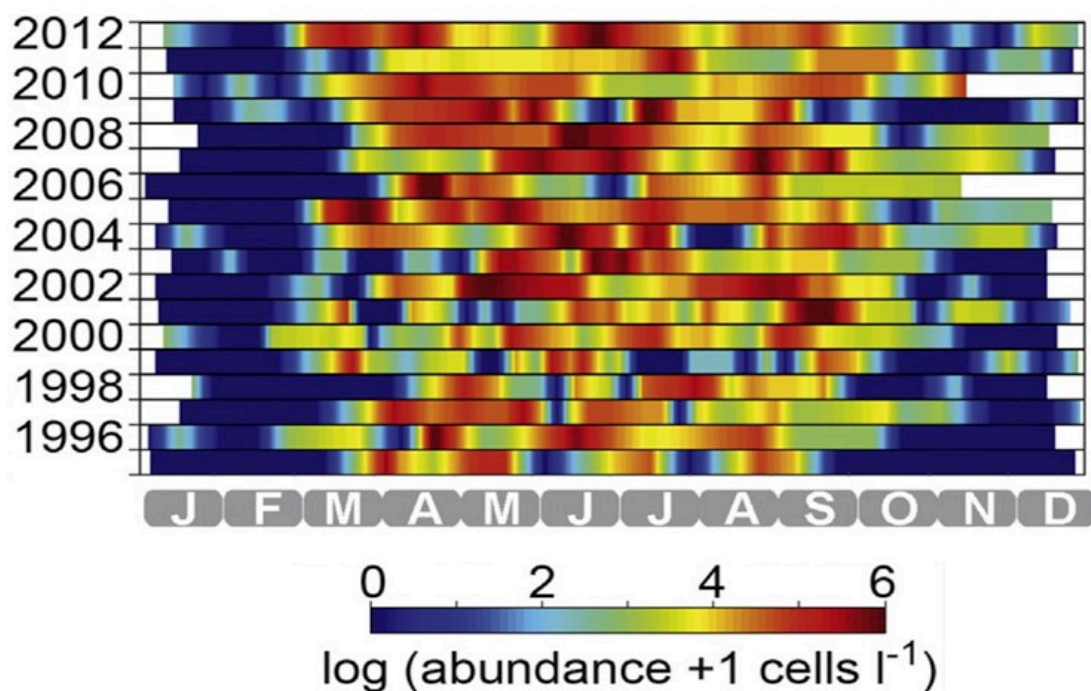


Figure 5 : Abondance de *Pseudo-nitzschia* spp en Rade de Brest de 1995 à 2012. Adaptée de Husson, 2016 (Husson *et al.*, 2016).

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## Objectifs de la thèse

Face à l'augmentation de blooms toxiques de *Pseudo-nitzschia* sp. et de la longue rétention de l'acide domoïque chez *P. maximus*, il apparaît nécessaire d'étudier et de développer des stratégies permettant d'accélérer la décontamination ou de limiter la contamination de cet animal. L'impact négatif des HABs provoqués par *Pseudo-nitzschia* sp. en rade de Brest est très préoccupant pour tout le secteur de la pêche et a ainsi conduit à la mise en place du projet « MaSCoET » (Maintien du Stock de Coquillages en lien avec la problématique des Efflorescences Toxiques). Ce projet, répondant à la demande de comités des pêches, est financé par l'association France Filière Pêche ainsi que par Brest métropole et est porté par le laboratoire DYNECO PELAGOS d'Ifremer en partenariat avec le LEMAR. Cette thèse s'intègre plus précisément dans l'action n°2 qui est de comprendre les processus de contamination et de décontamination de l'acide domoïque chez la coquille Saint-Jacques. Parmi les cinq objectifs de cette action, trois seront abordés dans ce manuscrit. En effet, (1) cette thèse s'intéresse au suivi *in situ* de la contamination et décontamination de *P. maximus* lors d'efflorescences de *Pseudo-nitzschia* sp. en rade de Brest permettant de mieux appréhender les sources de contamination possibles. Le monitoring des efflorescences toxiques a également permis d'échantillonner des coquilles Saint-Jacques naturellement contaminées afin (2) d'examiner le lien entre la contamination et la décontamination de l'acide domoïque et la taille de l'animal. Pour finir, le volet appliqué de cette thèse vise à (3) élaborer et tester un scénario d'accélération de la décontamination en conditions contrôlées à partir d'hypothèses basées sur la littérature existante. Chacun des chapitres de ce manuscrit développe un des objectifs mentionnés ci-dessus.

Après cette introduction générale permettant de faire un rappel des connaissances actuelles sur les efflorescences toxiques de *Pseudo-nitzschia* sp. et les phénomènes de contamination et décontamination chez la coquille Saint-Jacques ainsi que les problématiques engendrées par la fermeture des campagnes de pêche, ce manuscrit s'organise comme suit.

Le premier chapitre s'appuie sur un suivi temporel de sept ans sur le site de Lanvéoc en rade de Brest, à proximité du point de surveillance du réseau national REPHY. En plus de la collecte toutes les deux semaines de coquilles Saint-Jacques permettant un dosage d'acide domoïque dans leur glande digestive, un échantillonnage d'eau en surface et au fond de la colonne d'eau,

à l'interface eau sédiment, a été réalisé. Le nombre de cellules de *Pseudo-nitzschia* spp. ainsi que la quantification de l'acide domoïque total ont été dosés dans ces prélèvements.

Le deuxième chapitre de ma thèse est consacré à une expérience de deux mois au sein de l'Écloserie du Tinduff. Après un bloom toxique de *Pseudo-nitzschia australis* en début d'année 2021, nous avons pu pêcher une centaine d'animaux de deux classes de taille différentes naturellement contaminés par l'acide domoïque. Les taux de dépuraison ont été calculés pour chaque catégorie de taille lors de la période de décontamination à l'écloserie.

Le troisième chapitre abordé dans ce manuscrit porte sur l'aide à la décontamination de la coquille Saint-Jacques. Ce chapitre se concentrera sur des analyses de microbiologie et de metabarcoding, premières étapes nécessaires au développement d'un probiotique qui permettrait à la coquille Saint-Jacques d'améliorer sa capacité à dépuriser l'acide domoïque selon l'hypothèse que les bactéries jouent un rôle prépondérant dans la décontamination de cette phycotoxine. Cet axe est structuré en deux parties complémentaires et menées en parallèle :

- une première approche ciblée, afin de recenser les bactéries cultivables de la glande digestive de différents bivalves à dépuraison plus ou moins lente de l'acide domoïque. Ce volet a été réalisé à l'aide de techniques classiques de microbiologie de cultures bactériennes.

- une recherche sans *a priori*, par analyse metabarcoding sur toutes les bactéries présentes dans les glandes digestives de *P. maximus* et *M. edulis* ainsi que l'évaluation de la possibilité de modifier le microbiote de la glande digestive de la coquille Saint-Jacques par simple balnéation en présence de moules. Pour étudier ce deuxième point, une expérience de transfert de microbiote de la moule vers la coquille Saint-Jacques a été menée.

Une partie discussion générale ainsi qu'une ouverture sur les perspectives et hypothèses soulevées par ce travail viendront clôturer ce manuscrit.



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# CHAPITRE I

Lien entre la présence de *Pseudo-nitzschia* sp., de la toxine acide domoïque et de la contamination par l'acide domoïque de la coquille Saint-Jacques *P. maximus*. Suivi en rade de Brest de 2011 à 2018.

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## Préambule

Acquérir le maximum de connaissances sur les sources de contamination par l'acide domoïque chez les coquilles Saint-Jacques est d'une grande importance pour mieux comprendre les cinétiques de contamination et décontamination de *P. maximus*. Le suivi national effectué par le REPHY permet d'identifier et de suivre l'évolution des espèces de microalgues dans les eaux de surfaces permettant la sécurité des usagers de la mer ainsi que les consommateurs des produits issus de son exploitation. De tels réseaux existent également partout dans le monde, pour surveiller les régions touchées comme en Irlande (<https://www.marine.ie/>), en Écosse (<https://data.marine.gov.scot/>), en Angleterre (<https://www.afbini.gov.uk/>) et en Espagne (<http://www.intecmar.gal/lnrbm/gl/index.html>), régions fortement touchées par la contamination le long de la façade atlantique de l'Europe. Plusieurs réseaux de surveillance aux États-Unis couvrent également l'ensemble du littoral (<https://calhabmap.org/> et <https://oceanservice.noaa.gov/hazards/hab/east-coast.html>).

Toutefois, il a parfois été remarqué des discordances entre les épisodes de prolifération de *Pseudo-nitzschia* sp. et la présence d'acide domoïque dans l'eau (Smith *et al.*, 2018). Il a été rapporté que certains blooms n'entraînent pas de contamination importante, comme ce fut le cas en 2011 dans la baie de Californie où certaines valeurs en acide domoïque particulière étaient au-dessus de  $50 \mu\text{g.L}^{-1}$ , mais l'efflorescence de très courte durée n'a pas entraîné une hausse de mortalités animales (Stauffer *et al.*, 2012).

Mettre en évidence les raisons de ces discordances est important pour optimiser les détections des contaminations de la coquille Saint-Jacques en acide domoïque, notamment en rade de Brest où s'est déroulée notre étude.

C'est pourquoi depuis 2011 dans la zone de Lanvéoc, en Rade de Brest, à proximité du point de suivi national officiel (REPHY), un suivi hebdomadaire ou bimensuel est mené. Il permet de suivre la contamination des coquilles Saint-Jacques par l'acide domoïque ainsi que les concentrations, en surface et à l'interface eau-sédiment, des cellules de *Pseudo-nitzschia* sp., (au niveau de l'espèce quand cela était possible) et des concentrations en acide domoïque dissous. Ce chapitre 1 met en avant les résultats de ces échantillonnages de 2011 à 2018 et nous a permis d'observer la présence de *Pseudo-nitzschia* sp., au regard de la quantité d'acide domoïque dans les eaux de surface et de fond ainsi qu'avec la contamination des coquilles.

## Publication 1

Seven years monitoring of *Pseudo-nitzschia sp.* and domoic acid in surface and bottom waters of the bay of Brest and associated King scallop (*Pecten maximus*) contamination.

Margot Deleglise, Tania Hernandez-Farinas, Aude Leynaert, Gaspard Delebecq, Amélie Derrien, Aouregan Terre-Terillon, Beatriz Beker, Malwenn Lassudrie, Justine Castrec, Marc Long, Erwan Amice, Thierry Le Bec, Julien Thébault, Laurent Chauvaud, Caroline Fabioux, Hélène Hegaret

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**Seven years monitoring of *Pseudo-nitzschia* sp. and domoic acid in surface and bottom waters of the bay of Brest and associated King scallop (*Pecten maximus*) contamination.**

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### **Abstract**

Over the last decades, the French coasts and more particularly those along Brittany have been subjected to an increase of harmful algal blooms (HAB) of the genus *Pseudo-nitzschia* and its neurotoxin domoic acid. The king scallop (*Pecten maximus*) is among the most affected bivalves. Unlike most other bivalves, it depurates domoic acid very slowly, taking up to years in some cases. This leads to closures of fishing activities which can last sometimes for several years, thus endangering the economical equilibrium of the fishery sector.

Monitoring of *Pseudo-nitzschia* sp. in the surface water and contamination of *P. maximus* do not always correlate. Shellfish can be contaminated even though *Pseudo-nitzschia* sp. is not or not yet detected at the surface. Conversely, *Pseudo-nitzschia* sp. sampling does not always cause scallop contamination. This study aims to provide a more detailed description of the origins of the contamination of scallops.

Since 2011, a bi-monthly *in situ* survey has been deployed in the Bay of Brest (France) to assess for the presence of *Pseudo-nitzschia* sp. cells and domoic acid in surface and bottom waters, as well as a measurement of domoic acid in king scallops, thus allowing to correlate their

contamination with the presence of domoic acid and/or *Pseudo-nitzschia* sp. in surface and bottom waters.

Monitoring results pointed out that domoic acid can sometimes be detected in bottom water and in scallops even though no sign of *Pseudo-nitzschia* sp. is detected at the surface. Consequently, domoic acid contamination in scallops originates from domoic acid concentration both in surface and bottom waters, with a major contribution from domoic acid in bottom waters. These results highlight the importance of considering both surface and bottom water sampling when developing monitoring strategies of benthic bivalve species.

**Keywords:** *Pseudo-nitzschia*, domoic acid, *Pecten maximus*, water monitoring, harmful algae bloom

## 1. Introduction

The genus of diatom *Pseudo-nitzschia* sp., present in all waters of the globe (Bates *et al.*, 2019; Lelong *et al.*, 2012), is responsible for an increasing number of toxic contamination incidents (Husson *et al.*, 2016). To date, 29 species of the genus *Pseudo-nitzschia* sp. have been confirmed as producers of domoic acid (Bates *et al.*, 2018), a neurotoxin responsible for amnesic shellfish poisoning (ASP) (Trainer *et al.*, 2012). Domoic acid is a water-soluble amino acid, homologue of glutamic acid, a major excitatory neurotransmitter that enables synaptic transmissions (Doble *et al.*, 2000; Garattini *et al.*, 2000). Domoic acid can therefore disturb nervous signaling, particularly in vertebrates. Since the first fatal human poisoning reported on Prince Edward Island in Canada in 1987 (Bates *et al.*, 1989), this toxin has caused the death of many other organisms, from marine mammals (Scholin *et al.*, 2000; Bargu *et al.*, 2012) to birds (Work *et al.*, 1993). This toxin has the particularity of being easily, rapidly, and intensely bioaccumulated along the trophic chain (Bargu *et al.*, 2002; Fire *et al.*, 2010; D'Agostino *et al.*, 2017). Filter-feeding bivalves, as primary consumers, are the main vectors of domoic acid particularly for marine mammals and humans leading to ASP syndrome, as they can bioaccumulate large amounts of toxin in their tissues (Trainer *et al.*, 2012; Basti *et al.*, 2018). Domoic acid contaminations of exploited bivalve species are responsible for repeated and sometimes lengthy closures of their exploitation to avoid public health issues.

Although the king scallop (*Pecten maximus*) remains the first bivalve species fished in France, domoic acid contaminations over the past twenty years have raised concern for this economic sector. In 2018, 60,035 tons of scallops were fished in French waters (Ministry of Agriculture - Agreste- DGAMPA "GRAPH'AGRI 2019")<sup>2</sup> but dropped to only less than 40,000 tons in 2021 (Ministry of Agriculture - Agreste- DGAMPA "GRAPH'AGRI 2022")<sup>3</sup> after a large bloom of *Pseudo-nitzschia australis*. Scallops are able to accumulate large quantities of domoic acid, up to 3,000 mg DA.kg<sup>-1</sup> (James *et al.*, 2005; Blanco *et al.*, 2006), with a high degree of inter-individual variability (Bogan *et al.*, 2007; Campbell *et al.*, 2001). Unlike the majority of bivalve species, such as blue mussels for example, which depurate domoic acid very quickly (Blanco *et al.*, 2002), king scallops depurate very slowly, remaining contaminated for months or even years with toxin burdens above the international health safety threshold of 20 mg DA.kg<sup>-1</sup> (Blanco *et al.*, 2002; Blanco *et al.*, 2006).

Concentrations of *Pseudo-nitzschia* sp. and shellfish toxicity monitoring are thus essential in areas subjected to toxic *Pseudo-nitzschia* blooms. Monitoring is often supervised by a network of national reference laboratories for toxic phytoplankton and biotoxins. Such networks exist for example in Ireland (<https://www.marine.ie/>), Scotland (<https://data.marine.gov.scot/>), England (<https://www.afbini.gov.uk/>) and Spain (<http://www.intecmar.gal/lnr/bm/gl/index.html>), the regions affected by contamination along Europe's Atlantic margin. Several monitoring networks in the USA also cover the entire coastline (<https://calhabmap.org/> and <https://oceanservice.noaa.gov/hazards/hab/east-coast.html>). In France, this task was entrusted to the dedicated phycotoxin monitoring network managed by IFREMER (French Research Institute for Exploitation of the Sea) as part of the REPHY-REPHYTOX system (<https://bulletinrephytox.fr/accueil>) and is since February 2021, the responsibility of LABOCEA laboratory (<https://www.labocea.fr/>). For reasons of time and cost, water sampling used to identify and count phytoplankton in most monitoring is mainly carried out on surface or sub-surface water (1 m). In France, when *Pseudo-nitzschia australis* concentration in water samples exceed the alert threshold of 100 000 cells.L<sup>-1</sup>, sampling of shellfish is then performed as well to carry out toxin assays in the tissues.

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2 Agreste- DGAMPA "GRAPH'AGRI 2019 - [https://agreste.agriculture.gouv.fr/agreste-web/download/publication/publie/GraFra2019Chap7/GraFra2019\\_peche-maritime.pdf](https://agreste.agriculture.gouv.fr/agreste-web/download/publication/publie/GraFra2019Chap7/GraFra2019_peche-maritime.pdf)

3 Agreste- DGAMPA "GRAPH'AGRI 2022 - [https://agreste.agriculture.gouv.fr/agreste-web/download/publication/publie/GraFra2022Chap7/GraFra2022\\_peche-maritime.pdf](https://agreste.agriculture.gouv.fr/agreste-web/download/publication/publie/GraFra2022Chap7/GraFra2022_peche-maritime.pdf)

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Data issued from networks enable monitoring the presence of *Pseudo-nitzschia* sp. and contamination levels in scallops and other shellfish. However, observation of the monitoring data raises a number of questions. Under regular circumstances a high number of *Pseudo-nitzschia* sp. cells is a sign of future domoic acid contamination, however, there are events of *Pseudo-nitzschia* sp. proliferation that do not necessarily lead to an increase in domoic acid concentration in scallops. In June 2018, in the bay of Brest, *Pseudo-nitzschia* sp. bloomed up to 900 400 cells.L<sup>-1</sup> without increasing domoic acid levels in the scallops digestive gland. The French national surveillance network succeeded in identifying the *Pseudo-nitzschia* sp. complexes present during the bloom indicating that this bloom was due to non- or few toxic *Pseudo-nitzschia* sp. cells, from the *P. fraudulenta*, *P. delicatissima* and *P. seriata* complexes. The first two species are considered as non-toxic, while the *P. seriata* complex including *P. multiseriata* and *P. pungens* are known as less toxic.

The opposite has also sometimes been observed as well, as reported by Rowland-Pilgrim *et al.*, (2019) in early 2016 in Scottish waters, where an increase in domoic acid concentration in bivalves occurred without any increase in the concentration of *Pseudo-nitzschia* sp. cells between the surface and 10m below the surface.

Monitoring also showed that King scallops, *P. maximus*, are prone to regular episodes of recontamination that are not always linked to an increase in the biomass of toxic microalgae in the first 10m depth. These filter-feeding bivalves live on sediment at the water-sediment interface zone (Brand *et al.*, 2006). Studies have described the importance of microphytobenthos as a food source for *P. maximus* and other bivalves. The results of Marchais (2014) in an experiment with phytoplankton and microphytobenthos labelled with isotopic markers show the ingestion of microphytobenthos even in the presence of phytoplankton. The isotopic signature most frequently found in the digestive contents of *P. maximus* was that of microphytobenthos. Similar results have also been observed for other benthic feeders, such as *Argopecten irradians* (Davis and Marshall, 1961) and *Mercenaria mercenaria* (Porter *et al.*, 2013). In cases of prolonged contamination, the importance of the resuspension of organic matter particles from sediment into the water column at the water-sediment interface, and the role played by bivalves in this process has been studied. According to Grant *et al.* (1997), the giant scallop *Placopecten magellanicus* is capable of re-suspending particles in the water column that can remain in the environment for hours. Particles >5 µm are of manageable size for these filter feeders. This study also stated that scallops absorbed this re-suspended organic matter from the sediment with an efficiency rate of 40%. Considering the natural habitat of

these bivalves, it is important to question the origin of the domoic acid responsible for their contamination, when no increase in *Pseudo-nitzschia* sp. cells were detected at the surface.

The aim of this study was thus to identify if the long-term contamination of scallops could be partly attributable to the presence of *Pseudo-nitzschia* sp. cells or domoic acid at the surface and/or at bottom of the water column. To address this question, a 7-year monitoring of both domoic acid and *Pseudo-nitzschia* sp. cell concentrations both at the surface and at the bottom of the water column, at the water-sediment interface, as well as domoic acid concentration in King scallop digestive glands was performed in the Bay of Brest.

## **2. Materials and methods**

### **2.1 Study site**

Monitoring was carried out in the Bay of Brest (Brittany, France) at the Lanvéoc site (48°17'41.23"N - 4°27'12.63"W) (**Fig. 1**). This collection point was chosen for its proximity to a national REPHY monitoring point, but also a well-known, monitored, and instrumented location of the SOMLIT (Service d'Observation en Milieu Littoral from Centre National de la Recherche Scientifique) monitoring network.

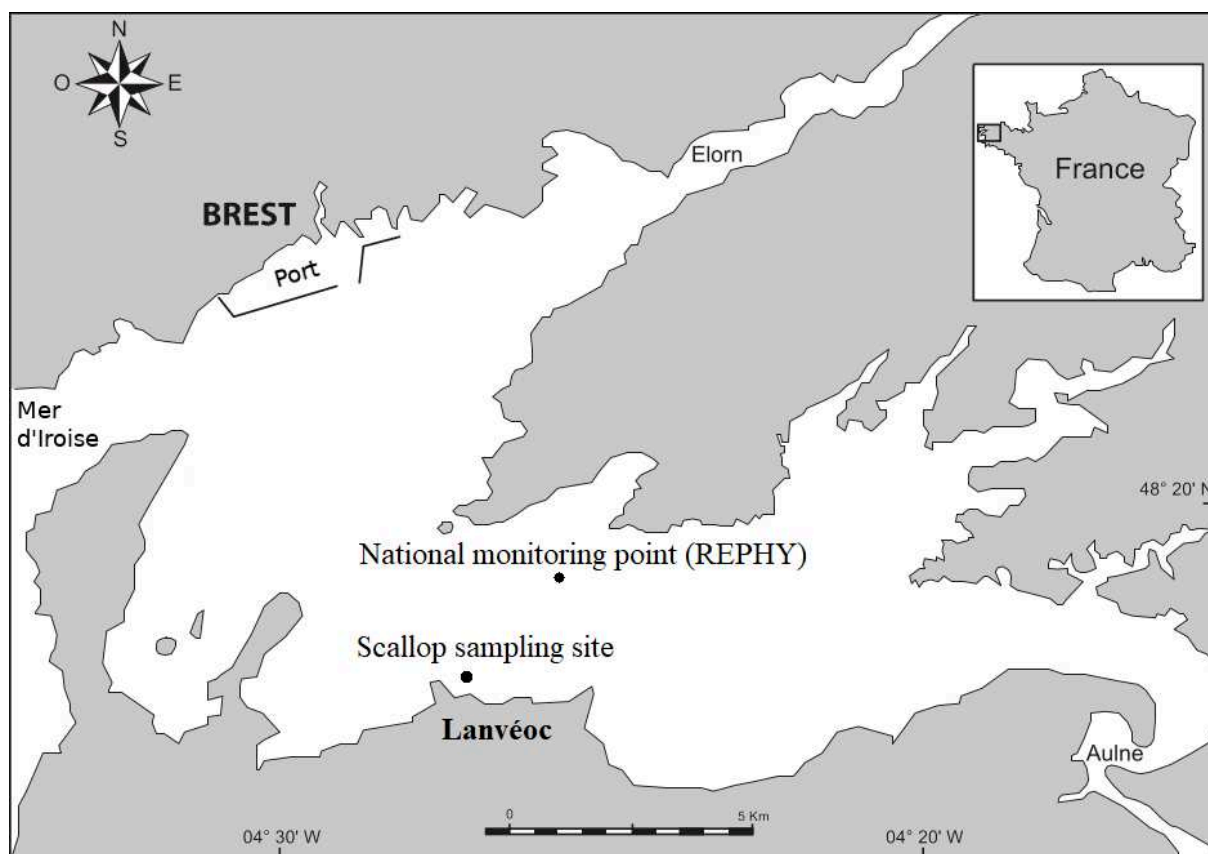


Figure 1: Map of the Bay of Brest with the location of the national monitoring site (REPHY station) and the site for scallops sampling at Lanvéoc.

## 2.2 Sample collection.

Scallops and water samples were collected every two weeks from Avril 2011 to December 2018. Water was collected at two points in the water column: first between 1 and 2 m below the surface and secondly, at the water-sediment interface hereinafter referred to as the bottom point. For each water sample, 1L of surface water was taken using a Niskin bottle and 1L of bottom water collected with a syringe by a diver.

For taxonomic identification, water samples were fixed directly after sampling on the boat by adding Lugol iodine solution (0.5% to 2% final concentration) and stored in the dark at the laboratory at  $-20^{\circ}\text{C}$  until phytoplankton identification analysis. In addition, 5 ml of water were also sampled for domoic acid analysis and directly stored in the dark at the laboratory at  $-20^{\circ}\text{C}$  prior to analysis.

Additionally, five adult scallops (9 cm +/- 2 cm) were collected by a diver on the seabed and stored at  $-20^{\circ}\text{C}$ .



### **2.3 *Pseudo-nitzschia* identification**

All water samples were carefully homogenized before settling (Utermöhl, 1958). A volume of 10 ml was used for the identification of phytoplankton phyla and placed in a sedimentation chamber to allow random distribution of phytoplankton after homogenization by gently turning the bottle 30 times. Cells larger than 20 µm were counted using a cell counter. Identification was performed using an inverted light microscope (Axio Observer. A1, Zeiss). As this study focused on the sources of bivalve contamination, only toxic microalgae of the genus *Pseudo-nitzschia* were selected for further analyses. As *Pseudo-nitzschia* sp. is difficult to identify at species level using microscopy, the specimens were separated into three different groups. The “pugens” group included the largest slivered cells, the “seriata” group for the larger asymmetrical cells and finally the “delicatissima” regrouped all thinner cells.

### **2.4 Domoic acid extraction and quantification**

#### **2.4.1 Domoic acid quantification in water samples**

Water samples previously frozen, were thawed on ice and sonicated using a Digital Sonicator - Ultrasonic processor (Misonix, Inc.) equipped with a microtip to disrupt cell membranes. Samples were kept on ice and processed 2 min alternating pulse on for 2 sec and off for 8 sec, to prevent domoic acid degradation. An amplitude of 50 Hz was applied. Each sample was then filtered through 0.2 µm cellulose acetate membrane filter (Minisart® Syringe Filter by Sartorius) and maintained at -20 °C until analysis.

Measurements of domoic acid concentration were performed using ASP ELISA kit, according to the manufacturer’s protocol (BiosenseLaboratories, Bergen, Norway). The absorbance was read using a spectrophotometer (POLARstar Omega, BMG Labtech) using a 450 nm filter.

A four-parameter logistic curve fitting model was created with the absorbance values obtained for the dilution range using the software included in the ELISA kit. The absorbance values of the samples were then plotted against the standard curve. The analyzed samples are classified according to their position relative to the standard curve as “too diluted”, “in range” and “too concentrated”. Out of range samples were run again after adjusting their dilution.

### 2.4.2 Domoic Acid quantification in digestive glands of scallops

Domoic acid was quantified only in digestive glands of scallop as this organ accumulates more than 80% of the total domoic acid load (Blanco *et al.*, 2020).

Three out of five animals sampled at each sampling time were used as replicates for this analysis. Each digestive gland dissected from frozen scallops was weighed and freeze-dried for four days, as described in McCarron *et al.*, (2007). Once dehydration was completed,  $200 \pm 5$  mg of freeze-dried tissue were rehydrated in MilliQ water according to the following formula ( $V_{H_2O} = m_{\text{final wet mass}} - m_{\text{lyophilisate weighed}}$ ). A volume of 450  $\mu\text{l}$  of MeOH/H<sub>2</sub>O (1/1; v/v) and 300 mg of 100-250  $\mu\text{m}$  diameter glass beads were added to the tube and then briefly vortexed. Samples were grinded using a Laboratory Mixer Mill MM 400 system (Retsch® Fisher Scientific, Illkirch-Graffenstaden, FR) for 3 min at 30 Hz and centrifuged 5 min at 15000 g at 4 °C. Supernatant was collected. These two steps were repeated a second time. The two supernatants obtained were pooled and the volume adjusted to 1 mL with the MeOH/H<sub>2</sub>O solution and then vortexed. A volume of 200  $\mu\text{l}$  of this crude extract was then filtered on a 0.2  $\mu\text{m}$  filter (Minisart® Syringe Filter by Sartorius) by centrifugation for 5 min at 4 °C. The resulting filtrate was transferred to an amber glass insert vial and stored at -20 °C pending HPLC analysis. Domoic acid quantification was then performed for each sample according to the HPLC method described by Corona *et al.* (2022) previously adapted by the LER-BO, IFREMER from the “EU-Hormonised Standard Operating Procedure for determination of domoic acid in shellfish and finfish by RP-HPLC using UV detection, 2008”.

## 2.5 Statistics

All data collected for the 2011-2018 period were plotted to visualize the relationship between scallop domoic acid contamination, the concentration of *Pseudo-nitzschia* in the water column (at the surface and at the bottom) and the amount of total domoic acid quantified in the water (at the surface and at the bottom). Cross-correlations (CCF) between these five variables were carried out. Cross-correlation is a simple and widely used way to evaluate the strength and direction of time-lagged relationships between two time series (*e.g.* abundance of *Pseudo-nitzschia* sp. at the bottom and the concentration of total domoic acid at the water sediment surface). This approach implies the determination of a regular temporal resolution (lag) for analysis. Two criteria were considered when choosing an “optimum” lag (*e.g.* daily, weekly, bi-

monthly, monthly): (i) avoid losing too much information by aggregating data and (ii) minimising the number of sample units with a missing value. The lag selected for optimal expression of correlations was established as two weeks, since samplings were mostly performed every 2 weeks. Furthermore, to reduce variability within the several different variables from this dataset, the square root method was used for data transformation. The significance threshold for the CCF of the time series study was set at 0.25. Finally, in this analysis, the estimation of the CCF considered the presence of missing values (NA) as described by Simpson *et al.* (2001).

### 3. Results

#### 3.1 2011-2018 monitoring data

Monitoring was performed bi-monthly from April 2011 to the end of November 2018 (**Fig 2**). Some data was missing from the dataset as the surveys tried to be as regular as possible, but due to variations in the frequency of water monitoring, it was not always possible to obtain corresponding data for phytoplankton and shellfish testing across all samples.

First, surface domoic acid measurement started only in September 2011. Secondly, no data on *Pseudo-nitzschia* sp. concentration or domoic acid in the water were recorded during 2013; only domoic acid concentrations in the scallops were recorded for this year. Finally, domoic acid concentration in water is also missing from February to April 2017 as well as from June to October 2017 and finally from November 2017 to early 2018.

Over this seven-year period, three distinct large *Pseudo-nitzschia* sp. blooms were identified in April 2014, June 2015, and April 2017 (**Fig. 2**).

In 2014, the maximum number of cells at the surface in April was reached on 3 April 2014 with 1700 cells.L<sup>-1</sup>. On the same date, *Pseudo-nitzschia* sp. cells was 54,870 cells.L<sup>-1</sup> at the bottom. The maximum concentration on the bottom reached 203,370 cells.L<sup>-1</sup> on 15 April 2014 while the maximum presence of *Pseudo-nitzschia* sp. on the surface peaked later at the end of May with 51,333 cells.L<sup>-1</sup>. In early April, a concentration of 110.64 pg.L<sup>-1</sup> of domoic acid was detected at the surface and a concentration of 565,881pg.L<sup>-1</sup> domoic acid was recorded at the bottom of the water column. The contamination found in the digestive glands of scallops rose

from 7 mg DA.kg<sup>-1</sup> at the end of February 2014 to a maximum of 33,421 mg DA.kg<sup>-1</sup> at the beginning of June 2014.

A year later, a significant increase in the presence of *Pseudo-nitzschia* sp. cells was recorded in June 2015. The number of *Pseudo-nitzschia* sp. cells at the surface reached a maximum of 213,484 cells.L<sup>-1</sup> while the domoic acid concentration in the surface water was 787.59 pg.L<sup>-1</sup>. *Pseudo-nitzschia* sp. presence at the bottom rose from 1,780 cells.L<sup>-1</sup> in mid-April to 64,448 cells.L<sup>-1</sup> in early June 2015. At the same time, domoic acid concentration rose to 725.17 pg.L<sup>-1</sup> at the bottom. Contamination in the digestive glands of the scallops did not increase significantly as would be expected following this proliferation of cells and even kept decreasing with a level of domoic acid measured at 1,462.7 mgDA.kg<sup>-1</sup>, after having reached 2,425 mgDA.kg<sup>-1</sup> in January 2015.

Lastly, in 2017 *Pseudo-nitzschia* sp. was observed from March onwards, with a peak reached in mid-April with the presence of 28,694 cells.L<sup>-1</sup> at the surface. On the same date, there was a very sharp increase in the concentration of total domoic acid in the water, with 289,809 pg.L<sup>-1</sup> at the surface. At the same time, 23,789 cells.L<sup>-1</sup> were observed at the bottom of the water column and a concentration of 10,998.5 pg.L<sup>-1</sup> of domoic acid was found at the bottom. This resulted in contamination of the digestive glands of the scallops, which rose from 498.4 mg DA.kg<sup>-1</sup> at the beginning of April to 3,177.7 mg DA.kg<sup>-1</sup> in mid-April. Maximum contamination in scallop digestive gland was observed one month later in May with 4,167.7 mg DA.kg<sup>-1</sup>. The concentration within the DG decreased slowly before rising again in 2017 consequently to the bloom decreasing until 2018, reaching 52 mg DA.kg<sup>-1</sup> on December 11, 2018. The total reopening of the fishery could be established in January 2019.

These major blooms resulting in the contamination of scallops have led to the closure for several months of the fishing activity in the Bay of Brest.

The 2014 bloom recorded concentrations of *Pseudo-nitzschia* sp. cells in the water column, ten times higher than during the 2017 bloom. The same factor applied for domoic acid concentration in the scallop's digestive gland.

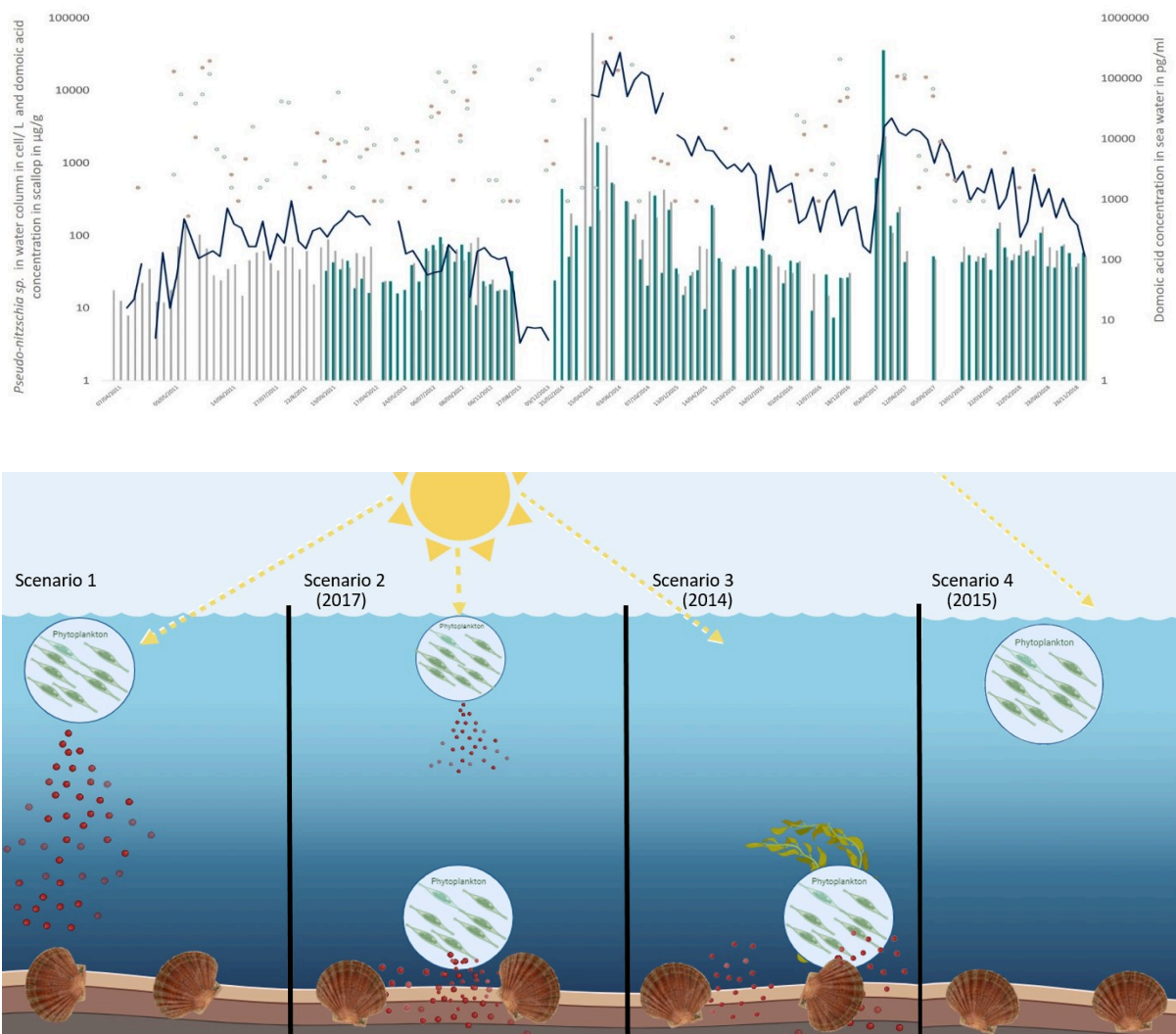


Figure 2: Global and combined representation of data from 2011 to 2018. Concentration of domoic acid in the surface water (blue bar), concentration of domoic acid in water at the bottom (grey bar), *Pseudo-nitzschia sp.* concentration in the surface water (blue dots), and *Pseudo-nitzschia sp.* concentration in the water on the seabed (brown dots), Concentration of domoic acid in the digestive gland of scallop *Pecten maximus* (blue curve, Mean of n=3). Schematic representation of all the different bloom scenarios, which may result in the presence of domoic acid and contamination of scallops *P. maximus*.

### 3.2 *Pseudo-nitzschia australis* presence

A focus was made on the determination of *Pseudo-nitzschia australis* species in water samples from the water column (Beker, personal comm.), because of its high toxicity. Results showed that the complex including *Pseudo-nitzschia australis* was present at the surface during the blooms of 2014 with a concentration ranging from 80 cells.L<sup>-1</sup> to 49,330 cells.L<sup>-1</sup> (Fig. 3). At the same time, a total cell density of *P. australis* ranged from 40 cells.L<sup>-1</sup> to 197,330 cells.L<sup>-1</sup> in the bottom water.

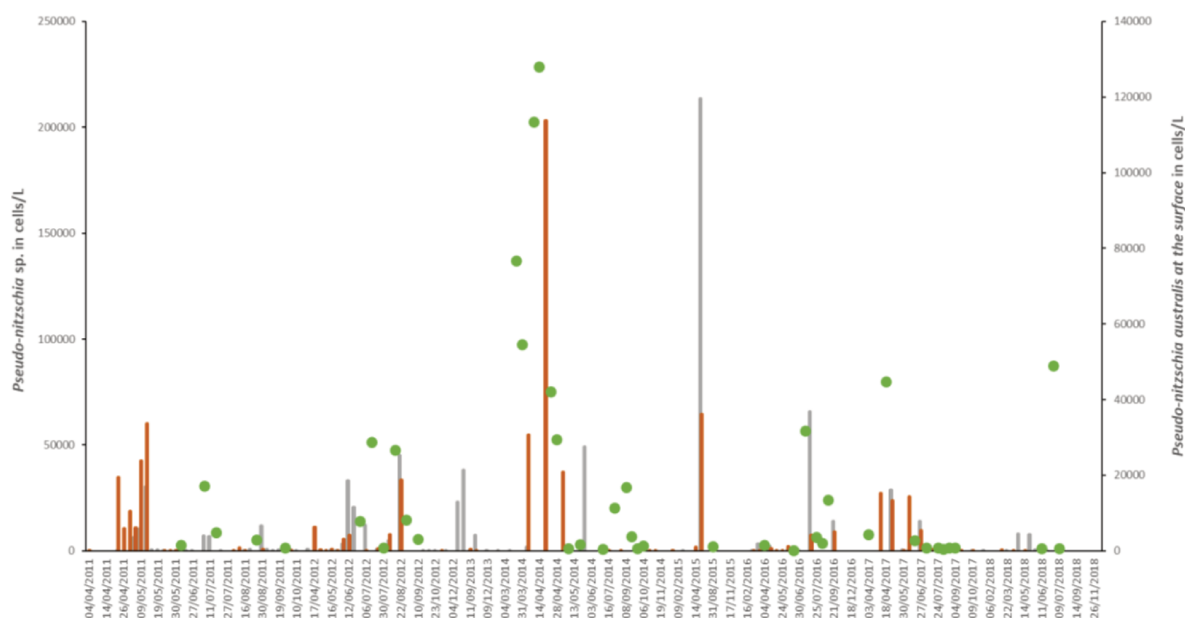


Figure 3: Total *Pseudo-nitzschia* sp. count at the surface (grey bars) and at the bottom of the water column (orange bars) during our monitoring, as well as *Pseudo-nitzschia australis* densities in cells.L<sup>-1</sup> at the surface of the water column as recorded by the REPHY national monitoring (green dots) from April 2011 to November 2018.

In 2015, *P. australis* complex was found at low levels in surface water with a single count of 40 cells.L<sup>-1</sup> in March 2015. The same pattern was observed in bottom waters with relatively low levels of 40 cells.L<sup>-1</sup> in January 2015 and 80 cells.L<sup>-1</sup> in April 2015.

In 2017, *P. australis* complex was observed at its peak at the end of June with 14,100 cells.L<sup>-1</sup> in surface water. In bottom water samples the same year, *Pseudo-nitzschia seriata* complex was recorded during several months from April to October. It reached its maximum concentration of 23,769 cells.L<sup>-1</sup> in mid-April, 2017. However, for these samples, less *P. australis* cells were identified.

These results were consistent with the *P. australis* identification reported by the national surveillance network (REPHY) in the vicinity of the laboratory sample collection point from the end of April 2011 to the beginning of July 2018.

### **3.3 Cross correlation**

To test the correlation between all variables, cross-correlation tests were performed on the dataset over the 2011-2018 period. These tests also enable highlighting any time lag between two correlated variables.

Tableau 1: Cross correlation between variables analyzed in pairs. CCF (cross-correlation factor) considers lags between two time-series. The lag was expressed fortnightly. Significativity at the threshold of 0.25 was retained. Five variables were analyzed: the concentration of *Pseudo-nitzschia* sp. at the surface (*Pseudo-nitzschia*<sub>S</sub>) and at the bottom (*Pseudo-nitzschia*<sub>B</sub>); the concentration of domoic acid at the surface (Domoic acid<sub>S</sub>) and at the bottom (Domoic acid<sub>B</sub>) and the concentration of domoic acid in the digestive gland of *P. maximus* (Domoic acid<sub>PM</sub>).

Variables	CCF (cross correlation factor)	Lag (fortnight)	Interpretation
<i>Pseudo-nitzschia</i> <sub>S</sub> vs Domoic acid <sub>S</sub>	0.26	0	Simultaneous positive correlation
<i>Pseudo-nitzschia</i> <sub>S</sub> vs <i>Pseudo-nitzschia</i> <sub>B</sub>	0.45	0	Simultaneous positive correlation
<i>Pseudo-nitzschia</i> <sub>S</sub> vs Domoic acid <sub>B</sub>	0.40	3	Positive correlation
<i>Pseudo-nitzschia</i> <sub>B</sub> vs Domoic acid <sub>S</sub>	0.53	-1	Positive correlation
	0.33	0	
Domoic acid <sub>S</sub> vs Domoic acid <sub>B</sub>	0.25	0	Positive correlation
	0.49	1	
<i>Pseudo-nitzschia</i> <sub>B</sub> vs Domoic acid <sub>B</sub>	0.66	0	Simultaneous positive correlation
<i>Pseudo-nitzschia</i> <sub>S</sub> vs Domoic acid <sub>PM</sub>	-	-	No significant correlation
Domoic acid <sub>S</sub> vs Domoic acid <sub>PM</sub>	0.26	-3	Positive correlation
	0.25	-2	
<i>Pseudo-nitzschia</i> <sub>B</sub> vs Domoic acid <sub>PM</sub>	0.37	-3	Positive correlation
	0.36	-2	
	0.33	-1	
Domoic acid <sub>B</sub> vs Domoic acid <sub>PM</sub>	0.83	-4	Positive correlation
	0.58	-3	
	0.74	-2	
	0.47	-1	



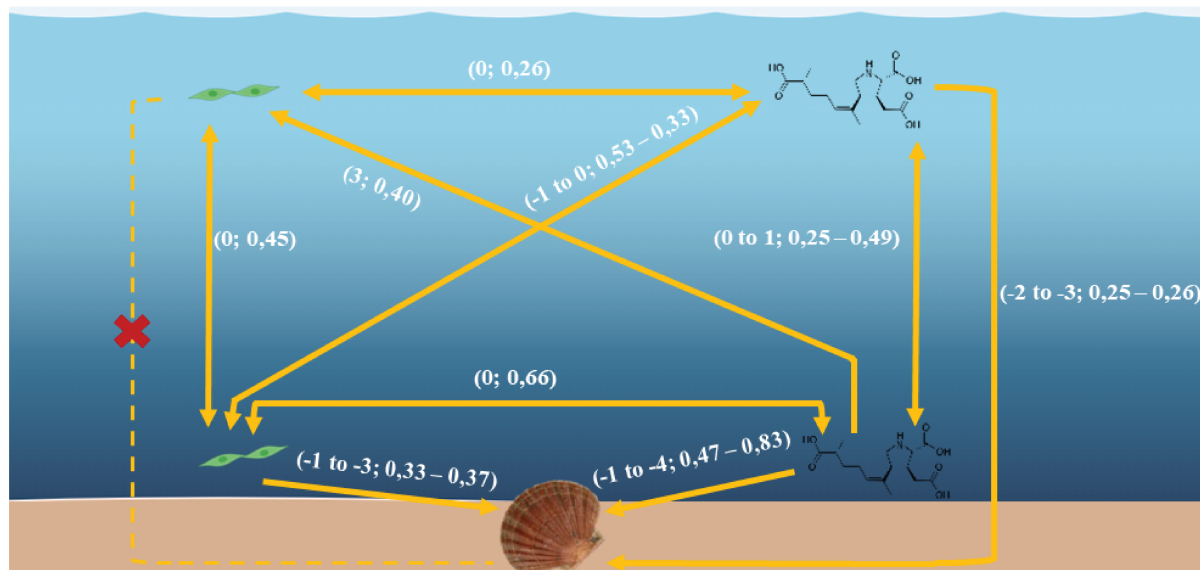


Figure 4: Diagram showing cross-correlation analyses between each variable (lag; CCF) obtained with data from Table 1.

For the seven years of monitoring, positive cross-correlations were found for almost all variables tested in pairs (**Table 1 and Fig. 4**). This implied that an increase in one variable was accompanied by an increase in the second. All cross-correlations were significant, except for the test between the presence of *Pseudo-nitzschia* sp. cells on the surface and the values of domoic acid in the scallops and were therefore considered as a lack of correlation.

The test carried out showed a lag of 0, reflecting a simultaneous increase, between the presence of *Pseudo-nitzschia* sp. cells and domoic acid on the surface (CCF of 0.26), *Pseudo-nitzschia* sp. and domoic acid on the bottom (CCF of 0.66), and the presence of *Pseudo-nitzschia* sp. on the surface and on the bottom (CCF of 0.45).

A significant correlation was observed between the presence of domoic acid in the surface and in the bottom water simultaneously (CCF of 0.25), but an even more significant CCF (0.49) was observed after a lag of 1. Thus, the increase in domoic acid values at the surface and at the bottom are likely to be observed at the same time but greater values of domoic acid at the surface are likely to be observed two weeks after the first observation of domoic acid at the bottom.

A negative lag was found for the correlations between domoic acid at the surface and domoic acid in the scallops, as well as for domoic acid at the bottom and domoic acid in the scallops, and between the presence of *Pseudo-nitzschia* sp. cells on the bottom and domoic acid in the scallops. This can be interpreted as an increase of the domoic acid values in scallops digestive gland between 1 to 3 fortnights after the increase of *Pseudo-nitzschia* sp. concentration at the bottom. The highest CCF (0.37) was for a lag of -3. As values of domoic acid at the bottom of the water column increased, the values of domoic acid in the scallops increased over a time period between 1 and 4 fortnights. In these cases, the highest correlations were observed in relation to the longest lags. In other words, domoic acid values in the digestive gland of scallops were highest after 2 months following the increase in domoic acid values at the bottom, with a CCF of 0.83.

The strongest correlations were observed between the values of domoic acid in scallop digestive glands and the presence of *Pseudo-nitzschia* sp. cells at the bottom of the water column, as well as domoic acid concentration at the bottom. Correlations between surface *Pseudo-nitzschia* sp. cell values and domoic acid values in scallops were the least robust of all variables.

#### 4. Discussion

The aim of this study was to improve our understanding of the relationship between king scallop *Pecten maximus* contamination by domoic acid and the concentration of *Pseudo-nitzschia* sp., its toxin domoic acid. The seven yearlong monitoring presented in this study illustrated different scallop contamination scenarios, highlighting the complexity of the system under study.

During this field monitoring, the hypothetical scenario in which *Pseudo-nitzschia* sp. toxic bloom was observed only at the surface, leading to contamination of the scallop digestive gland by domoic acid that may have descended into the water column, was not observed.

**The first model** is the simultaneous increase in *Pseudo-nitzschia* sp. cell density, both at the surface and the bottom, and domoic acid concentration in shellfish. This joint evolution leads to an accumulation of the toxin exceeding the sanitary safety threshold.

This pattern was observed in this study during the 2017 bloom. Indeed, the bloom of toxic *Pseudo-nitzschia* sp. cells occurred both at the bottom and the surface waters from April to June 2017. A higher cell density was observed in the bottom than in the surface waters, however the

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sharp increase in domoic acid in the water column that followed was more important at the surface. Several hypotheses could explain this pattern, the first being that the same bloom would be large enough to be present from surface to bottom waters. It could also be caused by the sedimentation of *Pseudo-nitzschia* sp. cells, which could be dead or still alive, towards the bottom of the water column, hence the transport of domoic acid from the surface to the bottom. A study by Sekula-Woods demonstrated that the rapid transport of domoic acid to bottom waters could explain high levels of domoic acid observed in benthic organisms (Sekula-Woods *et al.*, 2009). Another hypothesis that could explain this observation is that the recorded bloom was composed of at least two species of *Pseudo-nitzschia* spp., with the more toxic one present at the surface. Indeed, domoic acid concentration at the surface was 26 times higher than at the bottom. An increase in domoic acid contamination was also observed in scallop digestive gland. Their slow decontamination began but was marked by new episodes of recontamination in May 2017, associated with the presence of *Pseudo-nitzschia* sp. and domoic acid at the water-sediment interface. As a result, the fishery was closed for nearly two years at the beginning of 2017 and was only able to reopen fully for the 2019 campaigns. In addition, even if a higher number of cells identified at the bottom belonged to the “*seriata*” complex which includes *P. australis*, only a few cells were identified as this specific species. As mentioned before, cross-correlation test showed that the CCF between scallop contamination and the presence of *Pseudo-nitzschia* sp. cells and domoic acid at the surface were less important and significant than between the variables at the water-sediment interface.

**A second scenario** was highlighted in the course of this work. Blooms of *Pseudo-nitzschia* sp. did not always seem to correlate directly with domoic acid contamination of the digestive glands of bivalves sampled in the affected area. As observed during the *Pseudo-nitzschia* sp. bloom of 2015. Although the presence of *Pseudo-nitzschia* sp. was detected in greater density at the surface, this increase in algal cells did not, however, lead to a large increase in the concentration of domoic acid in the water column, nor a significant increase in the contamination of the scallops, as could have been expected. Domoic acid concentration in the digestive glands of scallops even continued to decrease during this time. The *Pseudo-nitzschia* spp. cells analyzed during this bloom under microscope in the laboratory belonged to the “*Pseudo-nitzschia pungens*” group at the surface and to the “*Pseudo-nitzschia delicatissima*” group at the bottom. These two groups are known to have low levels of toxicity (Baugh *et al.*, 2006; Trainer *et al.*, 2012), which can explain these low contamination rates.

These discrepancies between the presence of *Pseudo-nitzschia* sp. cells and scallops' contamination, when domoic acid is present were also reported by Rowland-Pilgrim *et al.* (2019) in the English and Welsh waters and by Delegrange *et al.* (2018) in the southern North Sea, who both observed that domoic acid concentration in shellfish tissue or water was not always correlated to absolute *Pseudo-nitzschia* sp. cell abundance. This was also highlighted by the absence of evident cross-correlation between the presence of *Pseudo-nitzschia* spp. at the surface and the scallop toxicity.

Results from this study follow the same pattern as already observed in other studies: not all *Pseudo-nitzschia* blooms result in the presence of domoic acid in the water. Indeed, not all *Pseudo-nitzschia* species are capable of producing domoic acid (Fehling *et al.*, 2005; Bates *et al.*, 2018; Lelong *et al.*, 2012). Furthermore, toxic *Pseudo-nitzschia* cells do not always produce toxins, and their production depends on a large number of external, but also intrinsic factors (Bates *et al.*, 2018; Thorel *et al.*, 2014; Lundholm *et al.*, 2018). Several species of *Pseudo-nitzschia* can therefore be found in the same location without always leading to domoic acid contamination. Previous studies in the literature have underlined the great difficulty in formally identifying the species of *Pseudo-nitzschia* sp. because of their morphological similarities (Bates *et al.*, 2018; Lundholm *et al.*, 2012). More precise identification of the species sampled could help determine if a bloom could lead to water and scallops contamination. Indeed, light microscopy determination requires a great knowledge of the species and is a time-consuming analysis. Formal identification is still problematic even though less expensive, and more time efficient methods are being developed. The most recent being the use of artificial intelligence deep learning models targeted for HAB detection (Guterres *et al.*, 2023). Molecular probes developed for specific species are also used with methods such as PCR (McDonald *et al.*, 2007), FISH (fluorescent in situ hybridization) (Kim and Sato, 2005; Ferrari *et al.*, 2023) or sandwich hybridization assays (SHAs) (Ayers *et al.*, 2005). These procedures are based on the genetic knowledge of each species and can help to differentiate them.

**Finally, the last contamination pattern** observed during this monitoring showed that contamination of scallop digestive glands appeared to be mainly due to a high concentration of *Pseudo-nitzschia* sp. cells and domoic acid at the bottom rather than at the surface. The data shown in this paper indicate that a *Pseudo-nitzschia* sp. bloom took place in 2014, starting in early April 2014, with a high proliferation of *Pseudo-nitzschia* sp. at the bottom of the water column. This led to a very significant increase in the quantity of domoic acid in the water at the bottom, while there was also an increase at the surface but to a much lesser extent. Heavy

contamination of the scallop digestive glands followed, well above the sanitary threshold, resulting in a closure of fishing activity. Cross-correlation revealed that following a strong increase in the content of *Pseudo-nitzschia* sp. cells and domoic acid at the water-sediment interface, the contamination of scallops increased over a period ranging from 2 weeks to 2 months, which may explain the very long contamination of scallops. Scallops could begin to accumulate the toxin as soon as the bloom and the production of domoic acid began and could increase for up to about 2 months afterwards. This was especially the case over the summer, when scallop contamination rose from 8,241 mg DA.kg<sup>-1</sup> at the beginning of July 2014 to 17,776 mg DA.kg<sup>-1</sup> in late August 2014. No sharp increase in domoic acid in the water column, at 1 m below the surface, where most monitoring occurs, nor in the presence of *Pseudo-nitzschia* sp. cells were noted over that summer. It took several months for these scallops to decontaminate. The fishery was only able to reopen at the end of January 2016, on condition that the scallops were enucleated (shucking is allowed at 4.6 mg DA.kg<sup>-1</sup> in muscle and gonads 2002/226/EC). The fishing activity of whole scallop resumed normally at the end of 2016, after domoic acid concentration reached below the sanitary threshold of 20 mg DA.kg<sup>-1</sup>. This episode of contamination in 2014 was thus mostly explained by the presence of toxic *Pseudo-nitzschia* sp. cells and domoic acid at the water-sediment interface. These results of the cross-correlation tests, showing a highly significant correlation factor between domoic acid contamination in scallops and the presence of *Pseudo-nitzschia* sp. and domoic acid at the water-sediment interface, clearly demonstrates the importance of considering the bloom of *Pseudo-nitzschia* sp. at the bottom when monitoring scallop domoic acid toxicity.

However, the question of the origin of the presence of *Pseudo-nitzschia* sp. cells and total domoic acid at the bottom of the water column, i.e. at water-sediment interface, has yet to be answered and several hypotheses should be investigated.

The most commonly accepted explanation for the presence of domoic acid at the sediment-water interface, is its transfer along the water column via mechanisms such as the formation of marine snow during bloom senescence (Schnitzer *et al.*, 2017). The presence of this toxin at the sediment-water interface could also be explained by the resuspension of particles trapped in the sediment. Punctual events, such as storms could play a significant role in the resuspension of domoic acid particles (Husson *et al.*, 2016). This phenomenon happened in February 2010 in the north-west of France, in the Pertuis Breton and in the Northern Bay of Biscay, after the 'Xynthia' storm (Husson *et al.*, 2016). This type of resuspension event was also observed during

studies on the east coast of the United States, along the Washington coast (Adams *et al.*, 2006). Other movements in the water column may be the cause of the resuspension of domoic acid. In 2006, Bogan *et al.* suggested that the long period of toxicity could be explained by pulses of toxic *Pseudo-nitzschia* sp. cells resuspended by upwelling events (Trainer *et al.*, 2009). Although the resuspension of toxic particles seems to be well explained, little is known about the possible ability of *Pseudo-nitzschia* sp. cells to retain their toxicity after a bloom and their descent into the water column.

The same lack of knowledge remains concerning the capacity of domoic acid to be preserved over time in sediment composition (Burns and Ferry, 2007). A possible phenomenon of dormancy in the sediment could also partly explain the contamination of scallops as well as the episodes of recontamination, while a toxic bloom was not detected at the surface.

Another hypothesis worth investigating would be that dead *Pseudo-nitzschia* sp. cells after a bloom, could still contain active/viable toxins as suggested by several studies (Sekula-Wood *et al.*, 2009,2011; Schnetzer *et al.*, 2017). The behavior of scallops and other bivalves could also play a role in domoic acid resuspension (Schnetzer *et al.*, 2017). It is plausible that in addition to the previously described water movements, the opening and closing of bivalve valves, especially *Pecten maximus*, could induce displacements sufficient to resuspend toxic *Pseudo-nitzschia* sp. cells that have sunk from the surface (Schnetzer *et al.*, 2017) or domoic acid that might have been adsorbed to the sediment (Burns and Ferry, 2007) as well.

According to our cross-correlation tests, the values of *Pseudo-nitzschia* sp. cells at the surface increase concomitantly with those of *Pseudo-nitzschia* sp. cells present at the bottom of the water column, and the same is also observed for the values of domoic acid at the surface and at the bottom. Densities of *Pseudo-nitzschia* sp. at the surface do not seem to correlate with contamination values in the digestive gland of scallops. The sinking of toxic *Pseudo-nitzschia* cells that could contaminate the shells therefore seems to be a hypothesis that is not supported by the data collected during this monitoring program.

However, *Pseudo-nitzschia* sp. densities and domoic acid concentrations at the bottom of the water column are both strongly correlated with scallop contamination values. The latter correlation is strongest after a lag of 2 months, which could correspond to a resuspension event of the toxin.

Furthermore, the significant cross correlation factor between the values of domoic acid at the surface and the values of domoic acid in scallop digestive gland with a lag of up to a month to a month and a half could be a proof of transfer of the toxin from the surface to the bottom. This hypothesis may be similar to scenario number 2 observed in 2017.

Another source of the presence of *Pseudo-nitzschia* sp. cells as well as domoic acid at the sediment/water interface is the possibility that blooms of toxic *Pseudo-nitzschia* sp. form directly deeper in the water column or at the sediment interface. This situation is highlighted by the 2014 results in this study when scallop contamination correlated strongly with *Pseudo-nitzschia* sp. cells and domoic acid at the bottom of the water column. As mentioned in several articles, ‘cryptic’ HABs could undoubtedly play an important role in explaining the discrepancies observed (Rines *et al.*, 2002; Mcmanus *et al.* 2007; Seegers *et al.*, 2015). These authors demonstrate that thin layers of *Pseudo-nitzschia* spp. near the bottom or even *Pseudo-nitzschia* spp. living alongside *Chaetoceros socialis* found deeper in the water column could be a source of unexplained toxicity. Those thin layers, also described by Velo-Suárez *et al.* (2008), could lead to HAB events and go undetected because they are located in discrete subsurface water. In an event of a toxic *Pseudo-nitzschia* sp. blooming, its expansion in this thin layer, could be a source for “seeding” a later surface HAB episode inducing a delay in its observation at the surface. Their involvement in contamination and recontamination episodes leading to long toxin retention in certain organisms such as *P. maximus* when coupled with water movements should not be overlooked.

### **The importance of developing bottom *Pseudo-nitzschia* sp. and domoic acid measurements in monitoring networks**

This study clearly highlights the great value of considering both subsurface and seafloor sampling when looking at shellfish contamination. Indeed, our results demonstrated some discrepancies between the detection of a *Pseudo-nitzschia* bloom at the surface and the presence of domoic acid at the water-sediment interface as well as in scallops. The absence of correlation demonstrated that assessing only *Pseudo-nitzschia* cell concentration at the surface may not be sufficient to predict scallop toxicity. These results thus demonstrate the importance of also considering the presence of *Pseudo-nitzschia* sp. cells and domoic acid at the water-sediment interface. This compartment should not be overlooked when monitoring for domoic acid contamination of bivalves or other benthic organisms.

Furthermore, Uhmau *et al.* (2018) have distinguished between dissolved (dDA) and particulate domoic acid (pDA) from *Pseudo-nitzschia* sp. and highlighted the contribution of each to the presence of domoic acid in the water column. They found that pDA corresponded to  $57.3 \pm 34.8\%$  and dDA to  $42.7 \pm 34.8\%$  to tDA pool measured in the Santa Barbara Basin, California from 2009 to 2013. Moreover, according to Novaczek *et al.* (1991) only less than 1% of dDA is incorporated into the tissues of the mussel *M. edulis* and this percentage rises to 6% for the food-borne domoic acid. It has already been shown that scallops filter and assimilate particles  $>5\mu\text{m}$  (Grant *et al.*, 1997). Therefore, particulate domoic acid filtration seems responsible for most of the bivalve contamination. On the contrary, the role of dissolved domoic acid in bivalve contamination is probably very limited, almost non-existent. Dissolved domoic acid could be, to a large extent, filtered and then excreted. Our study only investigated total domoic acid, it would however be interesting to differentiate between dissolved and particulate domoic acid and clearly identify the compartment responsible for scallop domoic acid contamination.

## 5. Conclusions

This study clearly highlights the great value of considering both subsurface and water-sediment interface waters for monitoring of scallop contamination by domoic acid.

Deeper water analysis or the use of sediment traps, when studying King scallops or benthic organisms, can shed light on the different sources of contamination. Further studies on the fate of domoic acid produced in surface waters or its persistence in the sediments, and the investigation of subsurface blooms of *Pseudo-nitzschia* sp. could help to determine the threat that toxin buildup poses to the environment. A better understanding of the species of *Pseudo-nitzschia* sp. responsible for shellfish contamination combined with the development of accurate techniques for taxonomic determination of the genus *Pseudo-nitzschia* sp. will help with the prediction of bivalve toxicity.

## 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.

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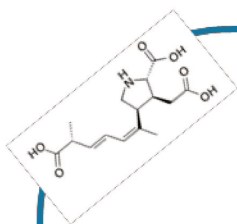
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**Take home message****A retenir**

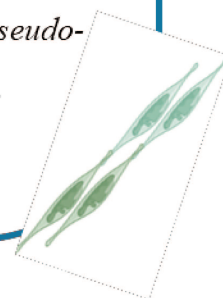
Les coquilles Saint-Jacques peuvent être contaminées selon trois scénarios différents:

1. un bloom de *Pseudo-nitzschia* sp. toxique en surface entraîne la production d'acide domoïque qui finit par contaminer la coquille Saint-Jacques.
2. la présence de *Pseudo-nitzschia* sp. toxique, à la fois à la surface et au fond, et une contamination par l'acide domoïque dans les coquilles Saint-Jacques.
3. une contamination de la coquille Saint-Jacques avec pour origine la présence de cellules de *Pseudo-nitzschia* sp. et d'acide domoïque total au fond de la colonne d'eau.

Dans le cadre d'études sur les sources de contamination des organismes par l'acide domoïque il est important de considérer la présence de la toxine et des cellules de *Pseudo-nitzschia* sp. dans les eaux de fonds au même titre que les eaux de surface.

L'espèce *Pseudo-nitzschia australis* est à l'origine des plus fortes contaminations enregistrées en rade de Brest.

Ce travail appuie la nécessité de pouvoir identifier les espèces de *Pseudo-nitzschia* sp. toxiques pour pouvoir les différencier de manière efficace.







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# CHAPITRE II

Lien entre la taille de la coquille Saint-Jacques et sa dépuración de l'acide domoïque.

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### **Préambule**

L'acide domoïque est une toxine amnésiante qui affecte de nombreux organismes, des mammifères aux oiseaux en passant par les crustacés et certains bivalves. Certaines espèces de copépodes, par exemple, modifient leur activité de broutage en sa présence. Les coquilles Saint-Jacques ne semblent pas être impactées par cette toxine. En effet, dans une étude de Liu et collaborateurs publié en 2008 ils ont démontré que des juvéniles de *P. maximus* exposés à de l'acide domoïque dissous dans une solution pendant 18 jours ne montraient aucun changement au niveau de leur taux de filtration, l'activité de leurs valves, leur réaction de redressement ou bien la sécrétion du byssus. Une étude menée par Bogan et collaborateurs (2007) a cependant mis en évidence que les coquilles Saint-Jacques de petite taille avaient un taux de contamination plus important que celles de grande taille. Une corrélation positive a également été mise en évidence entre les plus petites coquilles et une dépuration plus rapide de ces dernières.

Ce chapitre 2 porte sur l'analyse des taux de dépuration de *P. maximus* adultes de deux catégories de tailles différentes près une contamination naturelle et pendant une période de décontamination de deux mois en milieu contrôlé, à l'écloserie du Tinduff. Cette étude est la première à être menée sur une durée aussi importante.

## Publication 2

Domoic acid decontamination in adult King Scallops of two different size group: Insights from a two-month study

Margot Deléglise, Jean Vanmarldergem, Amélie Derrien, Eline Le Moan, Florian Breton, Caroline Fabioux, Hélène Hegaret & Jonathan Flye-Sainte-Marie

**Domoic acid decontamination in adult King Scallops of two different size group:  
Insights from a two-month study**

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## Abstract

Contaminations by the amnesic phycotoxin domoic acid is the cause of fishing closures worldwide. This can last several months in the case of the bivalve king scallop, *Pecten maximus*, which can accumulate high amounts of toxin and retain it in its digestive gland for months. Little is known about the response of this bivalve during domoic acid decontamination. The aim of this study was to investigate the effect of domoic acid depuration rate of two size categories of adult scallops naturally contaminated during a bloom.

The initial domoic acid burden of scallops from two different size classes ("small group" =  $8.33 \pm \text{SD } 0.66$  cm and "large scallops" =  $10.63 \pm \text{SD } 0.41$  cm) and final concentration of domoic acid in the digestive gland was analysed. Scallops from the "small group" were more contaminated during the bloom and at the start of the experiment than those from the "large group". With a concentration of  $1634 \pm 65 \text{ mg kg}^{-1}$  for the "large group" and  $2023 \pm 56 \text{ mg kg}^{-1}$  for the "small group". After 60 days of decontamination, the domoic acid burden was calculated at  $851 \pm 47 \text{ mg kg}^{-1}$  for the "large group" and  $1189 \pm 47 \text{ mg kg}^{-1}$  for the "small group".

At the end of the experiment, the scallops showed a depuration rate in the digestive gland of  $0.0018 \text{ day}^{-1}$  for large scallops and  $0.0029 \text{ day}^{-1}$  for the small scallops. These depuration rates in the digestive gland of *P. maximus* are low and in line with the slow depuration rates observed in scallops before. These results also provide additional information in line with previous studies, which show different contamination and depuration rates between scallops of different sizes.

**Keywords :** *Pecten maximus*, depuration rate, domoic acid, size

## 1. Introduction

Among the bivalves of great economic importance, the King scallop (*Pecten maximus*) is one of the most fished along the Atlantic margin of Europe with a production of 60 000 tons in 2019 (FAO Fisheries and Aquaculture Department). Scallops live partially buried at the water-sediment interface and as filter feeders, they are particularly exposed to harmful algal blooms (HABs). The king scallop *P. maximus* has been shown to contaminate rapidly and strongly during blooms of the toxic diatom *Pseudo-nitzschia* sp., producing the amnesic phycotoxin domoic acid. The contaminated scallops retain the toxin for long periods, sometimes for years

in the case of domoic acid (Blanco *et al.*, 2006). When the sanitary safety threshold fixed by EU Amendment 97/61 to Directive 91/492/EEC of  $20 \mu\text{g}\cdot\text{g}^{-1}$  of total flesh is exceeded, fisheries are closed resulting in major losses for this economic sector. Little is known about the causes of the long retention of domoic acid by *P. maximus*, and although some progress has been made in locating the toxin (Blanco *et al.*, 2002; Garcia-Corona *et al.*, 2022), 90% of which is retained in the digestive gland (Blanco *et al.*, 2006), there is a growing need for studies to better understand this mechanism of high contamination and slow depuration.

In comparison, oysters are less heavily contaminated and depurate domoic acid more quickly than king scallops. This difference could be partly explained by the fact that oysters, both juveniles and adults, can modulate their filtration, for example in the presence of *Pseudo-nitzschia multiseriata*, *Crassostrea virginica* has a lower clearance rate and increased selective rejection in pseudofeces, which contributes to reduce the ingestion of algal cells and a lower level of contamination (Mafra *et al.*, 2009; 2010). Similar results were also obtained for juvenile oysters *Crassostrea gigas* fed with *Pseudo-nitzschia australis* (Sauvey *et al.*, 2021).

Therefore, domoic acid contamination and accumulation, appears to be influenced by some variables including the size of the organisms but it appears that this mechanism is not the same for all organisms, particularly *P. maximus*.

Mafra *et al.* (2010) also studied the impact of bivalve size and observed higher contamination level of domoic acid in smaller oysters *Crassostrea virginica* explained by their higher toxin uptake rates. Body size did not seem to have the same importance in mussels' contamination, but both bivalve species showed a higher domoic acid depuration rate in smaller animals, with mussels being the species with the higher elimination rate for animals of comparable size (Mafra *et al.*, 2010). This was also highlighted by Novaczek *et al.* (1992) in mussels *M. edulis* of different size categories naturally contaminated with domoic acid. Conversely, two field studies, based on one time sampling, in the north-west of Spain and on the west coast of Scotland, showed no correlation between size and domoic acid contamination in *P. maximus* (Arévalo *et al.* 1998; Campbell *et al.* 2001). Another study with more frequent samplings, however, revealed that small scallops (7-8,5 cm) had faster contamination kinetics and depurated faster than large or commercially sized *P. maximus* scallops (10-11,5 cm) (Bogan *et al.*, 2007).

However no further investigation has been published on the physiological response of *P. maximus* during domoic acid decontamination. To investigate the role of size in *P. maximus* domoic acid contamination and depuration rate, a two months domoic acid monitoring was performed at a hatchery after a natural *Pseudo-nitzschia australis* bloom.

## 2. Materials and methods

### 2.1 Biological material

#### 2.1.1 Animals

A total of 90 wild and naturally contaminated scallops were dredged at Camaret-sur-Mer, France (48° 26' 33.1" N, 4° 35' 49.6" W 48° 18.38') on April 8th, 2021, after a *Pseudo-nitzschia australis* bloom. According to the national monitoring network (REPHY <https://envlit-alerte.ifremer.fr/>) on March 30th, 2021, *P. australis* concentration reached approximately  $1.1 \cdot 10^5$  cells.L<sup>-1</sup>, meanwhile, scallops sampled in the same area reached contamination, around 100 mg.kg<sup>-1</sup> of total scallop wet weight, thus highly above the sanitary threshold. On the day of sampling, all animals were transferred to the experimental facilities of Tinduff hatchery (Bay of Brest, France), scrubbed of all epibionts and biometric measurements of height and weight were taken for each individual.

Two sets of scallops were established based on two size categories, 45 animals for the “small group” (shell height =  $8.71 \pm$  SD 0.76 cm and total weight =  $125.52 \pm$  SD 30.11 g) and 45 for the “large group” (shell height =  $10.2 \pm$  SD 0.62 cm and total weight =  $198.27 \pm$  SD 32.77 g).

First, 15 individuals from the “small group” (shell height =  $7.49 \pm$  SD 0.81 cm, total flesh mass =  $28.56 \pm$  SD 9.13 g and total weight =  $85,966 \pm$  SD 28.48 g) and 15 from the “large group” (shell height =  $10.72 \pm$  SD 0.45 cm, total flesh mass =  $71.88 \pm$  SD 10.47 g and total weight =  $222.68 \pm$  SD 28.77 g) were sampled for initial domoic acid burden and biometry.



The remaining scallops were placed in two 800 L-fiberglass tanks filled with well-aerated sand as follow, 30 scallops from the "small group" (shell height =  $8.33 \pm \text{SD } 0.66$  cm, total weight =  $111.56 \pm \text{SD } 26.89$  g) in one and 30 scallops from the "large group" (shell height =  $10.63 \pm \text{SD } 0.41$  cm, total weight =  $221.35 \pm \text{SD } 21.38$  g) in the other. Both tanks received continuous supplies of filtered ( $0.2 \mu\text{m}$ ) seawater from the Bay of Brest and were maintained under standard hatchery conditions ( $16 \text{ }^\circ\text{C}$ , salinity = 35 ppt). All scallops were continuously fed  $10.10^9 \text{ cell.ind}^{-1}.\text{day}^{-1}$  a mix of *Tisochrysis lutea*, *Chaetoceros* sp. and *Skeletonema* sp.

Throughout the two-month monitoring period 22 animals died and a total of 38 individuals were analysed at the end of the experiment divided as follow, 22 large and 16 small scallops.

### **2.1.2 Algae cultures to feed scallops.**

Cultures of *Tisochrysis lutea*, *Chaetoceros* sp. and *Skeletonema* sp. were grown in separated 300 L-cylinders enriched with Conway medium (Walne, 1970) and with continuous light. *C. muelleri* cultures were also enriched in silicium.

## **2.2 Experimental setup and data collection**

### **2.2.1 Initial and final biometry**

Shell height (hinge to ventral margin distance, cm) was measured at the beginning and after the the 2-month experiment.

### **2.2.2 Toxin quantification**

Domoic acid burden in digestive glands of scallops were measured individually at the beginning of the experiment and at the end of the 2-month experiment for the 16 "small scallops" and the 22 "large one". Digestive glands were excised and stored at  $-20 \text{ }^\circ\text{C}$  before analysis. Domoic acid extraction was performed at the Ifremer station in Concarneau from frozen tissues following the procedure from Quilliam *et al*, (1989). Each digestive gland ( $200 \text{ mg} \pm 5 \text{ mg}$ ) dissected was placed in a volume of  $450 \mu\text{L}$  of  $\text{MeOH}/\text{H}_2\text{O}$  (1/1; v/v) and

300 mg of 100-250 µm diameter glass beads were added to the tube and then briefly vortexed. Samples were grinded using a Laboratory Mixer Mill MM 400 system (Retsch® Fisher Scientific, Illkirch-Graffenstaden, FR) for 3 min at 30 Hz/s and centrifuged 5 min at 15000 g at 4 °C. Supernatant was collected. These two steps were repeated a second time. The two supernatants obtained were pooled and the volume adjusted to 1 mL with the MeOH/H<sub>2</sub>O solution and then vortexed. A volume of 200 µL of this crude extract was then filtered on a 0.2 µm filter (Minisart® Syringe Filter by Sartorius) by centrifugation for 5 min at 4 °C. The resulting filtrate was transferred to an amber glass insert vial and stored at -20 °C pending HPLC-UV analysis. Domoic acid quantification was then performed for each sample according to the HPLC-UV method described by Corona *et al.* (2022) previously adapted by the LER-BO, IFREMER from the “*EU-Hormonised Standard Operating Procedure for determination of domoic acid in shellfish and finfish by RP-HPLC using UV detection, 2008*”.

### 2.3 Statistical analysis

The normality of the toxin burden data, collected from digestive glands of scallops of different size groups sampled at the start and the end of the experiment was assessed using the Shapiro–Wilk normality test. As the assumptions were met, samples followed a normal distribution, and a Bartlett test of homogeneity of variances revealed no homoscedasticity and was thus followed by a nonparametric Wilcoxon rank test, where shell height was fixed as a factor and level.

The rate of domoic acid depuration in the digestive gland of the scallops at the end of decontamination experiment was calculated according to Dusek Jennings *et al.* (2020) using the one-compartment exponential decay model,  $DA_t = DA_0 \cdot e^{-rt}$ , where  $DA_t$  is the domoic acid concentration after  $t$  days,  $DA_0$  represents domoic acid concentration at the end of the depuration,  $t$  is days elapsed, and the slope of the equation ( $r$ ) is the daily depuration rate.  $DA_0$  and the slope were estimated using linear regression after ln-transformation of domoic acid burdens (Álvarez *et al.*, 2020). All statistical significance were set at  $\alpha = 0.05$  using command lines in the R language (R v. 4.2.2, R Core Team, 2022). All values are expressed as mean  $\pm$  standard error (SE).

### 3. Results

#### 3.1 Toxin burden in small and large scallops' digestive glands at T0 and T60

Scallop digestive glands were initially contaminated at a mean domoic acid concentration (Mean  $\pm$  SE)  $1634 \pm 65 \text{ mg.kg}^{-1}$  for the “large group” (**Fig. 1**) and  $2023 \pm 56 \text{ mg.kg}^{-1}$  for the “small group” (**Fig. 1**). Initial domoic acid concentrations were significantly different between the small and large groups ( $p\text{-value} < 10^{-3}$ ). The concentration of domoic acid was higher in the digestive gland of scallops from the “small group” at T0.

At the end of the experiment, after 60 days of depuration, the average domoic acid burden in the digestive gland was (Mean  $\pm$  SE)  $851 \pm 47 \text{ mg.kg}^{-1}$  for the “large group” (**Fig. 1**) and  $1189 \pm 47 \text{ mg.kg}^{-1}$  for the “small group” (**Fig. 1**). Samples were significantly different between small and large groups at final time ( $p\text{-value} = 3.412 \cdot 10^{-5}$ ). The concentration of domoic acid was higher in the digestive gland of scallops from the “small group” at T0 and at T60 scallops from the “small group” still displayed a higher domoic acid concentration than scallops from the “large group”.

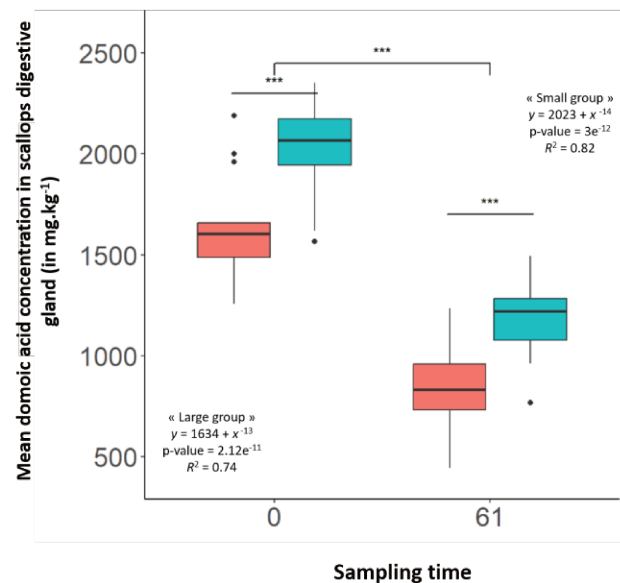


Figure 1: Domoic acid burden in the digestive gland in  $\text{mg kg}^{-1}$  of each scallop from the “large group” (in red) and the “small group” (in blue) at the initial (0 day) and final (60 days) times of the experiment. Untransformed domoic acid data are represented. Wilcoxon rank test, where shell height was fixed as a factor was used to assess the differences between each group at the initial and final time. All statistical significance were set at  $\alpha = 0.05$ . Samples were significantly different between small and large individuals at initial ( $p\text{-value} = < 10^{-3}$ ) and final time ( $p\text{-value} = 3.412 \cdot 10^{-5}$ ). The exponential daily domoic acid depuration rate is the slope of the regression line ( $R^2 \pm$  standard deviation).

### 3.2 Domoic acid burden and shell height

A significant negative correlation ( $p\text{-value} = 5.277 \cdot 10^{-5}$ ) was found between domoic acid burden and shell size at the start of the experiment (**Fig. 2A**) and between domoic acid burden and shell size at the end of the experiment after 60 days of decontamination ( $p\text{-value} = 6.95 \cdot 10^{-5}$ ) (**Fig. 2B**).

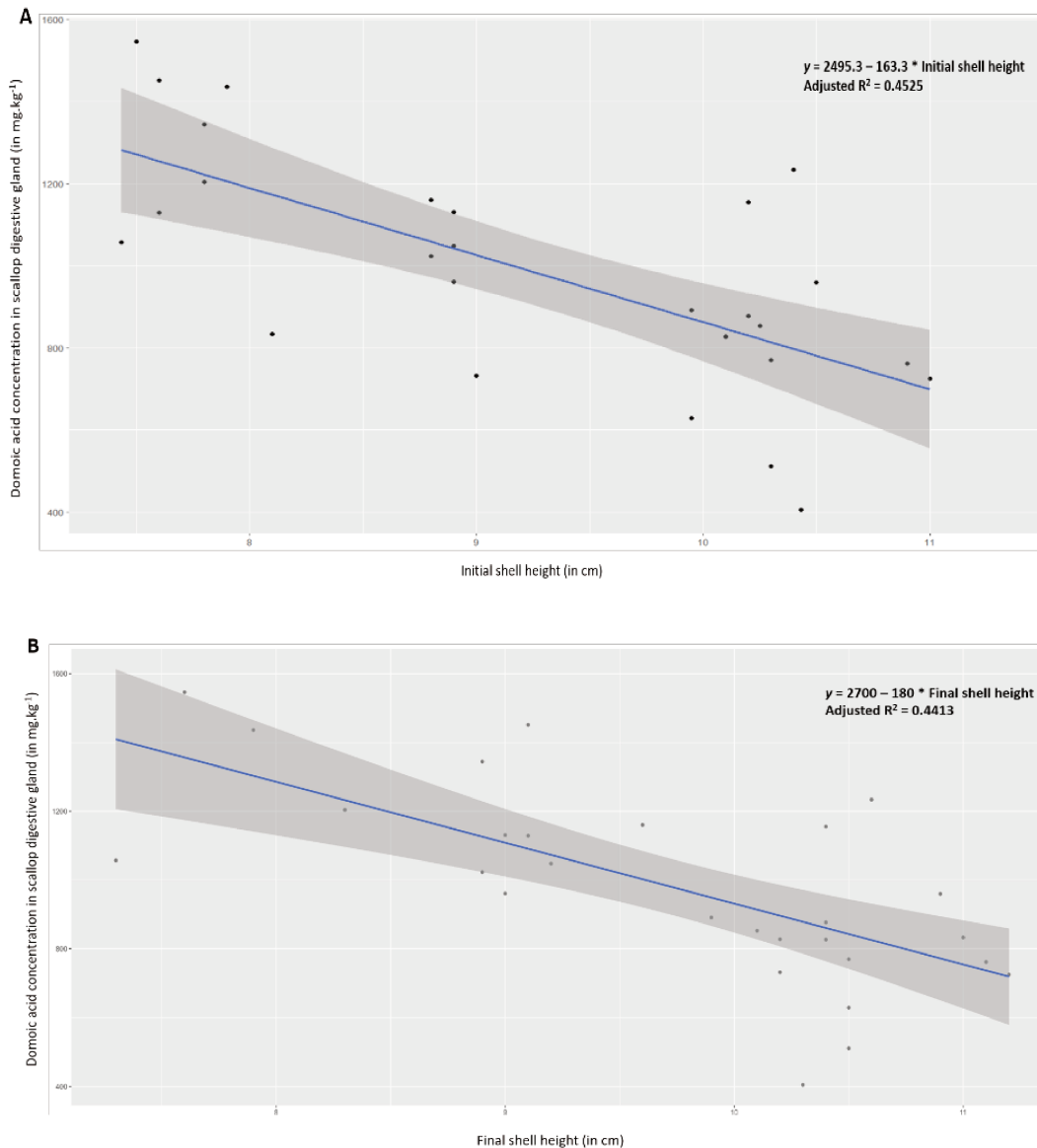


Figure 2: Significant negative correlation between domoic acid burden in the digestive gland and initial (A) or final height of scallops (B) of each scallop (grey dots) after 0 (n=60) or 60 days (n=38) of decontamination respectively. The  $F$ -test statistic revealed significant negative correlation at both times with  $p\text{-value} = 5.277 \cdot 10^{-5}$  and  $p\text{-value} = 6.95 \cdot 10^{-5}$ . The level of statistical significance was set at  $\alpha = 0.05$ .

### 3.3 Scallops depuration kinetic

Linear regressions were performed and gave a significant decrease with the following results for the “large group”  $\ln([DA]_{\text{large group}}) = -0.011 \text{ Time} + 7.39$  and the “small group”  $\ln([DA]_{\text{small group}}) = -0.008 \text{ Time} + 7.61$ . Given the average weight for scallop’s digestive gland from the “large group” and “small group”, final depuration kinetic for the digestive gland was evaluated at  $0.0018 \text{ day}^{-1}$  for large scallops and  $0.0029 \text{ day}^{-1}$  for small scallops (**Table 1**).

Table 1: Linear regression model was used to estimate the exponential daily domoic acid depuration rate. The slope of the regression line ( $R^2 \pm$  standard deviation) was reported for the “large” and “small” group. Data of domoic acid concentrations were ln-transformed and analyzed using the F-test statistic and degrees of freedom (DF) are reported. Significativity at the threshold of 0.001 (\*\*\*)

	Large group				Depuration rate in DG ( $\text{day}^{-1}$ )	Small group				Depuration rate in DG ( $\text{day}^{-1}$ )
	Coefficients					Coefficients				
	Estimate	Std. Error	t value	p-value		Estimate	Std. Error	t value	p-value	
(Intercept)	7.388348	0.060944	121.232	< 2e-16 ***		7.6066691	0.0374013	203.38	< 2e-16 ***	
Data_group\$Time	-0.011095	0.001296	-8.564	4.16e-10 ***		-0.0088288	0.0008534	-10.35	3.04e-11 ***	
Multiple R-squared	0.6769				0,0018	0.1449				0,0029
Adjusted R-squared	0.6677					0.7794				
F-statistic	73.34 on 1 and 35 DF			p-value: 4.157e-10		107 on 1 and 29 DF			p-value: 3.042e-11	

## 4. Discussion and conclusion

This study aimed to investigate the potential relationships between *P. maximus* size and domoic acid decontamination after a natural exposure to a *Pseudo-nitzschia australis* bloom.

### Domoic acid burden after 60 days of decontamination

Domoic acid burden was initially higher in smaller scallops, with a mean concentration of  $1740 \pm 171 \text{ mg.kg}^{-1}$ , compared to larger scallops with a mean of  $1695.5 \pm 350 \text{ mg.kg}^{-1}$ . Those results are consistent with the ones from Bogan *et al.*, (2007) where smaller scallops showed higher domoic acid concentrations during toxin uptake.

After 60 days of decontamination in the hatchery conditions, the average depuration rate of the digestive gland was estimated at  $0.0018 \text{ day}^{-1}$  for large scallops and  $0.0029 \text{ day}^{-1}$  for small scallops. These values are within the order of magnitude of the depuration rate of  $0.005 \mu\text{g.DA.day}^{-1}$  in the digestive gland of *P. maximus* after 60 days of depuration in the same conditions as in this study (Garcia-Corona 2023). Results are also consistent with the rate of  $0.007 \mu\text{g.DA.day}^{-1}$  and  $0.008 \mu\text{g.DA.day}^{-1}$  found during tests carried out on the digestive gland of *P. maximus* by Blanco *et al.*, (2002; 2006). Further analysis of the rest of the tissue (gills, adductor muscle, and gonad) should provide further information on the concentration of domoic acid in the total flesh and its depuration rate, which can range from 0.008 (Blanco *et al.*, 2002) to 2.9 (Liu *et al.*, 2007).

In this study, depuration rate appears to be higher for smaller scallops, which is consistent with the results obtained by Bogan *et al.*, 2007 where a positive correlation was demonstrated between a higher depuration rate and smaller scallops compared to commercial size animals. However, this correlation disappeared when only scallops greater than or equal to 100 mm shell height were analyzed.

Depuration rates for small or large scallops in this study remain well below those obtained for organisms considered as fast depurators of domoic acid, such as *Crassostrea gigas* with a depuration rate in the digestive gland of  $6.9 \mu\text{g.DA.day}^{-1}$  for animals placed at  $16 \text{ }^{\circ}\text{C}$  for 120 h in a continuous flow of filtered seawater (Jones *et al.*, 1995). Some other studies have also shown that clam and mussel species such as *Perna canaliculus*, *Mesoderma donacium* and *Ruditapes philippinarum* also have relatively fast whole tissues depuration rates of 2, 1.27 and  $0.44 \mu\text{g.DA.day}^{-1}$ , respectively (MacKenzie *et al.*, 1993; Álvarez *et al.*, 2015; Jennings *et al.*, 2020). Rapid depuration rate was also reported in *M. edulis* digestive gland with a rate of  $1.6 \mu\text{g.DA.day}^{-1}$  for mussels maintained at  $12 \text{ }^{\circ}\text{C}$  and fed with non-toxic *Isochrysis galbana* for 21 days (Mafra *et al.*, 2010). The Eastern oyster, *C. virginica*, showed a digestive gland depuration rate of  $0.88 \mu\text{g.DA.day}^{-1}$  (Mafra *et al.*, 2010). Some species of pectinids are also capable of rapidly depurating domoic acid from their digestive glands, such as *Placopecten magellanicus* and *Argopecten purpuratus*, with depuration rates of  $0.17 \mu\text{g.DA.day}^{-1}$  and  $0.9 \mu\text{g.DA.day}^{-1}$  respectively. Furthermore, *A. purpuratus*, depurates the toxin more rapidly via its transfer from the digestive gland to other organs and then into the environment (Álvarez *et al.*, 2020). This is a notable difference from *P. maximus*, where 90% of the toxic burden remains sequestered in

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the digestive gland for several weeks or even months (Blanco *et al.*, 2006) and to this day the mechanism behind this long retention period is not yet known.

### **Link between domoic acid burden and shell height**

Previous studies could not find any link between size and domoic acid depuration in scallops (Arevalo *et al.* 1998; Campbell *et al.* 2001; Bogan *et al.* 2007). Indeed, Arevalo *et al.* (1998) sampled 8-10 cm scallops (n=61 and n=72) twice and never found any significant correlation between size and domoic acid content. Campbell *et al.* (2001) were unable to demonstrate any correlation between the size (shell height > 90 cm) and the concentration of domoic acid in animals (n=170) collected on the same day at 3 different locations in Scotland. This may be due to occasional sampling (Bogan *et al.*, 2007) rather than temporal monitoring, or due to the small size range to observe a significant difference (Campbell *et al.*, 2001). Finally, Bogan *et al.* (2007) with samples (n=12) from a temporal monitoring over a year in Scotland found that smaller scallops (< 10 cm) decontaminated faster than larger scallops. This correlation disappeared when considering only individuals of commercial size  $\geq 10$ cm and this may also explain why Campbell *et al.* (2001) did not find any correlation with domoic acid burden in animals with a height higher than 9 cm. Similar results have also been described in mussels *M. edulis* contaminated with domoic acid, where smaller animals depurated faster than larger individuals (Novaczek *et al.*, 1992).

The link between domoic acid burden and shell height has been established in several previous studies, as well as in the present one, and were based on a large number of individuals analyzed and sampled regularly over the long term. A diverse size range appears to be another important variable to consider during a study.

### **Obstacles to long-term scallops monitoring**

This study provides a consistent dataset over a two-month depuration time for scallops sampled from the same location. To our knowledge, no such long follow-up had been carried out to monitor domoic acid depuration in scallops. The prolonged decontamination of domoic acid involves monitoring over several months, which is difficult to set up. It is especially

complicated due to the logistics involved in maintaining large numbers of individuals, whether in terms of space, food supplies. A high number of animals also implies harvesting them by dredging, but this fishing technique greatly weakens the animals, which can lead to numerous mortalities over a long monitoring period.

The sacrifice of animals in large numbers to obtain statistically robust results on the concentration of domoic acid in the digestive gland is also an obstacle to long-term monitoring. Although this may be facilitated by the application of biopsy, allowing to reduce the initial number of animals necessary for monitoring, this approach was tested prior to this study without giving satisfactory results.

To add complexity, the great inter-individual variability that exists in *P. maximus* poses real obstacles. It would be interesting to be able to follow the filtration and depuration rate during a contamination event, but this is limited by the fact that contaminated animals can only be obtained after a natural bloom or by contaminating them experimentally. Experimental contamination is difficult to set up due to the difficulty to cultivate toxic *Pseudo-nitzschia* sp. in large volumes as the conditions to trigger the production of domoic acid are not yet fully known (Cochlan *et al.*, 2023, Trainer *et al.*, 2012, Bates *et al.*, 2018, Lelong *et al.*, 2012).

## **5. Conclusion**

With the increase in HAB and associated contamination episodes driven by global warming (Tewari, 2022), understanding the long retention time of phycotoxins, such as domoic acid is therefore an important point that needs to be addressed.

In conclusion, this work sheds light on the complex relationship between the size of *Pecten maximus* and the depuration of domoic acid. These findings reveal that smaller scallops tend to accumulate higher initial concentrations of domoic acid, consistent with previous observations during toxin uptake. Over a 60-day period of depuration under controlled hatchery conditions, depuration rates in the digestive glands of both small and large scallops were observed. Notably, smaller scallops exhibited a slightly higher depuration rate compared to larger ones, although both rates remained relatively low in comparison with depuration rates in other bivalves. This highlights the importance of considering diverse size ranges and conducting long-term studies for a better understanding of the relationship between domoic acid and *P. maximus* to improve management strategies for this economically significant species.



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### **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.

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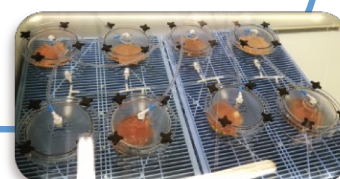
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**Take home message****A retenir**

- Aucun lien entre la charge en acide domoïque et les taux de filtration et de respiration n'a pu être mis en évidence, quelle que soit la catégorie de taille étudiée.
- Les plus petites coquilles ont un taux de dépuración de l'acide domoïque plus important ( $0.0029 \mu\text{g} \cdot \text{AD} \cdot \text{jour}^{-1}$ ) que les grosses coquilles ( $0.0018 \mu\text{g} \cdot \text{AD} \cdot \text{jour}^{-1}$ ) après 60 jours de dépuración.
- Les coquilles Saint-Jacques de petite taille ( $8.33 \pm \text{SD } 0.66 \text{ cm}$ ) ont accumulé plus d'acide domoïque au cours du bloom comparées aux coquilles de plus grande taille ( $10.63 \pm \text{SD } 0.41 \text{ cm}$ ) avec respectivement  $1740 \pm 171 \text{ mg} \cdot \text{kg}^{-1}$  et  $1695.5 \pm 350 \text{ mg} \cdot \text{kg}^{-1}$  d'acide domoïque dans la glande digestive. A la fin des deux mois de dépuración cette différence était toujours significative avec  $1189 \pm 189.3 \text{ mg} \cdot \text{kg}^{-1}$  et  $851 \pm 226.7 \text{ mg} \cdot \text{kg}^{-1}$  pour la concentration moyenne en acide domotique dans la glande digestive.



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# CHAPITRE III

Elaboration de scénarios d'aide à la dépuración de  
l'acide domoïque par la coquille Saint-Jacques

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## Préambule

Certaines études ont déjà été menées en jouant sur les facteurs tels que l'apport en lumière, en nourriture et sur l'effet de la température (Blanco *et al.*, 2006), pour modifier les paramètres physiologiques impliqués dans le processus de détoxification. L'utilisation d'un antioxydant permettant de booster la voie de détoxification a également été étudiée (Peña-Llopis *et al.*, 2014) sans résultat probant sur la glande digestive (Vanmaldergem *et al.*, 2023). Par ailleurs, ces essais représenteraient des coûts et une logistique trop importants à mettre en place pour les professionnels de la pêche. C'est pourquoi j'ai pensé à l'utilisation de bactéries directement en éclosion, sous forme de probiotiques (Ringø, 2020 ; Paillard *et al.*, 2022), afin d'accélérer le processus de dépuraison de l'acide domoïque. En effet, l'acide domoïque semble être piégé à plus de 80% dans la glande digestive de la coquille Saint-Jacques (Mauriz and Blanco, 2010) et l'hypothèse a été émise que le microbiote digestif pourrait jouer un rôle clé dans la faible contamination et/ou dépuraison rapide chez certains bivalves comme les moules *Mytilus edulis* ou la mye commune *Mya arenaria* (Stewart *et al.*, 1998). La coquille Saint-Jacques, comme d'autres dépurateurs lents, pourraient donc manquer de ces bactéries capables de dégrader l'acide domoïque et cette absence expliquerait, en partie, la longue rétention de *P. maximus*. La possibilité que *P. maximus* ne possède pas les bactéries permettant une dégradation de l'acide domoïque doit donc être étudiée.

Dans ce chapitre 3, deux approches complémentaires ont été menées en parallèle. La première pour répondre à la question de comparer quelles sont les bactéries cultivables que l'on trouve dans les dépurateurs rapides comme la moule, le pétoncle noir, ou la donace et dans la coquille Saint-Jacques, puisque seules les bactéries cultivables pourront être utilisées dans la création d'un probiotique. Il s'agit donc d'une approche ciblée. La seconde consiste à réaliser une liste complète des bactéries présentes dans la glande digestive de la moule et de la coquille Saint-Jacques afin de connaître les genres bactériens les plus abondants mais aussi de suivre l'évolution du microbiote de la glande digestive lorsque cette dernière est exposée à un apport en bactéries exogènes. Il s'agit donc d'une approche non ciblée réalisée par metabarcoding.

## **Publication 3**

Isolation and functional testing of three cultivable bacteria for domoic acid depuration.

Margot Deléglise, Hélène Hegaret, Adeline Bidault, Alix Drouart, Caroline Fabioux



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**Isolation and functional testing of three cultivable bacteria for domoic acid depuration.**

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**Abstract**

For many years the fishing activity has been subjected to the appearance of blooms of *Pseudo-nitzschia* sp. and its toxin domoic acid contaminating the trophic chain. This is particularly true along the French coast where domoic acid accumulates intensely and sometimes for several months in the digestive gland of King scallops *Pecten maximus*. This contamination leads to a significant loss of income for fishermen and this is why fishermen are looking for possibilities to accelerate the detoxification process.

The aim of this study was to isolate cultivable bacteria from the digestive glands of four bivalves species, contaminated with domoic acid following a natural bloom: three fast depurators (*Donax trunculus*, *Mymachlamys varia*, *Mytilus edulis*) and one slow depurator (*Pecten maximus*). At the end of this study, a collection of 601 isolates representing 32 different bacterial genera was created. Two bacterial strains of *Pseudoalteromonas* and *Cellulophaga* isolated from all fast-depurating bivalves were identified as potential candidates for their ability to decrease the concentration of total domoic acid in solution. A strain of the genus *Shewanella* found in the digestive gland of *P. maximus* was also chosen for testing on domoic acid in solution. The genus *Shewanella* can also have algicidal activity and could be used to mitigate blooms in addition testing bacteria directly found in the host digestive gland could limit the rejection that can be encountered when using a probiotic created from bacteria from another host.

No significant growth of the three bacterial strains seems to have been observed in 48H in a domoic acid solution. The concentration of domoic acid in solution was not significantly reduced after 48 hours in contact with the bacterial solution. The test should be performed again

by changing the concentration of bacteria as well as the exposure time in order to be able to consolidate the results obtained in this study.

These preliminary results intend to provide the first foundation for the development of probiotics to accelerate the depuration of king scallops.

**Keywords :** bacteria, domoic acid, *Pecten maximus*, depuration

## 1. Introduction

According to the Food and Agriculture Organization of the United Nations and the World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". In aquaculture, bacteria are used more and more (Ringø, 2020 ; Paillard *et al.*, 2022) for their ability to replace antibiotics in the fight against bacterial infections and for their growth and weight enhancement capability in a wide variety of species including prawns, crabs, finfish (see reviews Ringø *et al.*, 2020; Pérez-Sánchez *et al.*, 2013; Gomez-Gil *et al.*, 2000; Langlois *et al.*, 2021) and bivalve molluscs (Prado *et al.*, 2010; Paillard *et al.*, 2022; Sumon *et al.*, 2022). Strains from genera such as *Bacillus*, *Alteromonas* and *Streptomyces* are for example being used to promote growth of shrimps in shellfish aquaculture (Ringø *et al.*, 2020). Strains of *Phaeobacter inhibens* and *Bacillus pumilus* have also been identified to potentially protect *Argopecten irradians* and *Crassostrea virginica* larvae against *Vibrio spp.* challenges, as demonstrated by Sohn *et al.* (2016). Other bacteria, such as *Phaeobacter gallaeciensis*, *Alteromonas macleodii* 0444 and *Neptunomonas sp.* were identified for their activity against *Vibrio pectenicidia* in *Pecten maximus* larvae (Kesarcodi-Watson *et al.*, 2014). In the study by Kesarcodi-Watson *et al.* (2014), larval survival was elevated in all treatments and the largest larvae were observed in the control, *A. macleodii* 0444 and *P. gallaeciensis*. The beneficial effects of bacteria also extend to bioremediation, a method of pollutant degradation using living organisms. Bacteria have also been shown to reduce the concentration of phycotoxins *in vitro* but to date, the mechanism behind why and how bacteria use those toxins has not been specified. Indeed, tests carried out found that seven strains of *Pseudoalteromonas haloplanktis* isolated from the digestive tract of *Mytilus edulis* contaminated with Paralytic shellfish Toxins (PST) (Donovan *et al.*, 2009) were able to reduce by 90% the quantity of PST present in an algal extract of *Alexandrium tamarense*. Vasama *et*

*al.* (2014) found that two strains of freeze-dried powder *Lactobacillus rhamnosus* (GG, ATCC 53103 from Valio, Finland and LC-705, DSM 7061 from Ron Hull & Associates, Australia) removed up to 77% of saxitoxin and 33.3% of gonyautoxins 3 from a neutral pH 7.3 and acidic solution pH 2. These results are identical whether the bacteria are viable or not, suggesting that the mechanism for removal of the toxin is linked to binding and not to the metabolism of the bacteria (Vasama *et al.*, 2014). These strains had already been identified as capable of removing 60.3% of pure cyanobacterial peptide toxins microcystin-LR and microcystin-LF, 77.4% (Nybom *et al.*, 2008). Finally, certain lactic acid bacteria, such as *Lactocaseibacillus rhamnosus* (strain 1473) and *Paracoccus aerius* (strain 5038) isolated from various matrix (Parmigiano Reggiano cheese and seaweed, respectively) seem to be capable of degrading okadaic acid (OA) and tetrodotoxin (TTX). This effectiveness of the bacteria was also observed in a cell-free supernatant form, reducing OA by 30% and TTX by 24% (Martelli *et al.*, 2022).

In recent years, worldwide increase in toxic microalgal blooms impacted economic activity relating on bivalve harvesting (Moore *et al.*, 2019; 2020), since these filter-feeders can accumulate high amounts of toxin in their tissues, becoming toxic for humans when consumed. The genus *Pseudo-nitzschia* responsible for the production of domoic acid (Trainer *et al.*, 2012) that can be lethal to human, has been regularly observed in Europe (Husson *et al.*, 2016; Bogan *et al.*, 2007; Bresnan *et al.*, 2017). To protect consumers, the European Union has set a health threshold of 20µg of domoic acid per gram of shellfish flesh (Belin *et al.*, 2013). Above this limit, any shellfish harvest, issued from fishery or aquaculture, is banned (Campbell *et al.*, 2001; Bogan *et al.*, 2007; Husson *et al.*, 2016; Ryan *et al.*, 2017; Moore *et al.*, 2019).

Most bivalve species depurate domoic acid rapidly (Blanco *et al.*, 2002). For example, the blue mussel *M. edulis* depurates in a few hours or days high levels of domoic acid (Novaczek *et al.*, 1991,1992). Conversely, the King scallop *P. maximus* takes months or even years to depurate equivalent amounts of domoic acid (Blanco *et al.*, 2006; Lui *et al.*, 2007; Bresnan *et al.*, 2017).

Two studies proposed that bacteria from the digestive gland and feces of mussels *M. edulis* were capable of metabolizing domoic acid (Stewart *et al.*, 1998, Hagström *et al.*, 2007), such as strains of the *Moraxella* and *Alteromonas* genera (Stewart *et al.*, 1998), likely explaining the rapid depuration of contaminated mussels. Hagström *et al.* (2007) observed that the presence of mussel pseudo-faeces and bottom sediment rapidly removed most of the domoic acid toxin in solution without further identification of the bacterial strains. The authors suggested, after *in vitro* experiments, that the presence of these bacteria able to use domoic acid, could explain the

decrease of the toxin content in solution. On the contrary, they hypothesized that slow-depurating bivalves do not inhabit these bacteria. The use of these bacteria to facilitate the depuration of *P. maximus* therefore appears to be an interesting lead to investigate. Indeed, faster depuration could allow a reduction in the duration of closure of fishing activities in the event of a bloom of toxic *Pseudo-nitzschia* sp.

Considering that bacteria could be involved in the degradation of domoic acid in certain bivalves (Stewart *et al.*, 1998 ; Hagström *et al.*, 2007), and that the probiotic roles of bacteria in bivalves are now demonstrated (Sohn *et al.*, 2016), this study aimed to isolate cultivable bacteria from the digestive glands of four bivalve species, contaminated with domoic acid following a natural bloom: three fast depurators and one slow depurator. Bacteria isolated either from fast depurators or *P. maximus* were then tested for their ability to degrade domoic acid in solution. These preliminary results intend to provide the first foundation for the development of a probiotic to accelerate domoic acid depuration in slow depurators, such as king scallops.

## 2. Materials and methods

### 2.1 Sample collection

#### 2.1.1 Animals

Bivalves were sampled between the 30th of March and the 8th of April, 2021, in the northwest coast of Brittany, France, after a *Pseudo-nitzschia australis* bloom in Camaret-sur-Mer ( $1.1 \times 10^5$  cell.l<sup>-1</sup> according to REPHY monitoring network in Camaret-sur-Mer on the 30th of March <https://envlit-alerte.ifremer.fr/>). A total of 5 clams *Donax trunculus* (Linnaeus, 1758) and 5 blue mussels *Mytilus edulis* (Linnaeus, 1758) were sampled on March 30th, 2021, from the Kervel location as well as 10 variegated scallop *Mymachlamys varia* (Linnaeus, 1758) obtained from the Anse du Roz and Roscanvel locations (Brittany, France) collected on April 7th, and 8th, 2021. Finally, 10 king scallops *Pecten maximus* (Linnaeus, 1758) were dredged from Camaret (Brittany, France) on April 8th, 2021 (**Fig.1**).

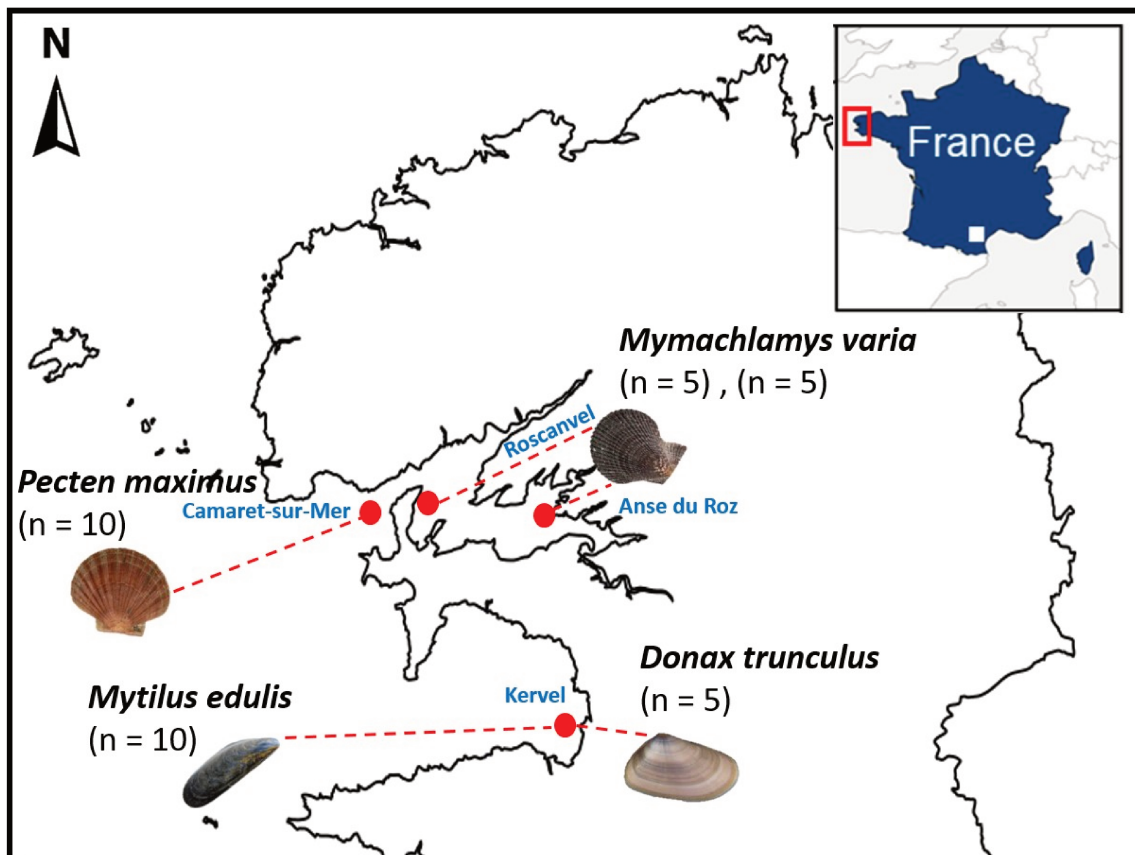


Figure 1. Sampling sites of the clams *D. trunculus* (n=5), the scallops *M. varia* (n=5) and *P. maximus* (n=10) and the mussels *M. edulis* (n=10) contaminated by domoic acid during a *Pseudo-nitzschia* bloom, in the northwest coast of Brittany, France between the 30<sup>th</sup> of March and the 8<sup>th</sup> of April 2021.

### 2.1.2 Culture of bacteria from digestive glands

For each bivalve species, digestive glands of all individuals were excised and pooled to obtain a total of 6 g of digestive tissue into a 5 mL-Eppendorf tube and grounded using an Ultra Turrax® with a microtip to obtain an homogenate for each species. An aliquot of 100  $\mu$ L of each digestive gland homogenate was diluted 1/10 with sterile seawater followed by dilution series from  $10^{-1}$  to  $10^{-4}$ . For each sample, 100  $\mu$ L of each dilution were spread on marine Reasoner's 2A agar plates (R2A), in triplicates. R2A mediums were adapted from Reasoner (1985) protocol with the addition of Sea Salts (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of  $22.5 \text{ g.L}^{-1}$  (Suzuki *et al.*, 1997). Agar plates were incubated for 7 days at 20  $^{\circ}$ C in the dark. Bacterial colonies obtained on each culture plate were analysed visually and classified according to several criteria: morphology, consistency, and colour. Colonies described as different according to these criteria were then streaked for isolation and

purification onto marine R2A plates. Isolated colonies were then cultured in 5 mL of marine R2A broth at 20 °C for 7 more days. A 500 µL aliquot of each bacterial culture was then frozen in 500 µL of 50% glycerol solution for storage at -80 °C. The remaining culture was centrifuged, and the pellet was stored at -20 °C for DNA extraction for identification.

When needed, the bacterial strain selected for exposure to dissolved domoic acid was inoculated into R2A broth at 20 °C from a glycerol stock maintained at -80 °C. Bacterial growth is assessed with a reading of the OD at 492 nm.

## **2.2. gDNA extraction from isolated bacterial colonies and 16s rRNA gene amplification**

DNA extraction from isolated bacterial colonies was performed using the Wizard® Genomic DNA purification kit (Promega Corporation, USA) according to the manufacturer instructions and DNA concentration and purity were assessed using a Nanodrop™ spectrophotometer.

The 16s rRNA gene used for bacteria identification was amplified by PCR using Universal primers 8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GTTACCTTGTTACGACTT-3') (Durand *et al.*, 2015). PCR were performed using a thermal cycler (Biosystem GeneAmp PCR System 9700) in 25 µL final reaction volume containing 1 X GoTaq Flexi buffer, 0.2 µM of each primer, 0.2 µM dNTP, 0.15 U of GoTaq polymerase enzyme (Promega, Charbonnières, France) and 1 µL of bacterial DNA.

PCR assay started by an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min 30 s, and a final extension step at 72 °C for 5 min. Each PCR reaction was run together with positive gDNA from *Vibrio tapetis* and negative controls (molecular grade water instead of DNA).

PCR products were purified with the Wizard® SV gel and PCR Clean-Up System (Promega, USA) and eluted in 30 µL of molecular grade water and stored at -20 °C.

## **2.3 16s rRNA gene Sequencing**

The 16s rRNA gene amplicons were verified by electrophoresis (1% agarose, 1 in 1X TBE) and cleaned with Wizard® Genomic DNA Purification kit (Promega, USA). Samples were then sent to GENEWIZE (Germany) for Sanger sequencing.

Sequences received were cleaned, trimmed, and aligned using Geneious software (v.2023 1.2, Biomatters, New Zealand) and analyzed with BLAST and NCBI reference database. A

sequence similarity greater than 94.5 % was considered sufficient to allocate the taxonomic identity at genus level (Yarza *et al.*, 2014).

#### **2.4 Extraction and quantification of total domoic acid from *Pseudo-nitzschia* cultures**

Two solutions were prepared from *Pseudo-nitzschia* cultures to test the capacity of selected bacteria to degrade domoic acid. The first solution (DA) containing domoic acid was obtained from a toxic *Pseudo-nitzschia australis* (strain PN-21-21) culture. The second solution (non-DA) did not contain any domoic acid and was obtained from *Pseudo-nitzschia fraudulenta* (strain PNF 20-1) culture. The cultures were provided by the UMR BOREA from the University of Caen (France). Upon arrival, *Pseudo-nitzschia australis* and *Pseudo-nitzschia fraudulenta* cultures were first sonicated using a Digital Sonicator - Ultrasonic processor (Misonix, Inc.) equipped with a microtip using an amplitude of 50 Hz. Samples were kept on ice and processed 2 min alternating pulse on for 2 sec and off for 8 sec, to prevent deteriorating domoic acid. Shreddings were then filtered through 0.2  $\mu\text{m}$  cellulose acetate membrane filter (Minisart® Syringe Filter by Sartorius), transferred into 15 mL Eppendorfs tubes and maintained at  $-20^{\circ}\text{C}$  until experiments. Measurements of domoic acid concentration of each solution obtained were performed using ASP ELISA kit, according to the manufacturer's protocol (Eurofins Abraxis, Warminster, PA). The absorbance was read using a spectrophotometer (POLARstar Omega, BMG Labtech) using a 450 nm filter. A four-parameter logistic curve fitting model was created with absorbance values obtained for the dilution of a domoic acid standard solution using the software included in the ELISA kit. The absorbance values of the samples were then plotted against the standard curve. Domoic acid stock solutions obtained from *Pseudo-nitzschia australis* and *Pseudo-nitzschia fraudulenta* respectively contained a final concentration of  $219,313 \text{ pg.mL}^{-1}$  and  $0 \text{ pg.mL}^{-1}$ .

For *in vitro* exposure of bacteria, 20 mL of solution at a concentration of  $17,545 \text{ ng.mL}^{-1}$  for toxic samples and  $0 \text{ ng.mL}^{-1}$  for non-toxic samples were obtained after dilution of the stock solution in sterile filtered ( $0.2 \mu\text{m}$ ) seawater.

## 2.5 *In vitro* test of domoic acid degradation by bacterial candidate

Candidate bacteria, identified from fast-depurating species and not found (or not part of the top genera) in scallop digestive gland, were recultured from glycerol stocks as described above.

The first two bacterial strains tested, *Pseudoalteromonas* sp. and *Cellulophaga* sp., were only found in the three fast-depurating species and not in scallops. They were thus isolated from the digestive gland of *M. edulis*.

The third candidate bacterial strain, *Shewanella* sp., was shared by *D. trunculus*, *M. varia* and *P. maximus* in this study. This genus has however been described in the microbiota of mussels (Pierce and Ward, 2019) even though it was not found in this study. This third bacterial strain was chosen to test the hypothesis that scallops possess the bacteria that metabolize domoic acid, but in a concentration too low to depurate the high quantities of domoic acid accumulated during a bloom. In fact, as the depuration of the scallop is effective at the start of its contamination, it can be assumed that these bacteria can use the domoic acid present in the environment. During a bloom, the quantity of domoic acid that the bacteria must assimilate would be too great compared with the number of bacteria capable of metabolizing this toxin. This could correspond to the plateau reached by the depuration of the scallop (Garcia-Corona *et al.*, in prep). The strain used for the test was isolated from the digestive gland of *P. maximus*.

A sample of each candidate bacterial strain was incubated in Zobell broth at 20 °C under agitation and in the dark for 24 hours. Bacterial growth of each strain was also monitored to see if bacteria multiplication was observed, using OD<sub>492 nm</sub> reading, using a Multiskan™ FC Microplate Photometer (ThermoFisher Scientific, USA). One milliliter of each bacterial cultures at a final concentration of 10<sup>6</sup> CFU mL<sup>-1</sup> (1 OD unit = 8 x 10<sup>8</sup> CFU mL<sup>-1</sup>) was distributed into each well of a 96-well plate, diluted into 1 mL of “DA” and “non-DA” solutions”, at a final concentration of 17,545 ng.mL<sup>-1</sup> for toxic solutions (from *P. australis* cultures) and 0 ng.mL<sup>-1</sup> for non-toxic samples (from *P. fraudulenta* cultures). The plate was incubated at 20 °C with agitation and in the dark. Samples taken after 24 and 48 hours of incubation and kept at -20 °C before analysis were divided as follows: 500 µL for the determination of particulate domoic acid and 500 µL which was sonicated and filtered for the determination of total domoic acid. A sample of 200 µL of each well was taken to monitor bacterial growth using a photospectrometer.



## 2.6 Domoic acid quantification

### 2.6.1 In animal samples

Right after bivalve sampling, each digestive gland was dissected and domoic acid extraction was performed as described by Quilliam *et al.* (1995) and stored at -20 °C pending analysis. Domoic acid quantification for *P. maximus*, *D. trunculus*, *M. edulis*, and *M. varia* was carried out at the Ifremer station in Concarneau on digestive gland. Samples were treated and analyzed by high performance liquid chromatography (HPLC-UV) at the Ifremer station in Concarneau as described in Garcia-Corona *et al.*, 2022.

### 2.6.2 In domoic acid solution incubated with bacteria.

Each mix present in the wells of the 96-wells plate was divided into two samples, as follows: 500 µL were filtered on 0.2 µm cellulose acetate membrane filter (Minisart® Syringe Filter by Sartorius) for dissolved domoic acid quantification and 500 µL were sonicated according to the protocol (see section 2.5.) and then filtered on a 0.2 µm filter in order to measure total domoic acid quantification. Domoic acid quantification was performed using an ELISA kit analysis following the protocol described above (see section 2.4).

## 2.7 Statistical analysis

Values of OD and domoic acid concentrations after incubation with several bacterial strains were analyzed using a Kruskal-Wallis Test where “condition” was fixed as a factor. Differences were considered statistically significant at  $\alpha = 0.05$  for all analyses. All analyses were done using R (R version 4.2.1; R Core Team 2022).

## 3. Results

### 3.1 Domoic acid concentration in bivalves.

Differences in the domoic acid accumulated in the digestive glands were found between the different shellfish species sampled after blooms of the toxic *P. australis* (**Table 1**). Higher concentrations of domoic acid were observed in the scallop *P. maximus*, followed by the ones in the digestive gland of clams *D. trunculus* and the scallops *M. varia* and the lowest were found in the blue mussels *M. edulis*.

Table 1. Domoic acid concentrations in the digestive glands of four bivalve species sampled in the northwest coast of Brittany, France between the 30<sup>th</sup> of March and the 8<sup>th</sup> of April, 2021 during a *Pseudo-nitzschia australis* bloom.

Species	Number of individuals	Sampling localisation (Brittany, France)	Domoïc acid concentration in the digestive gland (Mean +/- SE)
Clams ( <i>Donax trunculus</i> )	5	Kervel	9.02 ± 1.7 mg.kg <sup>-1</sup>
Variegated scallops ( <i>Mymachlamys varia</i> )	5	Roz	-
	5	Roscanvel	11.26 ± 2.8 mg.kg <sup>-1</sup>
Blue mussels ( <i>Mytilus edulis</i> )	10	Kervel	2.4 µg.kg <sup>-1</sup>
King scallops ( <i>Pecten maximus</i> )	10	Camaret-sur-Mer	1765,6 ± 252,5 mg.kg <sup>-1</sup>

### 3.2 Bacterial cultures

Out of the 213 sequences obtained, 155 could be identified (**Fig. 2**) the remaining 58 were not of good enough quality after sequencing to be used. Bacterial genera obtained were represented using pie charts for each bivalve species studied.

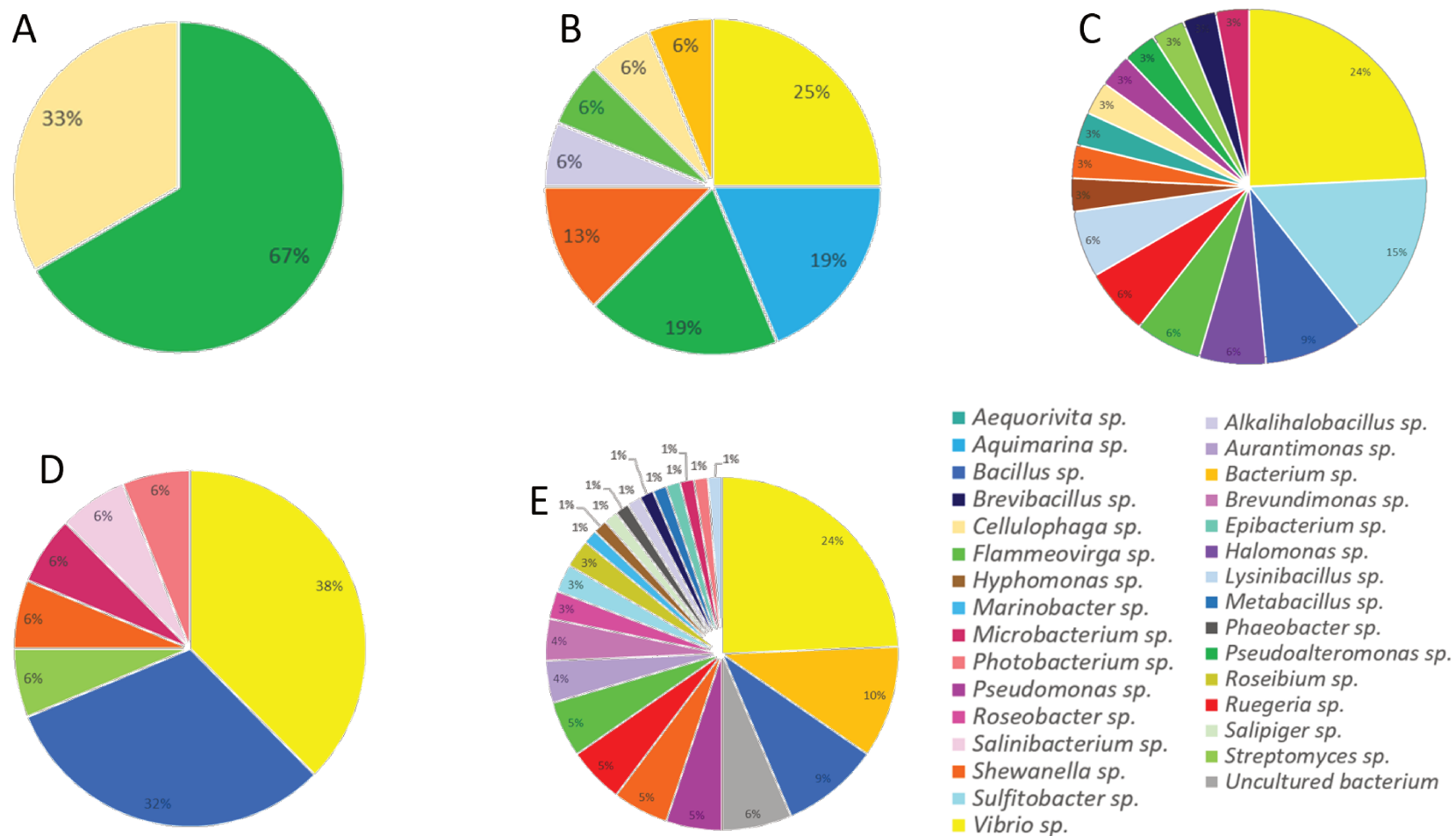


Figure 2. Repartition of cultivable bacterial genera isolated and characterized from digestive glands of four bivalve species sampled in Brittany, France, after a *Pseudo-nitzschia australis* bloom producing domoic acid. Pie charts represent percentages of bacterial genera isolated from each bivalve species: (A) blue mussels (*M. edulis*) from Kervel; (B) clams (*D. trunculus*) from Kervel; (C) variegated scallops (*M. varia*) from Roscanvel and (D) Anse du Roz and (E) King scallops (*P. maximus*) from Camaret-sur-mer.

Only two bacterial genera were found from the 16 sequences isolated from the digestive glands of *M. edulis* and identified as *Pseudoalteromonas* and *Cellulophaga* (**Fig. 2A**). The genus *Pseudoalteromonas* represented 67% of the strains identified.

A total of 8 different bacterial genera were observed out of the 23 sequences isolated from the digestive glands of clams *D. trunculus* (**Fig. 2B**). The major genera isolated were for 25% *Vibrio* sp., 19% *Aquimarina* sp., 19% *Pseudoalteromonas* sp., 13% *Shewanella* sp., 6% *Flammeovirga* sp., 6% *Alkalihalobacillus* sp., 6% *Cellulophaga* sp. and 6% *Bacterium* sp.

For variegated scallop *M. varia* sampled in Roscanvel (**Fig. 2C**), 33 usable sequences were analyzed, leading to 15 identified bacterial genera. A total of 24% were represented by the genus *Vibrio* sp., 15% by *Sulfitobacter*, 9% by *Bacillus*, 6% by *Flammeovirga*, 6% by *Ruegeria*, 6% by *Lysinibacillus* and 6% by *Halomonas*. These genera were followed by 3% of *Shewanella*, *Aequorivita*, *Cellulophaga*, *Pseudomonas*, *Pseudoalteromonas*, *Streptomyces*, *Brevibacillus*, *Microbacterium*.

For variegated scallops *M. varia* sampled in the Anse du Roz (**Fig. 2D**), 16 sequences were used to identify 7 bacterial genera. The main genera were *Vibrio* (38%) and *Bacillus* (32%), followed by *Shewanella* (6%), *Microbacterium* (6%), *Salinibacterium* (6%), *Photobacterium* (6%) and *Streptomyces* (6%).

From the 78 sequences available for the digestive glands of King scallops *P. maximus* (**Fig. 2E**), 23 bacterial genera were identified. The main genera were *Vibrio* (24%), *Bacterium* (10%), *Bacillus* (10%). Other genera isolated included *Pseudomonas* (5%), *Flammeovirga* (5%), *Shewanella* (5%), *Ruegeria* (5%), *Aurantimonas* (4%), *Brevundimonas* (4%), *Roseobacter* (3%), *Sulfitobacter* (3%) and *Roseibium* (3%). The other genera represented only 1% of the isolated bacteria.

The comparison of bacterial genera between the four bivalve species (**Fig. 3**) highlighted two genera: *Pseudoalteromonas* and *Cellulophaga*, isolated from DA-fast depurators: variegated scallops, mussels, and clams, but not from king scallops. There were no bacterial genera found in common between king scallops and blue mussels but the genera *Vibrio*, *Shewanella* and *Flammeovirga* were found in variegated scallops, clams, and King scallops. Furthermore, *D. trunculus* and *P. maximus* shared two additional bacterial genera in common: *Alkalihalobacillus* and *Bacterium*. Finally, variegated scallops and king scallops had 8 species

in common: *Sulfitobacter*, *Ruegeria*, *Pseudomonas*, *Lysinibacillus*, *Bacillus*, *Brevibacillus*, *Microbacterium* and *Photobacterium*.

Variegated scallops, clams and King scallop samples had bacterial genera that were not found in blue mussels. Variegated scallops' samples were the only ones to show the genera *Streptomyces*, *Halomonas*, *Aequorivita* and *Salinibacterium*. Clams' samples had one genus, *Aquimarina*, not shared with any other bivalve species studied here. As for king scallops, 10 genera were observed only in these samples: *Aurantimonas*, *Brevundimonas*, *Roseobacter*, *Roseibium*, *Metabacillus*, *Marinobacter*, *Hyphomonas*, *Salipiger*, *Phaeobacter* and *Epibacterium*.

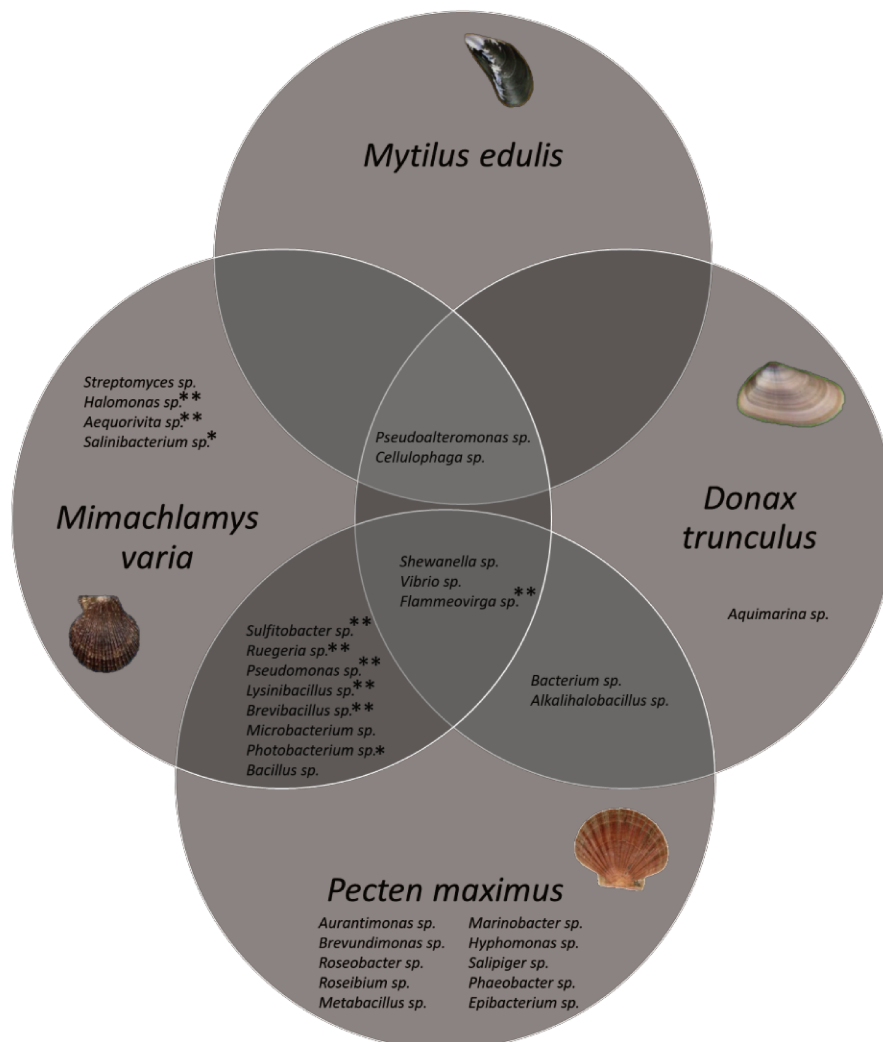


Figure 3. Venn diagram of the bacterial genus identified after digestive glands cultures on R2A agar plate for *Mytilus edulis*, *Donax trunculus*, *Pecten maximus* and *Mymachlamys varia*. Symbols distinguish bacterial genera present only in the *M. varia* sampled at Anse du Roz (\*) and only in those sampled at Roscanvel (\*\*).

### 3.3 In vitro test of domoic acid degradation by bacterial candidate

Two genera, *Pseudoalteromonas* and *Cellulophaga*., found in all three fast depurator bivalve species, and absent from king scallops, were chosen to be tested on solutions prepared from toxic (DA) or non-toxic (No-DA) *Pseudo-nitzschia* cultures to assess their potential to degrade or metabolize domoic acid. The *Pseudoalteromonas* sp. and *Cellulophaga* sp. strains used for *in vitro* degradation tests were obtained from *M. edulis* digestive gland samples. In addition, a strain of the genus *Shewanella* sp., isolated from the digestive gland of the King scallop, was also tested.

#### 3.3.1 Optical density (OD) and bacterial growth

A significant increase in OD between T0 and T48 was observed for the *Cellulophaga* (Kruskal-Wallis, p-value = 0.0463) and *Shewanella* (Kruskal-Wallis, p-value = 0.0463) strains exposed to dissolved domoic acid in solution. No significant increase in OD was observed for the *Pseudoalteromonas* strain after 48 hours of exposure (**Fig 4**).

A significant increase in OD between T0 and T48 was observed for the *Cellulophaga* (Kruskal-Wallis, p-value = 0.04311) and *Shewanella* (Kruskal-Wallis, p-value = 0.04953) strains without domoic. No significant increase in OD was observed for the *Pseudoalteromonas* strain after 48 hours of exposure (**Fig 4**).

The difference in OD between the three strains tested without domoic acid and recorded after 48 hours for the control without domoic acid showed a significant difference for each of the three strains, with respectively p-value = 0.0463 for *Cellulophaga* sp. and *Pseudoalteromonas* sp. and p-value = 0.04311 for *Shewanella* sp (**Fig 4**).

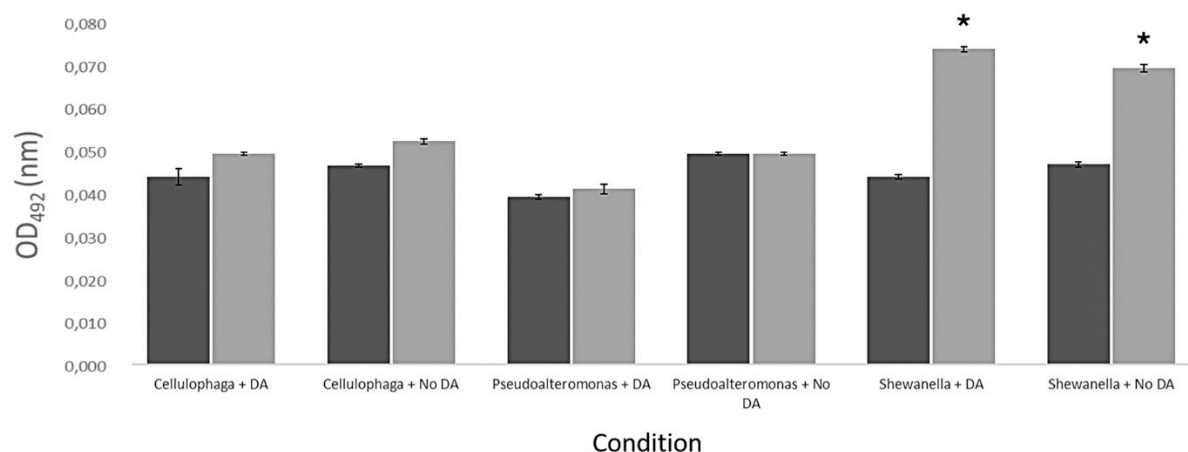


Figure 4. Bacterial growth for three different bacterial strains in solution with (DA) or without (No DA) dissolved domoic acid (n=3). OD at 492 nm was taken at the start of the experiment (black bar) and after 48 hours (grey bar) of exposition. All values are expressed as mean  $\pm$  standard error (SE).

### 3.3.2 Domoic acid concentration in solution

The mean domoic acid concentration for the control was  $25.01 \pm 1.13 \text{ ng.mL}^{-1}$  at the start of the experiment and  $14.27 \pm 0.28 \text{ ng.mL}^{-1}$  after 48 hours. All three bacterial strains exposed to dissolved domoic acid showed differences in domoic acid concentration compared to the control samples after 48 hours (p-value = 0.04953). Only the *Cellulophaga* strain had a domoic acid concentration different from T0 control samples (p-value = 0.04953). After 48 hours, no decrease in the concentration of dissolved domoic acid was observed for any of the bacterial strains used (Fig 5).

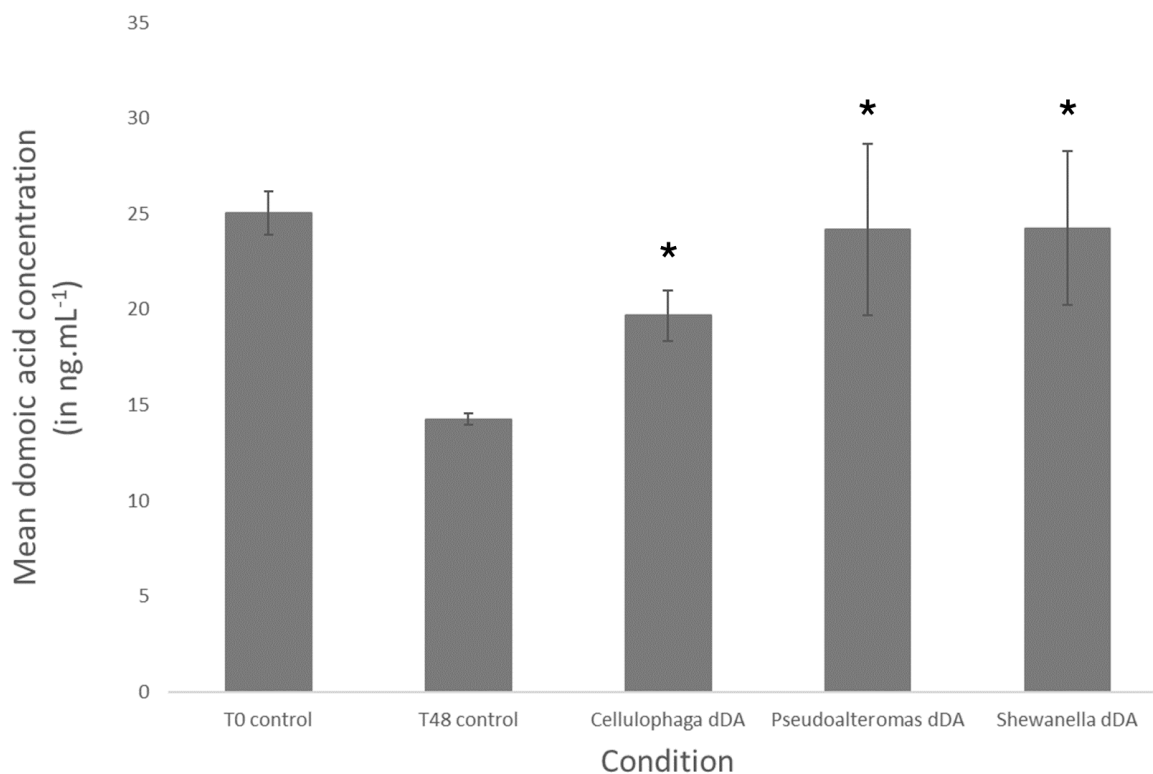


Figure 5. Domoic acid concentration after 48 hours of bacterial exposition in dissolved domoic acid solution. Three bacterial strains were used, *Cellulophaga* sp. from *M. edulis* digestive glands, *Pseudoalteromonas* sp. from *M. edulis* digestive glands and *Shewanella* sp. from *P. maximus* digestive glands. Controls for dissolved domoic acid concentration were also sampled at the beginning of the experiment (T0 control) and after 48 hours of exposure (T48 control). Samples (n=3) were filtered, and the results show the part of dissolved domoic acid (dDA). All values are expressed as mean  $\pm$  standard error (SE). Differences were considered statistically significant at  $\alpha = 0.05$  for all analyses.

#### 4. Discussion

This study consisted in a screening of cultivable bacteria from digestive glands of four bivalve species contaminated by domoic acid. Three bivalve species identified as fast-depurator of domoic acid and one species as a slow-depurator. The objective was to identify cultivable bacteria as potential candidates for their ability to decrease domoic acid concentration *in vitro*. Blooms of toxic microalgae are on the increase (Anderson *et al.*, 2021; Wells *et al.*, 2020), and the contamination of exploited species poses a real threat to the fishing industry (Brown *et al.*, 2019). Fishing activities are sometimes closed for several weeks in the case of contamination,



and this can extend to months or even years when the species concerned retains the toxin for a long time, as is the case with *P. maximus* and the domoic acid toxin (Campbell *et al.*, 2003). To this day the explanation for this long retention is still unknown, several scenarios for accelerating depuration have been studied, by modifying temperature, light, or food intake (Blanco *et al.*, 2006) or by supplementing with antioxidants (Pena-Llopis *et al.*, 2014; Vanmaldergem *et al.*, 2023) unfortunately without success for concentrations encountered during blooms. In the long term, the development of a probiotic seems to be the most feasible solution for the entire industry, which could be introduced at the larval stage in hatcheries.

### **Cultivable bacteria isolated**

This work identified a total of 30 cultivable bacterial genera in *M. edulis*, *D. trunculus*, *M. varia* and *P. maximus*. None was shared by all the four bivalve species studied. The greatest diversity was found in *P. maximus*, with 23 different genera of cultivable bacteria. This result can be partly explained by the higher number of digestive glands analyzed for *P. maximus* compared to the number of digestive glands used for *D. trunculus* and *M. varia*. However, the same number of digestive glands were analyzed for *P. maximus* and *M. edulis*, which presented the lowest diversity of cultivable bacteria isolated. Consequently, the difference in diversity between the two species does not depend only on this parameter.

Both species of bivalves were contaminated by domoic acid, and it can be hypothesized that exposure to domoic acid may have favoured bacteria species able of metabolizing domoic acid to the detriment of other bacteria species, leading to a depletion of cultivable microbiota in the mussel. In fact, the presence of these bacteria could explain the fast-depurating processus in *M. edulis*, *M. varia* and *D. trunculus*. This hypothesis has been formulated by Stewart *et al.* (1998) after demonstrating that bacterial strains of *Moraxella* sp., *Pseudomonas* sp. and *Alteromonas* sp. could use domoic acid in solution and appeared to be present only in the digestive gland of certain bivalves such as *M. edulis* and *Mya arenaria*. In addition, the authors pointed out that the bivalves *Placopecten magellanicus* and *Modiolus modiolus* considered as slow depurators, seemed to lack these bacteria.

A shift in the bacterial community in favor of other bacterial species or genera was observed in Manila clams *Ruditapes philippinarum* exposed to mercury in their environment, with a shift to mostly *Microbacteriaceae* (Leite *et al.*, 2017). This is also observed in the case of chronic

mycotoxicosis (HCC) diseases originating from a high dosage of mycotoxin contamination where specific bacterial species, such as *Helicobacter hepaticus*, have been found to correlate with the development of HCC in mice (Gargano and Hughes, 2014). This is also the case for diseases such as small bowel bacterial overgrowth (SBBO), a condition affecting humans and characterized by increased numbers of endogenous symbiotic bacteria in the small intestine that can lead to severe malabsorption syndrome (Sieczkowska *et al.*, 2016).

This change in microbiota composition may explain the faster depuration of *M. edulis* compared to *P. maximus* and in this study it is interesting to note that the samples from *D. trunculus* also have fewer bacterial genera identified and are also the least contaminated after mussels. This pattern was not found in *M. varia* from Roscanvel and the Anse du Roz, but it should be noted that no concentration of domoic acid was obtained for the animals from the Anse du Roz and that the animals from Roscanvel showed a contamination higher than for *D. trunculus* samples. *P. maximus* had the highest bacterial diversity and the highest domoic acid burden.

It cannot be ruled out that this low diversity may also be due to the choice of R2A medium for isolations, the composition of which would not have favoured the survival of certain bacteria in mussel's samples.

To our knowledge, there is no descriptive study of the bacterial genera or cultivable species present in the digestive system of these four bivalve species. However, many of the bacterial genera isolated in this study have been found in the digestive microbiota of bivalves. This is particularly true for the *Vibrio* genus, which is regularly found in bivalves and certain of its species have been implicated in diseases (Lambert *et al.*, 1998; Travers *et al.*, 2015 Pruzzo *et al.*, 2017). Numerous studies have been carried out on infections by the *Vibrio* genus affecting molluscs such as clams *Venerupis decussatus* and *D. trunculus*, abalone *Haliotis diversicolor* or oyster *C. gigas* (Gómez-León *et al.*, 2005; Cai *et al.*, 2006; Travers *et al.*, 2015; Prado *et al.*, 2015; Destoumieux-Garzon *et al.*, 2020). Grouped together under the term 'vibriosis', these infections can also affect *P. maximus* larvae and can lead to necrosis of the velar cells and a 100% mortality in 3 to 5 days (Nicolas *et al.*, 1996).

The genera *Pseudoalteromonas* and *Pseudomonas* have also already been found in the digestive gland and gut microbiota of bivalves, such as *C. gigas*, *M. galloprovincialis*, *Ruditapes* sp, *Perumytilus purpuratus*, *Perumytilus purpuratus*, *Perna uiridis*, *Sacupharca cornea* and *Venus mercenaria* (Vezzulli *et al.*, 2018 ; Akter *et al.*, 2023, Aranda *et al.*, 2012; Sun *et al.*, 2022;

Guillard, 1959) and have been found in the haemolymph microbiota of *P. maximus* (Desriac *et al.*, 2013). These genera are certainly part of the microbial core shared by these bivalves. Certain strains of the genus *Pseudomonas* were described by Stewart *et al.* (1998) as being capable of degrading domoic acid in solution, so their presence in the digestive gland of *M. varia*, a rapid depurator, is of interest. In addition, this genus is also present in *P. maximus*, a slow depurator, so the *Pseudomonas* strains present in scallops may be different from those found in fast depurators.

These results do represent the cultivable microbiota of the digestive gland at a specific time and cultivated on a particular medium and it has been proven that many factors such as temperature (Alma *et al.*, 2020), the presence of pollutants such as microplastics (Li *et al.*, 2020) or a change in diet (Pierce and Ward, 2019), can influence and modify the microbiota composition. These modifications may also be due to the environment, whether it being the geographic location of the animals, water composition or the presence of other organisms.

### **Bacterial candidates**

Potential candidate bacteria capable of metabolizing domoic acid, *Pseudoalteromonas* sp. and *Cellulophaga* sp. were isolated from rapid depurators in this study. If these strains demonstrate a domoic acid metabolizing activity, they could be tested as probiotics, enabling the scallops to depurate the toxin more quickly.

The genus *Pseudoalteromonas* is already widely studied for different properties, notably for its degradation actions on a mixture of PST. Indeed, Donovan *et al.* (2009) succeeded in isolating *P. haloplanktis* from the digestive tract of *M. edulis* contaminated with PST, which reduced *in vitro* the toxicity of the PST solution by 90% in three days. Since domoic acid is also a hydrophilic phycotoxin, it is possible that this bacterial genus also has a bioremediation action on the latter. Nonetheless, the results obtained in this study did not significantly demonstrate this activity under the conditions of this experiment but require additional analyses with a higher concentration of bacteria to confirm these results.

The genus *Cellulophaga* is known for its agarolytic activity (Skerratt *et al.*, 2002; Ramos *et al.*, 2017). A study has also shown that this bacterial genus is capable of switching on and off this activity, which makes it possible to promote the growth of certain species of associated microalgae (Skerratt *et al.*, 2002). Indeed, *Cellulophaga* sp. was shown to be non-virulent in the presence of its host *Pseudo-nitzschia pungens*, while its algicidal activity led to the decline

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of the culture of *Pseudo-nitzschia australis* (Sison-Mangus *et al.*, 2014). This mutualist interaction between the host algae and its bacteria also appears to influence domoic acid production, which is greater in the presence of the associated bacteria. The hypothesis being that the bacteria potentially feed on the domoic acid produced by the algae (Sison-Mangus *et al.*, 2014). We could therefore hypothesize that the *Cellulophaga* genus is capable of metabolizing domoic acid released into the environment.

The results obtained in this study did not demonstrate the activity of the *Cellulophaga* sp. strain we isolated on dissolved domoic acid solution obtained from a culture of toxic *Pseudo-nitzschia australis*. No growth of this *Cellulophaga* sp. strain was observed during the 48-hour experiment, neither did the total domoic acid content decrease after 48 h of incubation. However, these results need to be confirmed with a longer exposure time and higher bacterial concentrations to verify whether this action is dose-dependent and if similar results can be obtained with different strains of *Cellulophaga*.

Based on the criteria stated previously, the *Shewanella* genus has also been considered as a potential candidate. This genus is found in the gut microbiota of oysters *Crassostrea virginica* (King *et al.*, 2012), a bivalve species also considered to be a rapid depurator (Mafra *et al.*, 2010). Strains from this genus are known and used as a probiotic in fish aquaculture (García de la Banda *et al.*, 2012; Vidal *et al.*, 2016) and have shown to increase the phagocytic activity (Nayak, 2010). The genus *Shewanella* can also have algicidal activity, particularly on cyanobacteria, and could be used to mitigate blooms (Li *et al.*, 2014). Results obtained in this study did not demonstrate the activity of the *Shewanella* sp. strain on dissolved domoic acid solution. However, a small growth of this strain could be observed, resulting in an increase in the optical density after 48 hours in the presence of a solution with or without domoic acid but no decrease in total domoic acid content was monitored after 48 hours of incubation. The strain tested does not therefore appear to be capable of degrading domoic acid in the conditions of this study.

As previously stated, these results need to be confirmed with a longer exposure time, higher bacterial concentrations, and more replicates to verify these results. Different strains of these bacterial genera could also be tested because those used in this study may not have the activity of metabolizing domoic acid. As mentioned previously, the exposure time was probably not

long enough, for example in the study by Hagtröm *et al.* (2007) a decrease in the domoic acid concentration is only observable after 28 days of incubation.

It would perhaps be wise to resort to new isolations on media other than R2A, although it allows slower growth of bacteria and therefore a good evaluation of the diversity of the samples tested, such as marine broth more commonly used for the cultivation of marine bacteria. Finally, it would be interesting to be able to carry out smears of digestive glands not contaminated by domoic acid to evaluate the differences in bacterial diversity between digestive glands contaminated or not contaminated.

Nevertheless, further tests should be carried out because considering the information found in the literature on other phycotoxins (Vasama *et al.*, 2014; Donovan *et al.*, 2009) probiotics appear as the most cost-effective way to deal with the long retention of domoic acid in *P. maximus* but also other exploited species that may be impacted.

### **Probiotics elaboration**

This preliminary study made it possible to create a collection of bacteria and three have been tested for domoic acid degradation without conclusive results.

However, this is only the first step towards the creation and use of a probiotic cocktail. Indeed, even if the use of bacteria isolated from other species is very common in probiotics, more and more studies are being carried out to produce probiotics from bacteria present in the target species or a genetically close species (Gibson *et al.*, 1998). This is the reason why the *Shewanella* strain, isolated from the digestive gland of *P. maximus*, was also tested in this experiment, indeed if the activity of this strain would have been confirmed, this would make it possible to limit the rejection that can be encountered when using a probiotic created from bacteria from another host (Offret *et al.*, 2018; Gibson *et al.*, 1998). In fact, just like the grafting process in human medicine, rejection or a harmful ricochet effect on the organism being treated cannot be ruled out when there is a concentrated supply of exogenous bacteria (Gueimonde *et al.*, 2004). In the future, before any strains proven to be effective against domoic acid in solution can be used, several points would need to be checked.

First the precise identification of the species would be required, since certain species of *Pseudoalteromonas* sp. for example, can be harmful to *P. maximus* larvae (Kesarcodi-Watson *et al.*, 2014). Its use as a probiotic on *P. maximus* larvae will therefore depend on its efficiency on domoic acid degradation, but also its action on the larvae. It is essential to ensure that each

probiotic does not cause side effects or excess mortality in treated animals, whatever their life stage. This applies to each strain or mixture of strains considered.

To gain a better understanding of the action of domoic acid-metabolizing bacteria, it would also be interesting, on a second hand, to check whether this action remains even after lysis of the bacterial cells. In fact, certain strains of *Lactobacillus rhamnosus* keep the capacity to remove phycotoxins even though the cells are non-viable (Vasama *et al.*, 2014).

The use of probiotics to accelerate domoic acid depuration in *P. maximus* would also be a sustainable alternative if the changes made to the microbiota could be passed on to the offspring. This possibility was demonstrated by Fallet *et al.* in (2022), when survival of farmed oysters to the Pacific oyster mortality syndrome (POMS) increased when exposed to the microbiota of wild oysters. This improvement in survival was also transmitted to the next generation, which had not been exposed to wild oyster microbiota.

## 5. Conclusion

The proliferation of domoic acid-producing *Pseudo-nitzschia* sp. microalgae is an environmental and health problem, but it also leads to serious economic losses for the French and international scallop fisheries (Husson *et al.*, 2016). Finding ways to mitigate domoic acid action in solution but most importantly for bivalves such as *P. maximus* is of great importance for the future of this fishing industry and fishermen considering its long retention of the toxin that can lead to the closure of fishing for several months. This study is the first to focus on cultivable bacteria present in *P. maximus* contaminated with domoic acid and three rapid depurators. It made it possible to highlight potential candidates as well as the bacterial collection which will make it possible to consider different tests. Preliminary expositions have been carried out on domoic acid solution and new tests need to be conducted on other strains and with modified parameters in order to advance the search for a probiotic to accelerate the decontamination of domoic acid in *P. maximus*.

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**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 request.

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## Publication 4

Exploring microbiota transfer as a potential to mitigate the long retention of domoic acid in King scallop (*Pecten maximus*).

Margot Deleglise, Cyril Noël, Guillaume Barnouin, Adeline Bidault, Clément Offret, Malwenn Lassudrie, Hélène Hegaret, Caroline Fabioux



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**Exploring microbiota transfer as a potential to mitigate the long retention of domoic acid in King scallop (*Pecten maximus*).**

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## **Abstract**

Domoic acid contamination of marine resources can be an economic and sanitary obstacle for fisheries that needs to be addressed. Bivalves, as filter feeders, are among the most exposed organisms to *Pseudo-nitzschia* spp. blooms. The King scallop, *Pecten maximus*, is distinguished by its long retention of domoic acid, unlike other species such as mussels *Mytilus edulis*, which depurate very quickly from just a few hours to days. Two studies by Stewart *et al.* in 1998 and Hagström *et al.* in 2007 proposed that mussels could host in their digestive gland and their feces, bacteria capable of metabolizing domoic acid and thus inducing a decrease in its presence in the environment. The presence of such bacteria in King scallop digestive gland was questioned and the possibility of transferring them if they were absent was assessed. In this study, scallops contaminated with domoic acid were exposed to either mussel digestive gland

homogenate or mussel feces for two weeks. Changes in bacterial communities of scallop's digestive such as the appearance of amplicon sequence variant (ASVs) belonging to the genera *Maribius*, *Shewanella*, *Pseudoalteromonas*, *Colwellia* and *Roseobacter* were observed. This study focused on the composition of microbiota of mussels and scallops. In the long term, these results can be the foundation to initiate the development of probiotics as an accelerant of domoic acid depuration in King scallops.

**Keywords :** microbiota transfer, bacteria, domoic acid, *Pecten maximus*, *Mytilus edulis*

## 1. Introduction

Marine bivalves are an important resource for the world's population (Grant and Strand, 2019; Wijsman *et al.*, 2018). With the ever-increasing demand for this source of nutrition (Tan *et al.*, 2020), the exploitation and marketing of marine bivalves continues to grow (Costello *et al.*, 2020; Anderson *et al.*, 2011). While some species, such as oysters and mussels, are now widely cultivated, others, such as clams and scallops, are fished in the wild. Bivalves are filter feeders that feed on phytoplankton particles suspended in the water column (Shumway *et al.*, 1985; Jørgensen 1996) and are prone to accumulate high amounts of contaminants, including phycotoxins produced by some microalgae species. A worldwide problem that perfectly reflects this situation is domoic acid contamination.

This phycotoxin is produced by the microalga *Pseudo-nitzschia* sp., which is present in all the world's waters and whose blooms are becoming increasingly recurrent under the impact of global warming (Wells *et al.*, 2020; Gobble *et al.*, 2021; Tewari *et al.*, 2022). Domoic acid is a water-soluble amino acid, homologue of glutamic acid, a major neuro-excitatory for synaptic transmissions (Doble *et al.*, 2000; Garattini *et al.* 2000). Symptoms in humans caused by this toxin include nausea, vomiting and diarrhea, muscle weakness, disorientation, and memory loss, and in severe cases of intoxication, death. Domoic acid, like many toxins, has the particularity of bioaccumulating along the trophic chain (Bates *et al.*, 1998). Filter-feeding bivalves, as primary consumers, are therefore the first links in this chain and the main vector for cases of marine mammals, birds, and ultimately human poisoning due to their high consumption. Following an episode of contamination leading to the death of three people in Canada on Prince Edward Island in 1987 (Bates *et al.*, 2019) and to protect consumers, the

health authorities implemented a health safety threshold of 20 µg of domoic acid per gram of fresh total weight above which fishing or harvesting are closed.

All shellfish have not the same propensity to contaminate or even eliminate domoic acid. Some shellfish, such as blue mussels (*Mytilus edulis*), need only a few hours or days to depurate the toxin from their bodies (Mafra *et al.*, 2010; Novaczek *et al.*, 1991), while others, such as King scallops (*Pecten maximus*), take months or even years to depurate below the health safety threshold (Blanco *et al.*, 2006; Bogan *et al.*, 2006; Bresnan *et al.*, 2017). According to Mauriz and Blanco (2010), scallops concentrate more than 80% of the toxic burden in its digestive gland but little is known about the mechanisms involved in this long retention time in scallops. A few attempts to accelerate scallop depuration have been made in the past. The first hypothesis was tested by Blanco *et al.* in 2006, who demonstrated *in situ* that depuration seems to be 30% faster in the digestive gland when scallops are located in the water column. The authors hypothesized that a higher temperature, an increased luminosity, and a higher food consumption allowed scallops at the surface to depurate faster compared with scallops located on the substrate. This approach, although interesting, is not viable in the long term for fishermen, as it would require scallop stocks to be moved to an area combining these 3 conditions for a minimum acceleration of depuration. Another hypothesis was to test the antioxidant action of N-acetylcysteine. Indeed, Peña-Llopis *et al.* (2014) demonstrated an acceleration of fourfold of the standard depuration rate of domoic acid in adductor muscle of king scallops *P. maximus* exposed *in vitro* to N-acetylcysteine. In this study, the muscle was however very little contaminated by domoic acid. Vanmaldergem *et al.* (2023) thus investigated if this same antioxidant could accelerate the depuration of the digestive gland of naturally contaminated scallops. Scallops exposed to N-acetylcysteine did not show any significant acceleration in the depuration of the digestive gland. Furthermore, this approach would not be viable either for fishermen as this treatment would be impossible to implement in terms of cost, logistics and time. The use of treatment directly in the hatchery would be one way to limit costs and material requirements. This could be integrated in the form of a probiotic cocktail. A probiotic is, according to the definition of the Food and Agriculture Organization, “one or more living microorganisms which, when consumed in adequate quantities, confer a beneficial effect on the health of the host” (Food and Agriculture Organization of the United Nation 2006).

Certain bacterial genera or consortia of bacteria appeared capable of reducing the concentration of these toxins in the environment, including paralytic shellfish toxins (PST) (Smith *et al.*, 2001;

Donovan *et al.*, 2009; Vasama *et al.*, 2014), diarrhetic shellfish toxin (DSP) and tetrodotoxin (Svensson *et al.* 2009 ; Martelli *et al.* 2022) or microcystin (Thees *et al.* 2019, Ramani *et al.* 2012, Christoffersen *et al.* 2002). However, the exact role of these bacteria has not yet been defined, and it is not clear whether they degrade or metabolize the toxins.

Two studies have investigated the hypothesis that some bacteria could metabolize or degrade domoic acid. The first, by Stewart *et al.* (1998), mentions bacterial genera, *Alteromonas*, *Pseudoalteromonas* and *Moraxella*, capable of growing in the presence of domoic acid. These three genera were isolated using a domoic acid enrichment technique on a simple culture medium. Samples of digestive glands from various bivalve species were tested on this medium and the bacteria grown were considered capable of growing and biodegrading domoic acid. Bacteria with a significantly higher growth and biodegradation efficiency were isolated from the gills and/or digestive gland of blue mussels *M. edulis* and soft-shell clams *Mya arenaria*, two bivalve species recognized as domoic acid fast depurators. Conversely, the study of Stewart *et al.* (1998) stated that bivalves such as sea scallops *Placopecten magellanicus* and red mussels *Modiolus modiolus*, known as slow domoic acid depurators, rarely had this type of bacteria. The authors therefore concluded that these indigenous bacteria could be a factor facilitating the elimination of the toxin in *M. edulis* and *M. arenaria*. A second study by Hagström *et al.* (2007) showed that the fecal matter of mussels *Mytilus galloprovincialis*, as well as bottom sediment collected from a river where *Pseudo-nitzschia multiseriata* blooms regularly occur, led to the most rapid domoic acid deterioration. The authors hypothesize that this faster degradation was associated with a higher number of bacteria and enzymes capable of degrading the toxin, both unidentified.

The use of bacteria to accelerate the depuration of *P. maximus* therefore appears as a hypothesis worth investigating.

From human health effects (Gerritsen *et al.*, 2011; Gill and Garner, 2004) to their use in aquaculture (Ringø *et al.* 2020; Irianto *et al.* 2002), bacterial supplementation, known as probiotics, has been gaining in interest (Prado *et al.* 2010 ; Dittman *et al.* 2017 ; Langlois *et al.* 2021). In aquaculture, they are most often used to increase the size of treated shellfishes or fish (Abasolo-Pacheco *et al.*, 2017 ; Amin *et al.*, 2020; Douillet *et al.*, 1994 ; Hines *et al.*, 2022), but also to boost their immunity and thus strengthen their resistance to viruses and/or harmful bacteria reducing the use of antibiotics which are becoming a threat to the environment and can lead to the resistance to antibiotics (Cabello *et al.*, 2006). To date, few studies have been carried

out on the use of bacteria as potential probiotics for scallops *P. maximus*. The few experiments already carried out involved the use of several bacterial strains at the larval stage of the scallop in hatchery (Genard *et al.*, 2014; Ruiz-Ponte *et al.*, 1999; Kesarcodi-Watson *et al.*, 2012, 2014). These results showed that scallops larvae were receptive to exogenous bacteria and that the latter could have a protective effect on *P. maximus* larval development (Ruiz-Ponte *et al.*, 1999). Few other studies have been published on the characterization of bacteria associated with the different larval stages (Sandaa *et al.*, 2003) and the microbiota associated with the gonads of *P. maximus* (Lasa and Mira *et al.*, 2016).

Scallops therefore seem to tolerate certain bacteria for their immune protection and their growth. We can hypothesize that it could be possible to supplement *P. maximus* with bacteria from *M. edulis* that could accelerate scallops' decontamination. Contaminated scallops could be supplemented with microbiota issued from other bivalves considered to be rapid depurators, such as blue mussels. The approach presented in this article is designed to be rather quick, easy to implement and less expensive for fishing professionals. This experiment consisted of exposing naturally contaminated scallops to bacteria present either in mussel digestive gland homogenized material or directly in mussel feces. The initial postulate was that scallops, by filtering these different inputs, could incorporate the exogenous bacteria present. The bacterial composition of the digestive gland of treated scallops was monitored and the domoic acid load present in this organ was also measured, to test if some bacteria could be used to accelerate the depuration of *P. maximus*.

## 2. Materials and methods

### 2.1 Animals

A total of 159 one-year-old scallops ( $5.72 \text{ cm} \pm 8.1 \text{ cm}$  shell length;  $9.12 \text{ g} \pm 0.7 \text{ g}$  total weight), produced by the local Tinduff hatchery, were maintained in the Bay of Brest (Britany, France) for 10 months, and harvested on June 11, 2021, after an exposure to a natural bloom of *Pseudo-nitzschia* producing domoic acid.

On the same date, 468 mussels (*Mytilus edulis*) coming from another location of the bay of Brest, the "Petit Minou" beach in Plouzané were also sampled. Scallops and mussels were transferred to the lab the day of the sampling.

## 2.2. Microalgal cultures

The microalgae *Tisochrysis lutea* (clone *T.iso*) was used as forage algae to feed scallops and mussels during the experiment. Cultures, obtained from the Argenton hatchery (Ifremer, Brittany, France), were produced in 300L cylinders containing 1 mm-filtered seawater enriched with Conway medium (Walne, 1966) at 18 °C, with continuous light. *T.iso* was harvested in a stationary phase for the feeding experiments.

## 2.3. Experimental design

A two-week experiment was designed to test the possibility of a transfer of the microbiota from mussels to scallops and its potential effect on domoïc acid depuration by scallops. The 159 scallops were randomly distributed in nine tanks of 20 L. Three conditions were tested with 3 tanks for each condition : i) a control condition with only scallops (n=16 scallops per tank), ii) a condition with the addition of digestive gland homogenate from 20 mussel every 24 hours in the tanks (n=16 scallops per tank), and iii) a condition consisting in a co-culture of scallops and above them, mussels placed on a rail to exposed scallops to mussel feces (n=16 scallops and n=32 mussels per tank) (**Fig. 1**).

In each tank, the influx of water and food (*T-iso* microalgae was maintained at  $10^5$  cells.mL<sup>-1</sup>) was ensured continuously thanks to 2 peristaltic pumps maintaining the flow rate at 14 mL.min<sup>-1</sup> to ensure a complete renewal of the water in each tank every 24 h. Room temperature was set to 16.5 °C and the photoperiod (light: dark) to (14 h:10 h).

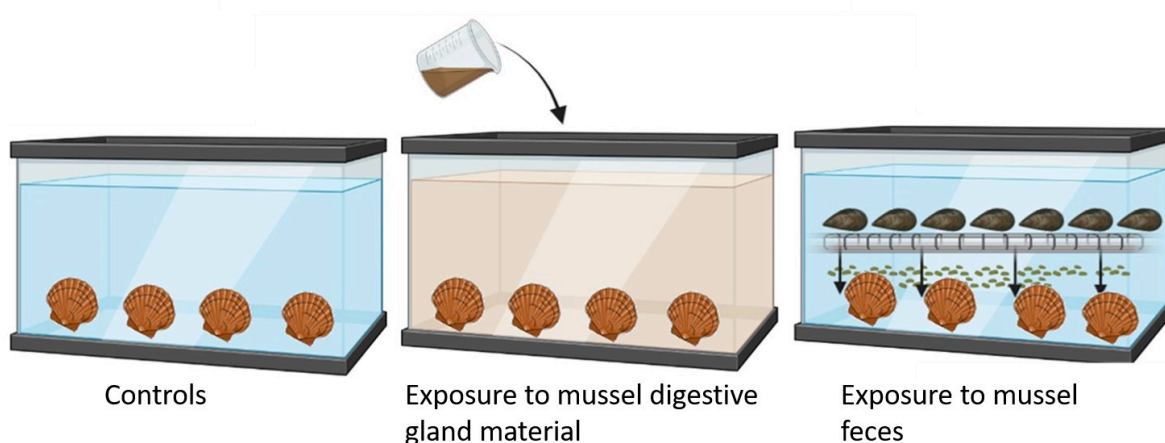


Figure 1. Experimental design to test mussel digestive-microbiota transfer to scallops.

## 2.4 Sample collection.

Scallops and mussels were sampled at the beginning of the experiment (D0, n=15 for each bivalve species), after 7 days (D7, n=3 animals per tank) and 14 days (D14, n=3 animals per tank). Dissections of the digestive glands from scallops and mussels were performed using sterile instruments. A part of 50 mg of each digestive gland was stored in sterile cryotubes at -80 °C for bacterial identification and another part of 200 mg ± 5 mg was stored at -20 °C for domoic acid analysis. Mussels and scallops' feces were also collected at D7 and D14 with sterile pipettes and stored in cryotubes at -80 °C pending bacterial genomic DNA extractions. Water of each tank (3 L) was filtered on 0.22 µm glass microfiber filters (Whatman™) at each sample time and kept in sterile cryotube at -80 °C before bacterial genomic DNA extraction. Following each sampling, the water in the tanks was renewed to avoid the development of biofilm.

## 2.5 DNA extraction protocol

### 2.5.1 From digestive gland and feces

Bacterial genomic DNA (gDNA) extractions from digestive gland and feces samples of scallops and mussels were performed according to the protocol described in Offret *et al.*, 2020, combining the use of phenol-chloroform-isoamyl (PCI) alcohol extraction with a DNA extraction kit (PowerLyser Powersoil DNA Isolation, Qiagen, USA).

Quickly after thawing on ice, 40 mg of digestive glands were recovered from the cryotube using a sterile scalpel and placed in a sterile 1.5 mL eppendorf tube. After adding 150 µL of TNE, 20 µL of SDS (20%) and 8 µL of protein K (20 mg.mL<sup>-1</sup>), the tube was vortexed and incubated for 30 min in a water bath at 45 °C. After centrifugation (10 min, 10 000 g at room temperature) the supernatant was recovered and stored at 4 °C. On the remaining pellet, a second digestion was performed by adding 100 µL TNE, 13 µL SDS and 5 µL proteinase K. The sample was incubated for 1 h at 45 °C after being vortexed. Then the digestate was centrifuged 10 min at 10,000 g and the obtained supernatant was pooled to the first one. The resulting pool (200 µL) were then transferred to a PowerBead tube from the PowerLyser kit to be mechanically lysed with 750 µL of Bead solution and 60 µL of the C1 solution. The FastPrep24™ (2x45 sec) was used to shake all samples. Each sample was centrifuged for 30 s at 10,000 g at room temperature and 700 µL of supernatant was collected in a new tube with 700 µL of phenol-chloroform-isoamyl alcohol. After mixing and centrifugating for 15min at 16,000 g at 4 °C, the upper aqueous phase was collected in a tube containing a volume of chloroform and mixed for 45 s

before being vortexed for 15 min at 16,000 g at 4 °C. The aqueous phase was recovered and mixed with 0.7 volume of ice-cold isopropanol and stored overnight at -20 °C.

Samples were centrifuged 30 min at 16,000 g at 4 °C and the pellet recovered. This pellet was suspended in 500 µL of TNE buffer and 250 µL of C2 solution and vortexed before incubation at 4 °C for 5 min and centrifuged again for 1 min at 10 000 g at room temperature. The supernatant was transferred into a tube containing 200 µL of C3 solution and incubated for 5 min at 4 °C and then centrifuged for 1 min at 10 000 g at room temperature. The supernatant (750 µL) was collected and added to 1200 µL of C4 solution. After vortexing, the mixture is added to a SpinnFilter from the kit and centrifuged for 1 min at 10 000 g and the filtrate discarded. Then the C5 solution (500 µL) is added to the SpinnFilter and the filtrate again discarded after a 1 min centrifugation at 10,000 g. The Spinnfilter was transferred to a collection tube and 100 µL H<sub>2</sub>O added to the filter and centrifuged 30 sec at 10,000 g. This step was repeated a second time and the DNA recovered from the bottom of the tube was stored at -20 °C.

### 2.5.2 From water samples

Bacterial genomic DNA (gDNA) from water samples was extracted from the 0.22 µm glass microfiber filters, according to the following protocol. Half a filter was put into a tube containing approximately 0.7 g of 100-300 µm diameter glass beads and 1 mL of 1X TEN lysis buffer (composed of 10 nM Tris-HCl (pH 10.5), 1 nM EDTA, 150 nM NaCl and 0.5 % SDS) along with 100 µL of 20 % SDS and 100 µL of 10 % Sarkosyl. After bead-beating using the FastPrep24™ for 4x45", 1 mL of supernatant was collected into a new tube containing 1 mL of PCI (25 :24 :1). The aqueous phase was recovered and centrifuged at 16,000 rpm for 15 min at 4 °C. Then the upper aqueous phase was transferred to a new tube with 1 mL of Chloroform and mixed by repeated inversion and centrifuged at 16,000 rpm for 15 min at 4 °C. Were added, 40 µL of 3M Sodium Acetate and 0.7 vol of ice-cold isopropanol to the upper aqueous phase in a new tube. The samples were placed overnight -20 °C and then centrifuged to recover the gDNA pellet 14,000 rpm for 20 min at 4 °C.

The pellet was resuspended with 0.5 mL of cold 75 % ethanol and centrifuged at 14,000 rpm for 5 min at 4 °C. After allowing the pellet to air dry, the gDNA was resuspended in 100 µL of sterile water and stored at -20 °C prior to analysis.



## 2.6 Metabarcoding analyses

gDNA concentration of all samples was checked by Quantifluor kit (Promega, USA) according to the manufacturer's protocol. A total of 144 samples were chosen and sent to Génome Québec Innovation Centre, Montreal, Canada, for sequencing. For each sample, 16S rRNA amplicon libraries were generated using primers 341F (5'-CCTACGGGNGGCWGCAG -3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') targeting the 16S V3-V4 region (Herlemann *et al.* 2011). Paired-end sequencing with a 250 bp read length was performed on a MiSeq system (Illumina) with a sequencing fraction of 0.014 for each sample.

Raw sequencing data were processed using the SAMBA (<https://gitlab.ifremer.fr/bioinfo/workflows/samba> ; v4.0.0) workflow created by the SeBiMER (Ifremer's Bioinformatics Core Facility). Amplicon Sequence Variants (ASV) were constructed using QIIME 2 (v2020.2) (Bolyen *et al.* 2019) and DADA2 (Callahan *et al.*, 2016) with the optimal trimming parameters defined by FIGARO (Weinstein *et al.*, 2019) (no trimming of forward and reverse reads; maxEE=2). Contaminated sequences were removed with *microDecon* (v 1.0.2) (McKnight *et al.* 2019). Using a Bayesian classifier trained with the SILVA database (v.138) and the QIIME 2 feature classifier (Wang *et al.* 2007) taxonomic assignment of ASVs was done. Community analysis and statistics were done on R (R version 4.2.1) (R Core Team 2022) using the *phyloseq* packages (v 1.40.0) (McMurdie and Holmes 2013) and *vegan* (v 2.6-4) (Oksanen *et al.* 2022).

## 3. Results

### 3.1 Sequencing results

Data analysis and samples information were reported (**Table 1**).

Table 1. Summary of sequencing information for the different sample types and numbers as well as the number of samples used for final analysis.

Sample type	Sampling time	Number of sample sent for sequencing	Number of sample used for analysis
Water	D0	1	1
	D7	9	9
	D14	10	10
Scallops digestive gland	D0	15	5
	D7	27	25
	D14	25	24
Scallops feces	D7	9	9
	D14	9	9
Mussels digestive gland	D0	15	11
	D7	9	9
	D14	9	9
Mussels feces	D7	3	3
	D14	3	3
Number of sequences generated from all 144 samples	14,293,511		
Number of high quality reads	8,304,995		
Reads quality	58%		
Average reads per sample retained for analyses	65,912		

### 3.2 Bacterial composition without preconceptions

The first analysis on metabarcoding data was to check that there were no major differences in the bacteria present in the water supplied to experimental tanks throughout the experiment, which could have led to bias in the analyses of the microbiota in the digestive glands of the exposed animals. Alpha diversity in water samples was assessed using Observed, Chaos1, InvSimpson and Shannon indexes (**Supplementary Figure 1**) and a Tukey significance test (HSD) was performed to identify any differences. The test gave no significant differences between the samples, revealing a stable ASVs composition in the water samples over time.

$\alpha$ -diversity of ASVs from bivalve's digestive gland, feces and water was assessed. The diversity index for scallops' feces samples was the highest for both Shannon and inverse Simpson's indices. Shannon and inverse Simpson's indices both indicated that total bacterial diversity was significantly different between scallops and mussel's digestive gland (Tukey's test, Shannon: d.f. = 2.2;  $p = 3.74e^{-09}$  and Simpson's inverse: d.f. = 0.43;  $p = 5.70e^{-09}$ ).

For the following analyses, the ASVs found specifically in the water tank samples were subtracted from the ASVs used in the analysis. The results obtained therefore only reflect bacterial ASVs that may have been transmitted by mussel feces or digestive gland homogenized material.

### 3.2.1 The ten most abundant genera

An NMDS (not shown) on data from the digestive glands of scallops and mussels at the start of the experiment showed that the samples were significantly different between the two bivalve species (Adonis statistical test:  $R^2=0.12$ ,  $p\text{-value}=0.001$ ).

Following this result, an UpsetR plot (not shown) revealed that at the start of the experiment, 179 bacterial genera, corresponding to 777 ASVs, were present only in the mussel digestive gland. Scallops, on the other hand, possessed 12 specific bacterial genera represented by 94 ASVs. The two bivalves shared 64 bacterial genera represented by 74 ASVs.

The ASVs were used to represent the 10 most abundant genera in the digestive gland samples at the beginning of the experiment (**Fig. 3**). The digestive glands of mussels (**Fig. 3A**) and scallops (**Fig. 3B**) share common bacterial genera. The genera *Blastopirellula*, *Mycoplasma*, *Prauserella*, *Rhodopirellula* and *Rubripirellula* are among the 10 most abundant genera shared by the two bivalve species. However, others are only found among the 10 most abundant genera in one of the two bivalves studied. For the mussel digestive gland samples, the genera *Maribius*, *Alteribacillus*, *Bythopirellula*, *Candidatus\_Xenohaliois* and Unknown *Rhodobacteraceae* could be observed among the 10 most abundant. Whereas for the scallop digestive gland samples, the genera *DEV007*, *Haglioglobus*, *Planctomicrobium*, Unknown *Alphaproteobacteria* and Unknown *Chlamydiales* were among the 10 most abundant observed. This last genus, *Chlamydiales*, was the most abundant in 3 of the 5 scallops samples analyzed.

For ease of representation the ASVs presented here and identified from the various samples were reduced to the 100 most abundant genera.

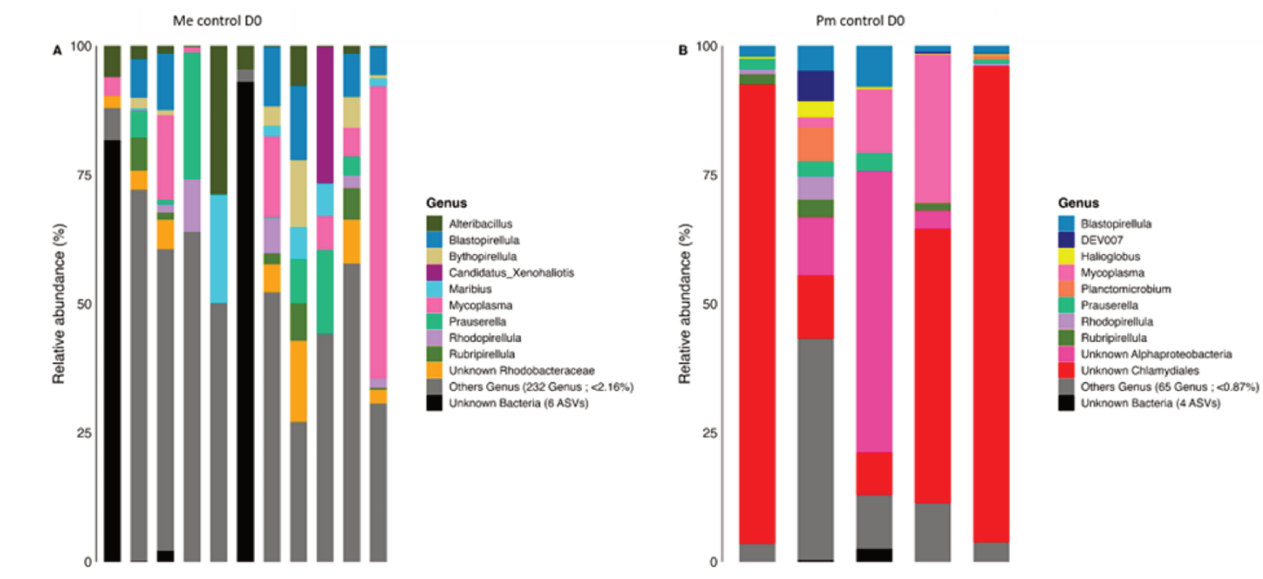


Figure 3. Representation of the 10 most abundant ASV genera at the beginning of the experiment (A) in the mussel digestive gland and (B) in the scallop digestive gland. Each individual sampled is represented by a bar.

The same representation was used to show the 10 most abundant genera in the digestive gland of scallops after 7 and 14 days of exposure to either mussel digestive gland homogenate or mussel feces (**Fig. 4**). The most abundant genus found among the different samples of scallop digestive gland was again *Chlamydiales* observed in all samples analyzed. In addition to this genus, two others, *Halioglobus* and *Mycoplasma*, also present at the start were found in the samples after 7 and 14 days of exposure to the different conditions. The top ten most abundant genera among the digestive glands of controlled or exposed scallops were completed by the genera *Colwellia*, *SAR324\_clade*, *Spirochaeta\_2*, *Sulfitobacter.*, Unknown *Alphaproteobacteria*, Unknown *Legionellaceae*, Unknown *Rhodobacteraceae*.

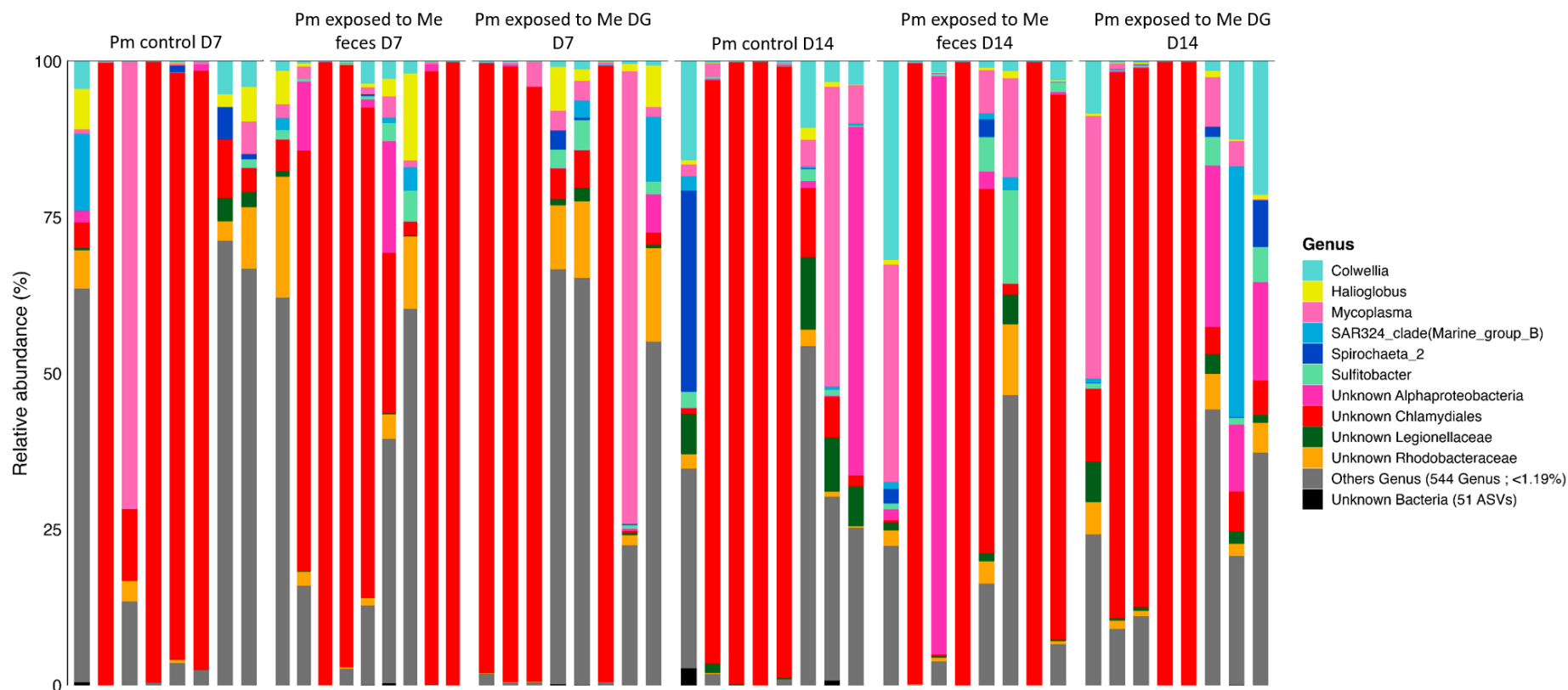


Figure 4. Representation of the 10 most abundant bacterial genera in the scallop digestive gland for the control scallop (Pm Control) and the scallops exposed to mussel's digestive gland homogenate (Pm exposed to Me DG) or feces (Pm exposed to Me feces) after 7 (D7) and 14 (D14) days. Each individual sampled is represented by a bar.



**ASVs that appeared in scallops' digestive glands after mussel exposure.**

The relative abundance of the top 100 ASVs not present in scallop digestive gland at the start of the experiment or in the control animals and shared only by scallops exposed to mussels' feces (**Supplementary Fig. 2A**) or digestive gland (**Supplementary Fig. 2B**) was observed after 7 or 14 days of experiment.

Out of the 737 ASVs identified in scallop's digestive gland and shared with mussels' feces and the ones with the highest relative abundance, 4 were recognized as belonging to the *Colwellia* genus, 2 to the *Sulfitobacter* genus, 2 to the *Halarcobacter* genus, 2 to the *Maribius* genus and *Polaribacter* genus and 1 to the *Pseudoalteromonas* genus and *Roseobacter* genus (**Supplementary Fig. 2A**).

After 7 days of exposure, the ASVs that belonged to the genera *Rosebacillus*, *Halioglobus*, *Unknown Marinifilaceae* had the highest relative abundance with 0.4%, 0.88% and 0.9% respectively. The relative abundance of these ASVs seemed to have decreased after 14 days of treatment. The ASVs with the highest relative abundance after 14 days of treatment were part of the genera *Halioglobus* (0.6%), *Colwellia* (3.8% and 3.9%), *Rhodobacteraceae* (0.6%), *Paludibacteraceae* (0.6%), *Polaribacter* (0.3%), *Halodesulfovibrio* (0.35%), *Phaeobacter* (0.6%), *Sulfitobacter* (1.6%) and *Halarcobacter* (0.3%).

For scallops exposed to mussel digestive gland homogenate, a total of 611 ASVs specifically shared between these scallops and the digestive glands of mussels were found and the top 100 were represented (**Supplementary Fig. 2B**). Some of the genera found are the same as for the condition with mussel feces. In fact, 3 ASVs belonged to the *Colwellia* genus, 2 to the *Sulfitobacter* genus, 3 to the *Pseudoalteromonas* genus and 1 to the *Roseobacter* genus. However, no ASVs representing the genera *Sulfitobacter*, *Halarcobacter*, *Polaribacter* or *Maribius* were found in the top 100. In addition, 2 ASVs were identified as belonging to the genera *Shewanella* and *Halomonas*.

The ASVs with the highest relative abundance after 7 days of exposure to mussel digestive gland microbiota were part of the genera *Mycoplasma* with 3%, *Spirochaeta\_2* with 0.5% and *Tenacibaculum* with 0.6%. They were not found after 14 days of treatment. The ASVs with the most relative abundance then, belonged to the genera *Citrobacter* (0.8% and 0.4%), *Shinella* (0.4%) and *Unknown Pseudomonadaceae* (0.5%).

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### **Evolution of ASVs which were present in control scallops DG but increased with mussel exposure.**

The ASVs shared between all scallop digestive glands and mussel's microbiota and whose relative abundance increased after exposure were also monitored and represented. A plot was made for the two exposure times for each condition.

This increase concerned 53 ASVs for scallops exposed to mussel fecal microbiota after 7 and 14 days (**Supplementary Fig. 3A**). The genera associated with these ASVs included *Colwellia*, *Halomonas* and *Mycoplasma*. The ASV of the *Mycoplasma* genus was the most abundant after 14 days of exposure, with a relative abundance of over 30% in the treated scallops compared with 4% in the control ones.

Scallops exposed to mussel digestive gland homogenate had 60 ASVs found with a higher relative abundance after treatment (**Supplementary Fig. 3B**). These ASVs were part of the genera *Unknown Rhodobacteraceae* (0.4%), *Methylotenera* (0.3%), *Limibaculum* (0.29%), *Paraglaciecola* (0.28%), *Unknown Pseudomonadaceae* (2.8%) et *Roseobacter* (0.35%).

## **4. Discussion**

The aim of this study was to monitor changes in the microbiota of the digestive gland of scallops *Pecten maximus* contaminated with domoic acid and exposed to exogenous bacteria from mussels, considered to be rapid depurators.

### **Most abundant genera at T0**

The first step in this study was to compare the microbiota of the digestive glands of *M. edulis* and *P. maximus*. This allowed the identification of similarities and differences between the two bivalves' digestive glands microbiota and their major bacterial composition.

Analyses at the start of the experiment showed that these two species share five of their ten most abundant genera with *Blastopirellula*, *Mycoplasma*, *Prauserella*, *Rhodopirellula* and *Rupripirellula*.



The *Mycoplasma* genus is also widespread in bivalves such as oysters, mussels and abalones (Akter *et al.*, 2023; Griffin *et al.*, 2020; Villasante *et al.*, 2020). It is generally reported as an infectious agent in the same way as *Rickettsia* and *Chlamydia* (Harshbarger *et al.*, 1977; Paillard *et al.*, 2004). However, it has also been described as symbiotic, in the digestive tract of mussel *M. galloprovincialis* (Akter *et al.*, 2023) for example or in the digestive gland of the freshwater mussel *Villosa nebulosa* (Aceves *et al.*, 2018). It was also dominant in *Crassostrea. gigas* digestive gland (Offret *et al.*, 2020) and present in *Ruditapes philippinarum* digestive gland as well (Offret *et al.*, 2023). Finally, it is one of the genera that predominates in the digestive glands of healthy red abalone (*Haliotis rufescens*) compared to abalone suffering from Whitening Syndrome (Villasante *et al.*, 2020). Few studies have been carried out on *Prauserella* sp., but it has been isolated from the tissues of the stony coral *Galaxea fascicularis* (Wu *et al.*, 2014). The last two genera shared by the two bivalve species are *Rhodopirellula* and *Rubripirellula*. Both belong to the phylum Planctomycetes, which is frequently found in bivalves (Zheng *et al.*, 2021; Pierce *et al.*, 2019). Planctomycetes are also found in greater abundance in marine snow and are therefore aggregated rather than in free form and are capable of adhering to diatoms during blooms (Morris *et al.*, 2006; DeLong *et al.*, 1993) and marine sediment (Storesund *et al.*, 2013; 2020).

This study also revealed a greater number of different bacterial genera and ASVs in mussel digestive gland samples compared to scallop samples. With almost 8.3 times more ASVs corresponding to almost 15 times more bacterial genera, the bacterial diversity of mussel digestive glands from this study is greater than that found in scallop digestive glands. Differing microbiota between *M. edulis* and *P. maximus* can be explained in part by a microbiota specific to these bivalve species (Paillard *et al.*, 2022) but also by a different geographical origin (Offret *et al.*, 2020; Del Rio-Lavín *et al.*, 2023). Thus, both intrinsic and extrinsic factors shape the bacterial community in organisms. It should also be noted that only scallops were contaminated with domoic acid, which could also lead to a selection of particular bacteria present in the digestive gland.

The composition of the top 10 most important bacterial genera in mussel's digestive gland, not present in the top 10 genera in the digestive gland of *Pecten maximus*, includes the genus *Maribius*. Few studies have been conducted on this genus; however, it is found in the digestive tract of the shrimp, *Litopenaeus vannamei* (Sha *et al.*, 2016) and is considered a bacterial candidate for hydrocarbon mitigation pathway genes (Guibert *et al.*, 2012).

Of the 10 most abundant genera found in scallops, only *Chlamydia* have been studied more extensively. Collingro *et al.* (2020) have published on the wide variety of hosts for this genus. Bivalves are no exception, with *Chlamydia* observed in both ctenidia and the mantle (Renault *et al.*, 1995) of the oyster *Crassostrea gigas* and in digestive cells (Grizel, 1987). As shown by the studies of Cajaraville and Angulo (1991), *Chlamydia* sp. are also found in the duct cells of the digestive gland of the mussel *M. galloprovincialis* and in the gut epithelium of *P. maximus* larvae. Following immunohistological analysis of these tissues, *Chlamydia* sp. was described as looking like ‘inclusion bodies’ in digestive cells of mussels (Cajaraville and Angulo, 1991). Studies have looked at the interactions of *Chlamydia* sp. with the immune system of its host. They focused on species infecting humans, such as *C. pneumoniae*, which appears to protect infected cells from apoptosis (Rajalingam *et al.*, 2001) or *C. trachomatis*, that have been shown to accumulate in non-degradative autophagosomes (Al-Younes *et al.*, 2004).

Although the presence of *Chlamydia* sp. in mussels *M. edulis*, which are fast depurators, has been confirmed, it appears to be much lower than in scallops. This has also been observed in this study with an initial abundance of *Chlamydia* sp. almost 49 times greater in *P. maximus* samples than in *M. edulis* samples.

A recent study by García-Corona *et al.* (2022) detected the presence of inclusion bodies comprising a domoic acid signal in the cytoplasm of digestive cells of scallops. These are thought to be late-autophagosomes. The hypothesis put forward in this paper is that the toxin, once integrated into the early-autophagosomes, cannot be digested by the lysosomal machinery, leaving the toxin trapped within these now labeled late-autophagosomes. We also know that domoic acid is a homologue of glutamic acid (Doble *et al.*, 2000; Garattini *et al.*, 2000). Glutamic acid is found in buffers used to transport and preserve *Chlamydia* spp. (Campbell and Kuo, 2009; Warford *et al.*, 1984; Liechti *et al.*, 2013); it must likely enable the bacterial cells to withstand a certain amount of stress. It is therefore possible that domoic acid could have the same effect on *Chlamydia* spp.

If there is an interaction between *Chlamydia* sp. and domoic acid, and if during its inclusion, the bacteria also enclose this toxin, then a major infection with *Chlamydia* sp. could be one hypothesis for the long retention of domoic acid in *P. maximus*. Further research, such as for example colocalization between domoic acid and *Chlamydia* sp. on cryo-immuno histological sections of scallop’s digestive glands, needs to be undertaken to investigate this hypothesis.

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### **Bacterial composition of *P. maximus* digestive gland after exposure to mussel microbiome**

The main objective of this experiment was to monitor the microbiota of the digestive gland of scallops after addition of bacteria from mussel microbiota, using two administration methods (feces or digestive gland) to see if any shift could occur.

#### **Top 10 genera in *P. maximus***

After exposure to the mussel microbiota, the composition of the top 10 most abundant bacterial genera in *P. maximus* samples revealed some changes not observed in control scallops. Indeed, while the genera *Hagliglobus*, *Mycoplasma*, *Unknown Chamydiales* et *Unknown Alphaproteobacteria* have been preserved, the others have been replaced by the genera *Colwellia*, *SAR324\_clade*, *Spirochatea\_2*, *Sulfitobacter*, *Unknown Legionellaceae* et *Unknown Rhodobacteraceae*.

The *Colwellia* genus is present in the digestive glands of control scallops however ASVs of this genus, after 14 days of exposure to mussel microbiota, were observed in slightly higher relative abundance in exposed scallops. It is therefore likely that the abundance of this genus was favored by the contribution of mussel microbiota. This genus has been isolated from several different environments such as seawater, sediments (Marx *et al.*, 2009, Nogi *et al.* 2004) and marine animals such as the intestines of sea urchin (Christiansen *et al.*, 2018) and has already been found in the blue mussel *M. edulis* (Kim *et al.*, 2017). This bacterial genus is known for its ability to digest agar (Xu *et al.*, 2017) and carrageenan (Christiansen *et al.*, 2018) and has been investigated for its ability to degrade hydrocarbons (Mason *et al.*, 2014).

The genus *Sulfitobacter* is considered to be a rather ubiquitous marine bacterium. It has been isolated from various marine species and seawater and has the ability to degrade gelatin (Ivanova *et al.*, 2004) as well as dimethylsulfoniopropionate (Kwak *et al.*, 2014). More interestingly, it is a genus described as having symbiotic interactions with diatoms and more specifically with *Pseudo-nitzschia* sp. The species *S. pseudonitzschiae* has been isolated from various *Pseudo-nitzschia* spp. and an increase of up to 35% in the growth of *P. multiseriis* has been observed in the presence of this bacterium, which metabolizes the taurine produced by the algae (Heliwell *et al.*, 2022). This type of bacteria also protects algae from certain viruses (Amin *et al.*, 2012).

Few studies have been carried out on *SAR324*, it is a ubiquitous and to this date uncultivated genera. It has been recorded in surface and bottom waters in the North Pacific (Boeuf *et al.*, 2021) and is found host-specifically in the abalone *Haliotis diversicolor* (Huang *et al.*, 2020).

Finally, the genus *Spirochaeta* is found in the intestinal microbiota of bivalves such as *C. gigas* and *M. galloprovincialis* (Akter *et al.*, 2023), and in the composition of the microbiota of the crystalline stylet of several bivalves such as *Arctica islandica*, *Cerastoderma edule*, *Crassostrea gigas* and *Laternula elliptica* (Husmann *et al.*, 2010). It has been shown that some species of this genus are capable of degrading xylan or cellulose (Hespell, 1994). Furthermore, Sohaskey and Barbour (2000) have demonstrated that the *Spirochaeta aurantia* species has a carboxylesterase activity, and the authors hypothesize that this activity could be beneficial to the bacteria due to its living environment. Indeed, in turbid environments such as marine, marsh and muddy waters, this could help it to inactivate toxic compounds. In mammals, carboxylesterases are also known to detoxify xenobiotic compounds (Wang *et al.*, 2018).

#### **ASVs appearance with a focus on genera identified for their domoic acid degrading activity.**

Scallops exposed to mussel feces appear to share 737 ASVs with mussel feces microbiota. In scallops exposed to mussel digestive gland homogenate the appearance of 611 ASVs shared with the mussel was observed. As these ASVs are not found specifically in water and are shared by mussels, the hypothesis that they have been transmitted via the mussel microbiota is plausible. Among the ASVs that can be considered as transferred from mussel microbiota, some are affiliated to genera of interest. This is the case of *Pseudomonas* sp. An ASV belonging to this genus was found in the top 100 ASVs of the digestive glands of scallops after 14 days of exposure to the digestive gland microbiota of *M. edulis*. Stewart *et al.* (1998) isolated this genus from the digestive system of *M. edulis* and identified it as one of the genera able to grow in a domoic acid-enriched environment and therefore capable to use it. This is also the case for the *Alteromonas* sp. genus. The genera found in Stewart *et al.* (1998) were able to induce the disappearance of up to 57% of the domoic acid enriched substrate in 14 days at 20 °C. The authors also point out that the presence of these bacteria in bivalves such as *M. edulis* or *M. arenaria* is related to their ability to rapidly eliminate domoic acid. Therefore, these bacteria are candidates worth considering for the depuration of domoic acid in *P. maximus*. The identification of these strains down to species level has not been made public, so studies enabling formal identification of the strains should be carried out.

Another interesting transfer is that of ASVs belonging to the genus *Pseudoalteromonas*. In this study, ASVs of this kind are observed to appear in the digestive glands of scallops after 7 and 14 days of exposure to mussel microbiota, whether in the condition with digestive gland homogenate or in the presence of feces. These ASVs were therefore absent from the digestive glands of control scallops. This potential transfer is most interesting indeed, this genus has a toxin degradation activity that has already been demonstrated for other phycotoxins. Donovan *et al.* (2009) found that 7 strains belonging to the species *P. haloplanktis* isolated from the digestive tract of *M. edulis*, reduced the toxicity of an algal extract of a paralytic shellfish toxin (PST) mixture by  $\geq 90\%$  within 3 days. It is therefore possible that strains of this genus are capable of metabolizing other phycotoxins, including domoic acid. This genus is therefore a good candidate to help scallop's domoic acid depuration.

## 5. Conclusion

This monitoring of ASVs present in mussel microbiota and found after 7- or 14-days exposure in scallop digestive glands provides constructive evidence of the possibility of modifying scallop microbiota via the transfer of fecal microbiota and mussel digestive tract. For some ASVs, their presence in the digestive gland of scallops seems to persist over the 14 days of simple exposure to mussel microbiota. Nevertheless, their possible implantation within *P. maximus* digestive system needs to be assessed to know whether these bacterial genera are simply part of the transient microbiome. The use of bacteria as probiotics from rapid depurators such as *M. edulis* seems a relatively realistic approach to implement. The possibility of exposing scallop larvae, in hatchery, to a probiotic cocktail containing bacteria found only in rapid depurators and metabolizing domoic acid could increase the depuration capacity of this toxin in scallops.

## 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 request.

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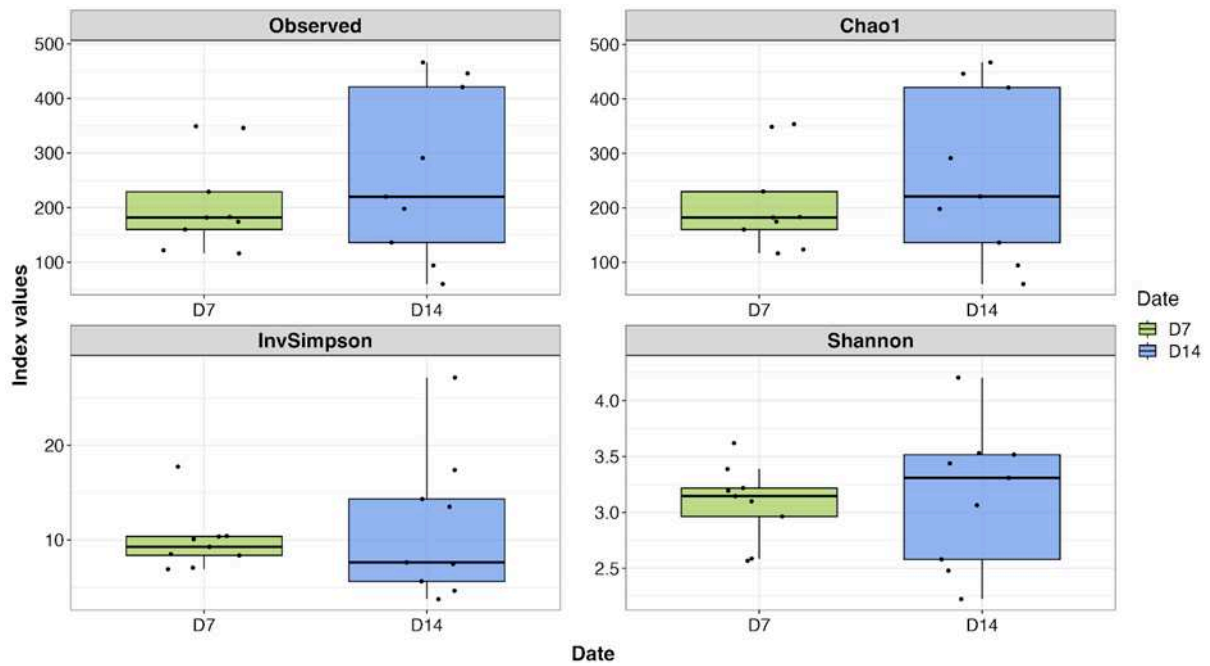
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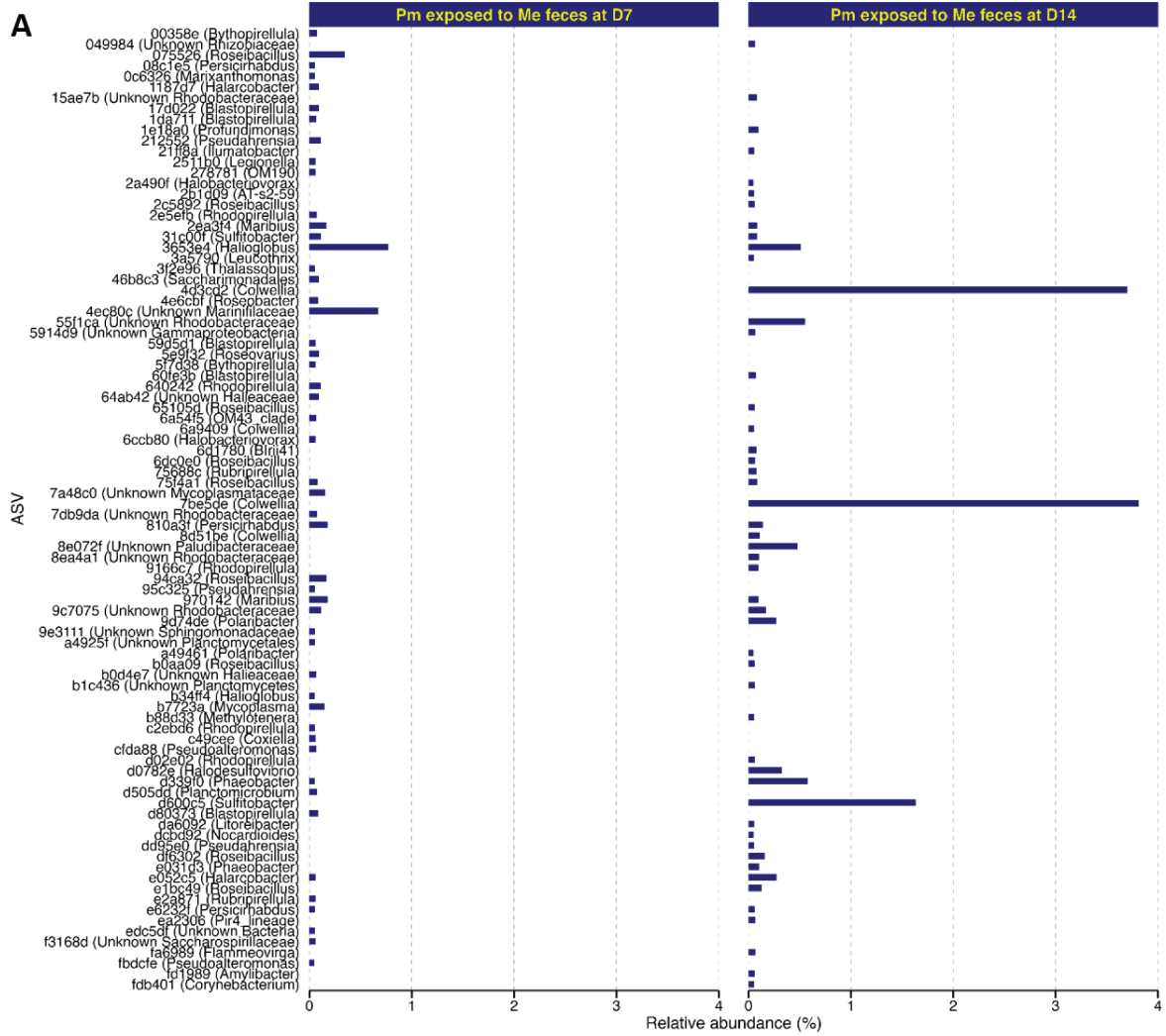


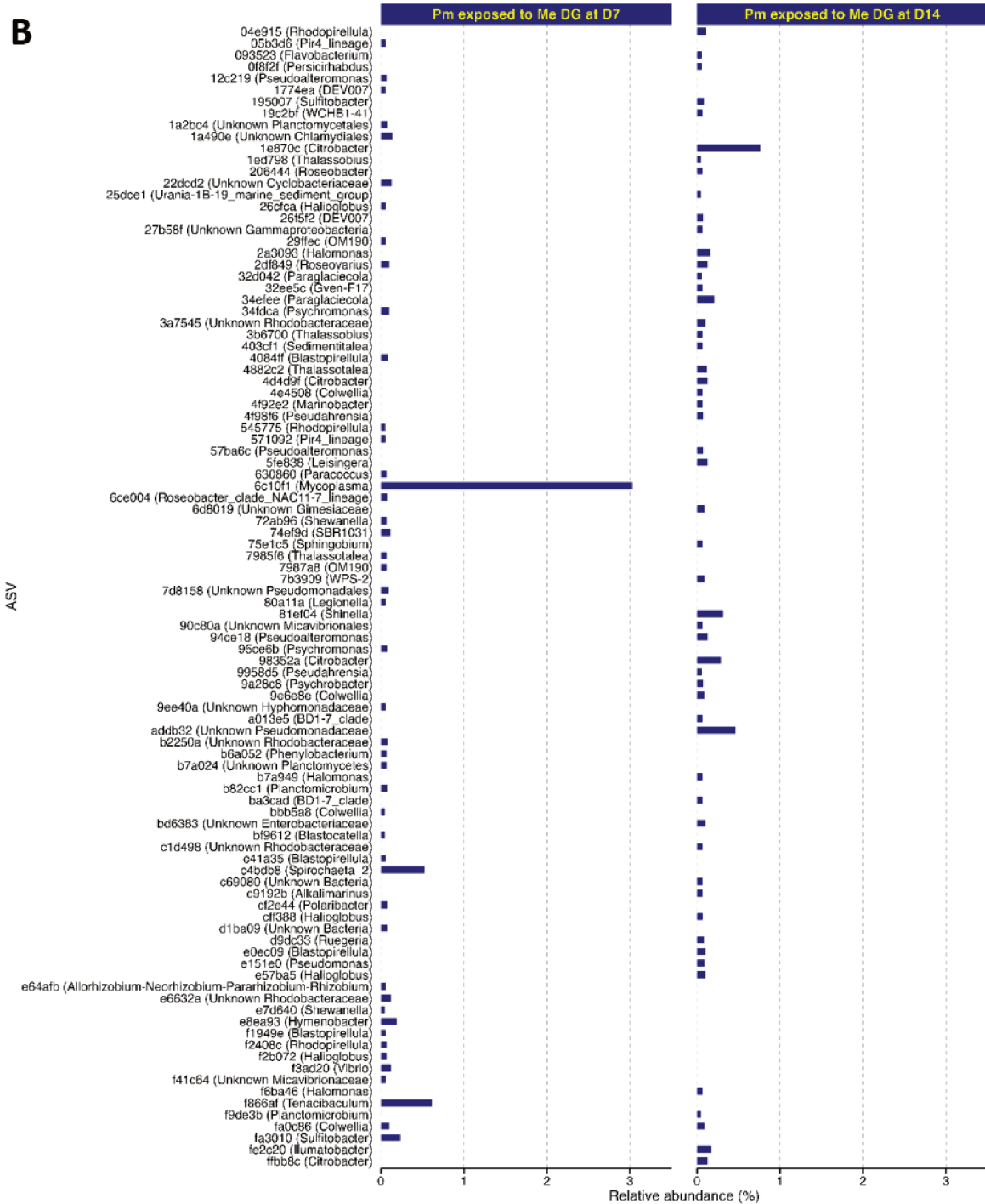
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## Supplementary figure

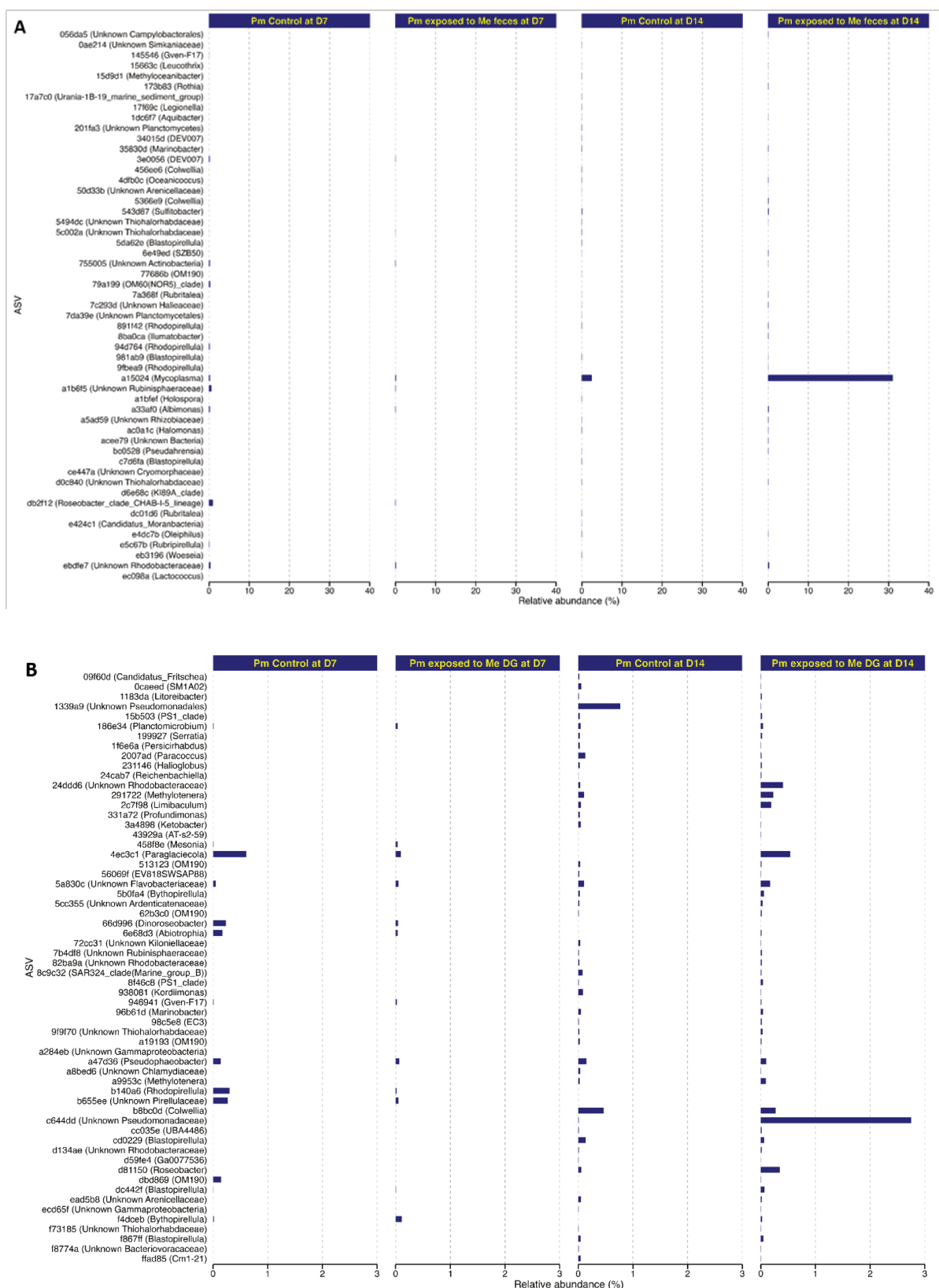


Supplementary Figure 1. Alpha diversity in water samples (n=18) assessed using Observed, Chao1, InvSimpson and Shannon indexes and a Tukey significance test (HSD) to identify any differences. The Tukey test gave no significant differences between the samples after 7 (green boxplot) and 14 days (blue boxplot) of experiment.





Supplementary Figure 2. Barplot of specific top 100 bacterial ASVs found in scallops after exposure to (A) mussel feces at D7 and at D14 and (B) to mussel digestive gland at D7 and at D14.



Supplementary Figure 3. Barplot of the evolution of the relative abundance of ASVs found in control and exposed scallops' digestive gland and shared with mussel feces (A) or mussel digestive gland (B) and showed an increase during mussel microbiota exposition.

## Take home message



## A retenir

### Etude par isolement bactérien

Un transfert de microbiote par simple exposition des coquilles Saint-Jacques aux fèces ou au broyat de glande digestive de moules semble possible après sept et quatorze jours d'expérience.

L'implantation des bactéries dans la glande digestive de *P. maximus* n'a pu être observée lors de cette expérience et leur présence reste transitoire.

La présence de ces bactéries n'a pas conduit à une dépuración accélérée de l'acide domoïque chez ces coquilles naturellement contaminées.

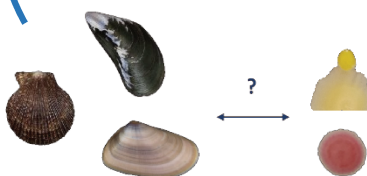
### Etude par métabarcoding

Une différence de composition du microbiote existe entre les dépurateurs rapides et la coquille Saint-Jacques.

Une collection de 32 genres bactériens isolés à partir de *P. maximus*, *M. edulis*, *D. trunculus* et *M. varia* a été réalisée après leur exposition naturelle à l'acide domoïque.

Une étude préliminaire pour tester la capacité de dégradation de l'acide domoïque de trois souches bactériennes de cette collection a été menée et les résultats doivent être analysés.

Les souches de *Cellulophaga* et de *Shewanella* semblent capables de croître dans un milieu en présence d'acide domoïque et apparaissent donc comme des souches à tester dans d'autres conditions.





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# **DISCUSSION GÉNÉRALE ET CONCLUSION**

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La contamination des bivalves par l'acide domoïque constitue un risque sanitaire et économique pour l'industrie de la pêche. Les fermetures de pêches dues à cette contamination entraînent une perte significative de revenus pour les professionnels partout dans le monde et notamment le long de la côte Atlantique de l'Europe de l'Irlande à l'Espagne (Chadsey *et al.*, 2012; Moore *et al.*, 2019; Petroff *et al.*, 2021; Ritzman *et al.*, 2018). La coquille Saint-Jacques *Pecten maximus*, en raison de sa rétention prolongée de la toxine, est particulièrement touchée, nécessitant parfois plusieurs années avant que la concentration en acide domoïque ne revienne sous les seuils de sécurité (Blanco *et al.*, 2006, 2002). La possibilité de décortiquer le muscle, qui accumule moins d'acide domoïque que la glande digestive, en usine spécialisée existe. Cette étape permet parfois de sauver une partie de la production mais cela entraîne des coûts supplémentaires tant pour les exploitants que les consommateurs et, avec une réorganisation nécessaire dans les usines qui peut être difficile à mettre en place (Décision de l'Union Européenne 2002/226/ EC). Cependant, cela est moins rentable financièrement que la vente de la coquille entière.

L'augmentation des efflorescences de la microalgue *Pseudo-nitzschia* sp. depuis les années 2000 (Amzil *et al.*, 2009; Husson *et al.*, 2016) accroît le risque de contaminations fréquentes par l'acide domoïque, mettant en danger la rentabilité des pêcheries à la coquille Saint-Jacques. Le risque de voir le taux d'acide domoïque contenu dans la glande digestive de la coquille Saint-Jacques ne redescendre que rarement sous le seuil de sécurité sanitaire est donc réel.

L'exposition aux cellules toxiques de *Pseudo-nitzschia* sp. est le principal facteur expliquant la contamination. Face à ce problème, des scénarios pour accélérer la décontamination ont été testés, tels que l'utilisation d'antioxydants et l'exposition à la lumière, une augmentation de la température et un apport en nourriture. Cependant, les coûts associés à ces scénarios sont prohibitifs pour les pêcheurs. Il est donc nécessaire d'explorer des techniques offrant une efficacité accrue avec un investissement minimal. Pour accélérer le processus de dépuración, des suivis *in situ*, ainsi que la compréhension des liens entre la taille de la coquille Saint-Jacques, sa contamination par l'acide domoïque et sa rétention prolongée, sont essentiels. La piste des bactéries émerge comme une voie prometteuse et peu contraignante pour les pêcheurs. Cependant, le manque de connaissances sur les cinétiques de contamination et de décontamination constitue un obstacle à l'élaboration de stratégies d'aide à la dépuración. La piste des bactéries émerge comme une voie prometteuse et potentiellement peu contraignante pour les pêcheurs.

L'ensemble des travaux menés durant cette thèse ont permis de démontrer que :

- (1) En rade de Brest, la contamination par l'acide domoïque des coquilles Saint-Jacques n'est pas toujours corrélée au bloom de *Pseudo-nitzschia* sp. observé en surface. Nos résultats, à la suite d'analyses de données de terrain depuis 2011, montrent que l'origine des contaminations peut-être plus complexe et met en évidence l'importance du compartiment à l'interface eau-sédiment, qui peut représenter une source de contamination conséquente (Chapitre 1).
- (2) Les coquilles Saint-Jacques de plus petite taille s'étaient plus contaminées que les coquilles de plus grande taille au cours d'un bloom naturel de *Pseudo-nitzschia australis*. et le suivi de ce lot de coquilles pendant deux mois en éclosérie a démontré que les individus de petite taille dépuraient plus rapidement la toxine de leur glande digestive que les individus de grande taille (Chapitre 2).
- (3) Le transfert de microbiote de glande digestive et de fèces entre deux espèces de bivalves a été démontré comme possible. De plus, notre travail d'isolement microbiologique et de recherche de bactéries candidates a permis d'obtenir une banque de souches provenant de dépurateurs rapides et de *P. maximus* en vue de possibles tests de dégradation de l'acide domoïque (Chapitre 3).
- (4) Le genre bactérien *Chlamydia*, très présent dans la glande digestive des coquilles Saint-Jacques d'après nos analyses en métabarcoding, pourrait être associé à la longue rétention de l'acide domoïque par la coquille Saint-Jacques (Chapitre 3).

### Les différents schémas de contamination de *P. maximus* en rade de Brest

L'objectif de cette étude était d'améliorer notre compréhension de la relation entre la concentration de *Pseudo-nitzschia* sp., sa toxine, l'acide domoïque, et la contamination de la coquille Saint-Jacques, *Pecten maximus*. Les résultats de sept ans de monitoring en rade de Brest nous ont permis de souligner la complexité de l'environnement étudié et de mieux identifier l'origine des contaminations de la coquille Saint-Jacques par l'acide domoïque (chapitre 1). Trois scénarios de contamination de la coquille Saint-Jacques semblent se dégager de ce suivi (**Fig. 6**).

Le premier (**Fig. 6**, scénario 1) met en évidence la présence d'un bloom de *Pseudo-nitzschia* sp. toxique en surface entraînant la production d'acide domoïque qui en sédimentant dans la colonne d'eau finit par être filtré par les bivalves tels que la coquille Saint-Jacques entraînant leur contamination.

Le deuxième scénario (**Fig. 6**, scénario 2) est l'augmentation simultanée de la densité cellulaire de *Pseudo-nitzschia* sp. toxique, à la fois à la surface et au fond, et de la concentration d'acide domoïque dans les coquilles Saint-Jacques. Dans ce scénario, les observations mettent en évidence l'apparition de cellules toxiques de *Pseudo-nitzschia* sp. à la fois dans les eaux de surface et de fond.

Ce suivi à grande échelle révèle que la contamination des coquilles Saint-Jacques est plus fortement corrélée avec la présence d'acide domoïque au fond de la colonne d'eau. Si ce résultat peut sembler évident étant donné l'habitat dans lequel *P. maximus* évolue, la présence de cellules de *Pseudo-nitzschia* sp. au fond est plus intrigante.

En effet, le troisième scénario de contamination (**Fig. 6**, scénario 3) de la coquille Saint-Jacques trouve son origine dans des efflorescences qui semblent provenir directement du fond. L'origine de la présence de cellules de *Pseudo-nitzschia* sp. et d'acide domoïque total au fond de la colonne d'eau n'a pas encore été résolue et plusieurs hypothèses ont été émises et mériteraient d'être étudiées.

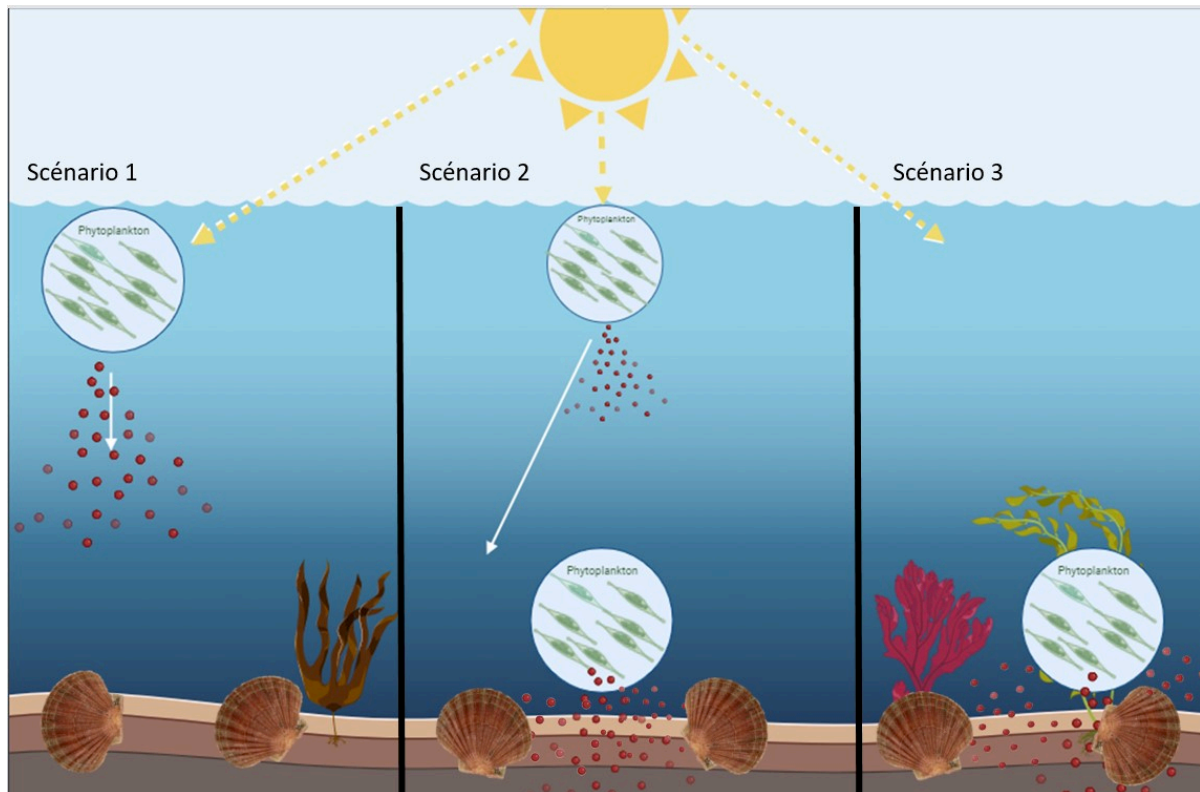


Figure 6 : Représentation schématique des trois scénarios de contamination par l'acide domoïque après un bloom de *Pseudo-nitzschia* sp. Le bloom phytoplanctonique produit de l'acide domoïque (sphères rouges) qui vient contaminer les coquilles Saint-Jacques posées sur le sédiment ou enfouies. La taille du cercle de cellules de *Pseudo-nitzschia* sp. est proportionnelle à la concentration en cellules algales. (Réalisé avec l'outil en ligne Biorender <https://app.biorender.com/>)

Parmi ces hypothèses, la présence d'acide domoïque à l'interface eau-sédiment pourrait s'expliquer par la remise en suspension de particules piégées dans le sédiment. Des événements ponctuels, tels que des tempêtes, pourraient jouer un rôle important dans la remise en suspension de l'acide domoïque. Ce phénomène s'est produit en février 2010 dans le nord-ouest de la France, dans le Pertuis Breton et dans le nord du Golfe de Gascogne, après la tempête Xynthia" (Husson *et al.*, 2016). Bien que la remise en suspension des particules toxiques semble bien expliquée, on sait peu de choses sur la capacité éventuelle des cellules de *Pseudo-nitzschia* sp. à conserver leur toxicité après une efflorescence. Le même manque de connaissances subsiste concernant la capacité de l'acide domoïque à être conservé au fil du temps dans les sédiments, même s'il a été démontré que l'acide domoïque peut s'adsorber sur les sédiments en fonction de leur composition (Burns and Ferry, 2007). Un éventuel phénomène de dormance

des cellules de *Pseudo-nitzschia* sp. dans les sédiments pourrait également expliquer en partie la contamination des coquilles Saint-Jacques ainsi que les épisodes de recontamination, alors qu'un bloom toxique n'a pas été détecté en surface. Une autre hypothèse méritant d'être étudiée serait que les cellules mortes de *Pseudo-nitzschia* sp. après un bloom, pourraient encore contenir des toxines actives/viables comme le suggèrent plusieurs études (Schnetzer *et al.*, 2017; Sekula-Wood *et al.*, 2011, 2009). La présence de cellules de *Pseudo-nitzschia* sp. et d'acide domoïque à l'interface sédiment-eau peuvent également s'expliquer par la possibilité que des efflorescences de *Pseudo-nitzschia* sp. toxiques se forment directement plus en profondeur dans la colonne d'eau ou à l'interface des sédiments.

Il arrive parfois que les efflorescences de *Pseudo-nitzschia* sp. ne soient pas en corrélation directe avec la contamination par l'acide domoïque, c'est le scénario rencontré en rade de Brest, en 2015. Ces événements sont associés à des proliférations de deux groupes de *Pseudo-nitzschia*, "pungens" et "delicatissima", connus pour avoir des niveaux de toxicité faibles (Baugh *et al.*, 2006; Trainer *et al.*, 2012). Ce résultat met en lumière l'importance de développer et d'étendre l'emploi de méthodes d'identification des espèces cryptiques comme *Pseudo-nitzschia* sp, telles que les sondes moléculaires développées pour des espèces spécifiques (Ferrari *et al.*, 2023; Kim and Sako, 2005; McDonald *et al.*, 2007).

Pouvoir suivre en temps réel la concentration en acide domoïque ou la prolifération de *Pseudo-nitzschia* sp. dans le milieu serait également un atout de taille dans la prévention des épisodes de contamination. Ce genre de dispositif a été mis en place par le Monterey Bay Aquarium Research Institute (MBARI - <https://www.mbari.org/technology/environmental-sample-processor-esp/>), il permet la mesure d'acide domoïque particulaire quasiment en temps réel à l'aide de l'Environmental Sample Processor (ESP) (Moore *et al.*, 2021) et certains résultats obtenus dans le cadre de suivis ont été publiés par Thukral et collaborateurs (Thukral *et al.*, 2023). Dans cette étude, des échantillons d'eau analysés ont révélé une correspondance entre la concentration en acide domoïque total et l'expression du gène *dabA* responsable de la biosynthèse d'acide domoïque. De plus, une corrélation positive entre les gènes *dab*, les molécules intermédiaires biosynthétiques de l'acide domoïque et l'abondance de *Pseudo-nitzschia* sp. a pu être effectuée. L'utilisation de cette technologie pourrait permettre une réponse plus rapide face au début de bloom et une anticipation facilitée pour les pêcheurs.

Notre étude met clairement en évidence la grande importance des eaux de fond dans la contamination de *P. maximus* mais également d'autres organismes benthiques exposés aux blooms. Le monitoring des contaminations des coquilles Saint-Jacques par l'acide domoïque dans le cadre de recherches nécessite donc la considération des eaux de fond au même titre que les eaux de surface. L'utilisation de trappes à sédiments renforçant les connaissances sur les sources de contaminations par l'acide domoïque sera un atout certain pour les prochaines études tout comme l'utilisation à grande échelle de méthodes moléculaires permettant une caractérisation précise des espèces et la surveillance rapide de l'acide domoïque.

### **Impact de la taille des coquilles Saint-Jacques sur la contamination et la décontamination de l'acide domoïque**

Au cours de notre étude, les coquilles les plus petites semblent avoir dépuré plus rapidement que celles de grande taille (Chapitre 2), ces résultats sont cohérents avec ceux obtenus précédemment par Bogan et collaborateurs (Bogan *et al.*, 2007a) lors d'une étude sur un pool de 12 individus renouvelé tous les mois pendant un an et qui montrait que les petites coquilles Saint-Jacques décontaminaient plus rapidement que les grandes. Cependant, d'autres études réalisées avec des animaux prélevés à une seule occasion, par Campbell et collaborateurs (Campbell *et al.*, 2001) Arévalo et collaborateurs (Arévalo *et al.*, 1998) ne font état d'aucune relation entre la concentration en acide domoïque et la longueur de la coquille de *P. maximus*. Il n'existe à ce jour pas de consensus autour de cette question, et la variabilité inter individuelle apparaît comme un facteur qui pourrait expliquer ces différents résultats. En effet, la coquille Saint-Jacques présente des cinétiques de contamination et de décontamination qui varient fortement d'un individu à l'autre (Bogan *et al.*, 2007b), il est donc important de noter qu'il existe une très grande variabilité inter-individuelle mais aussi entre groupes d'animaux de différentes tailles, qui pourrait être expliqué par des différences entre les taux de filtration de ces individus (Bogan *et al.*, 2006). En conséquence, les coquilles de petite taille se contamineraient plus et plus rapidement que les coquilles les plus grosses (Bogan *et al.*, 2007a), chapitre 2) et dépureraient plus rapidement également (Bogan *et al.*, 2007a). Cette longue dépuration affecterait donc plus fortement les individus de taille commerciale. Pouvoir suivre de façon efficace des coquilles Saint-Jacques de groupes de tailles différentes sur le long terme permettrait d'approfondir les connaissances déjà acquises sur ce thème.

La piste des paramètres écophysiologicals pouvant jouer un rôle dans la contamination et la décontamination par l'acide domoïque des individus de tailles différentes semble importante à étudier. Obtenir les informations concernant les taux de filtration et de respiration permettrait de savoir si ces paramètres jouent un rôle dans les cinétiques de contamination et de décontamination. Si tel était le cas alors, influencer sur ces paramètres physiologiques pourrait être une voie pour accélérer la dépuración des coquilles Saint-Jacques après un bloom.

En effet, la présence de la toxine peut induire une réponse physiologique adaptative chez certains organismes contaminés. Par exemple, en présence de cellules algales toxiques du genre *Pseudo-nitzschia* et donc d'acide domoïque, les huîtres *Crassostrea virginica* diminuent leur taux de filtration ce qui leur permet de consommer moins de cellules toxiques (Mafra *et al.*, 2009, 2010a, 2010b). Le résultat de cette baisse de filtration pourrait entraîner une contamination moins importante que pour d'autres espèces de bivalves comme la coquille Saint-Jacques. L'huître *C. virginica* est également capable de procéder à un tri sélectif des particules toxiques en augmentant sa production de pseudo fèces (Mafra *et al.*, 2009). L'étude de Sauvey et collaborateurs (Sauvey *et al.*, 2021) sur le taux de filtration chez des larves de coquilles Saint-Jacques lors d'une contamination par l'acide domoïque n'a révélé aucun changement dans leur taux de filtration. Il a pourtant été démontré que *P. maximus* était capable de moduler son taux de filtration en présence de certaines microalgues toxiques, puisque ce mécanisme a été observé lors d'une contamination aux toxines paralysantes produites par la microalgue toxique *Alexandrium minutum* (Even *et al.*, 2022). A ce jour aucune explication n'a été avancée concernant cette différence de réponse physiologique entre l'exposition à la toxine PST et celle à l'acide domoïque mais il se pourrait que la coquille Saint-Jacques soit moins sensible à l'acide domoïque qu'aux PST.

### **Lien entre la présence de *Chlamydia* et la longue rétention de l'acide domoïque ?**

La présence très importante de *Chlamydia* dans la glande digestive de coquille Saint-Jacques contaminée par l'acide domoïque mise en évidence par nos analyses métabarcoding pourrait constituer un début de réponse à la longue rétention de la toxine dans cet organe. En effet, même si ce genre a été également retrouvé chez d'autres bivalves dépurant rapidement la toxine, comme *M. edulis* (Svårdh, 1999), les ASVs retrouvés au cours de nos travaux chez ces deux bivalves ne sont pas identiques (Chapitre 3). Cela traduit donc une différence au niveau de l'espèce de *Chlamydia* présente chez la moule et chez la coquille Saint-Jacques. De plus, la proportion d'ASVs de *Chlamydia* est 49 fois plus importante chez *P. maximus* que chez

*M. edulis*. Lors d'une infection, les bactéries font face à la réponse du système immunitaire de l'hôte qu'elles colonisent (Wang *et al.*, 2018). Afin de déjouer cette protection, les bactéries déploient de nombreuses stratégies afin d'échapper au système immunitaire (Hooper, 2009). Un de ces mécanismes leur permet de s'encapsuler (**Fig. 8B**). La création d'une capsule, composée de polysaccharides, offre une protection contre la phagocytose occasionnée par le système immunitaire (Nanra *et al.*, 2013). Cet état permet la persistance des cellules bactériennes ainsi que leur survie lors de conditions défavorables subies par leur hôte, comme l'absence d'acide aminé ou la sécrétion de molécules de la réponse immunologique (Hogan *et al.*, 2004). C'est notamment le cas de l'interféron- $\gamma$ , sécrété par les cellules du système immunitaire et qui font entrer *Chlamydia trachomatis* dans sa phase de persistance (**Fig. 7**) en permettant l'expression d'une enzyme dégradant le tryptophane nécessaire à la croissance de la bactérie (Taylor and Feng, 1991). Cette privation d'acide aminé entraîne alors *C. trachomatis*, en état de persistance, à relarguer une protéine, la tryptophane synthase, permettant de convertir un substrat différent en tryptophane (Fehlner-Gardiner *et al.*, 2002; McClarty *et al.*, 2007). Ceci permet la création d'un stock de tryptophane qui sera utilisé par la bactérie pour son métabolisme. Sachant que l'acide domoïque est considéré comme un acide aminé, et que les *Chlamydia* ont la capacité d'utiliser ce mode d'évasion immunitaire, il est tout à fait possible de suggérer que ce mécanisme entre en jeu lors d'une contamination par l'acide domoïque.



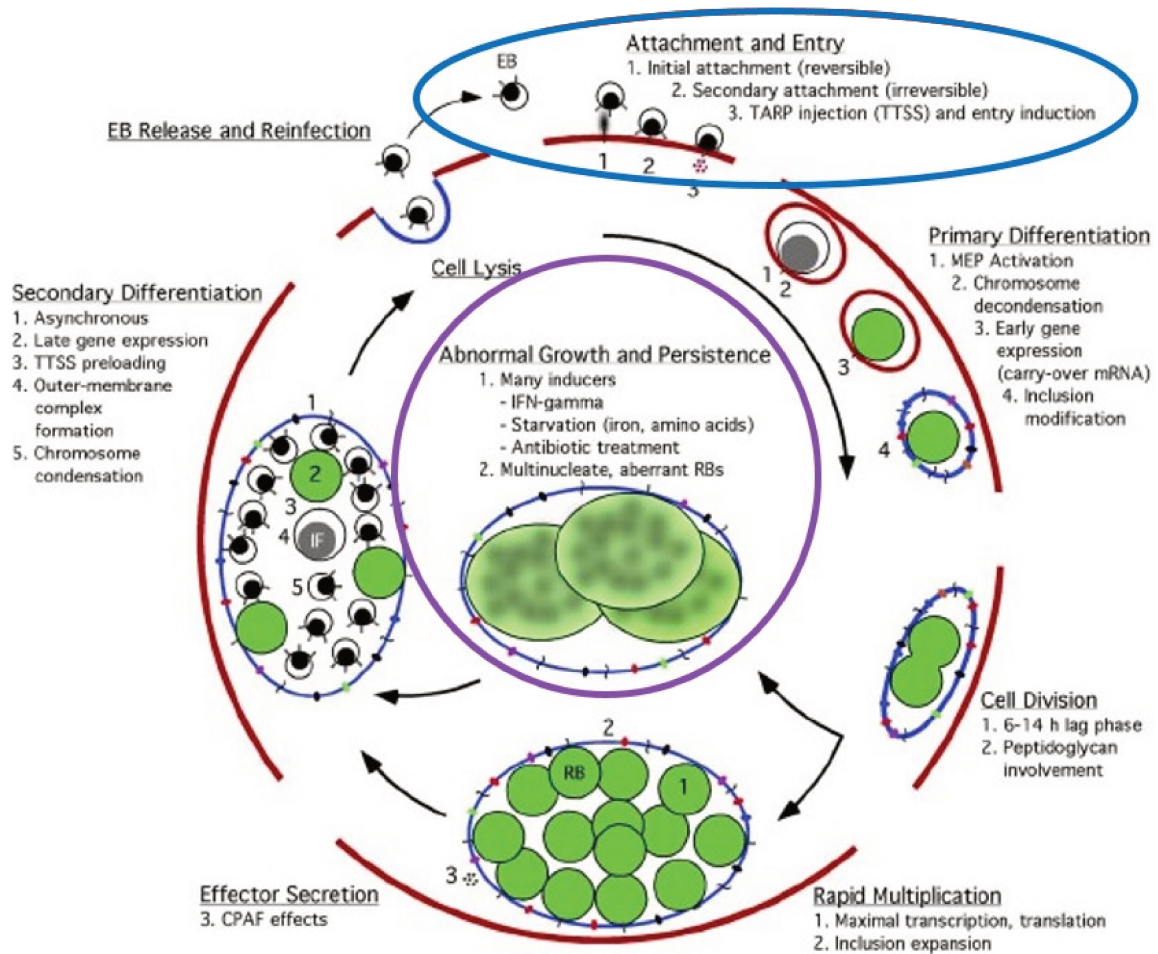


Figure 7 : Représentation schématique du cycle de développement de la chlamydia adapté de AbdelRahman et Belland (AbdelRahman and Belland, 2005). L'ellipse bleu clair en haut à droite indique la phase d'entrée de *Chlamydia* sp. dans la cellule hôte sous sa forme de corps élémentaire (EB : elementary body) qui peut être inactivée par l'utilisation de bactéries de la souche *Lactobacillus crispatus*. Le cercle violet distingue la phase de persistance enclenchée par *Chlamydia* en cas de stress pouvant être multiple (interféron gamma, carence en fer ou en acides aminés tel que le tryptophane, traitement antibiotique). Lors de cette phase les bactéries peuvent présenter plusieurs nucléus.

Pourrait-il être possible que chez *P. maximus*, où la présence de *Chlamydia* est prédominante, l'acide domoïque soit utilisé par cette dernière pour sa croissance ? Peut-on envisager que dans le cadre d'un bloom de *Pseudo-nitzschia* sp. produisant de l'acide domoïque, une réponse immunitaire face au stress imposé par ce changement de condition, entraîne alors la sécrétion de molécules provoquant l'entrée en persistance des *Chlamydia* qui, en retour, secréteraient une protéine capable de stocker l'acide domoïque ? La toxine alors séquestrée dans la glande digestive, organe chargé en bactéries, ne serait dépurée qu'au fil du temps lors de son utilisation par les bactéries pour continuer leur cycle de vie.

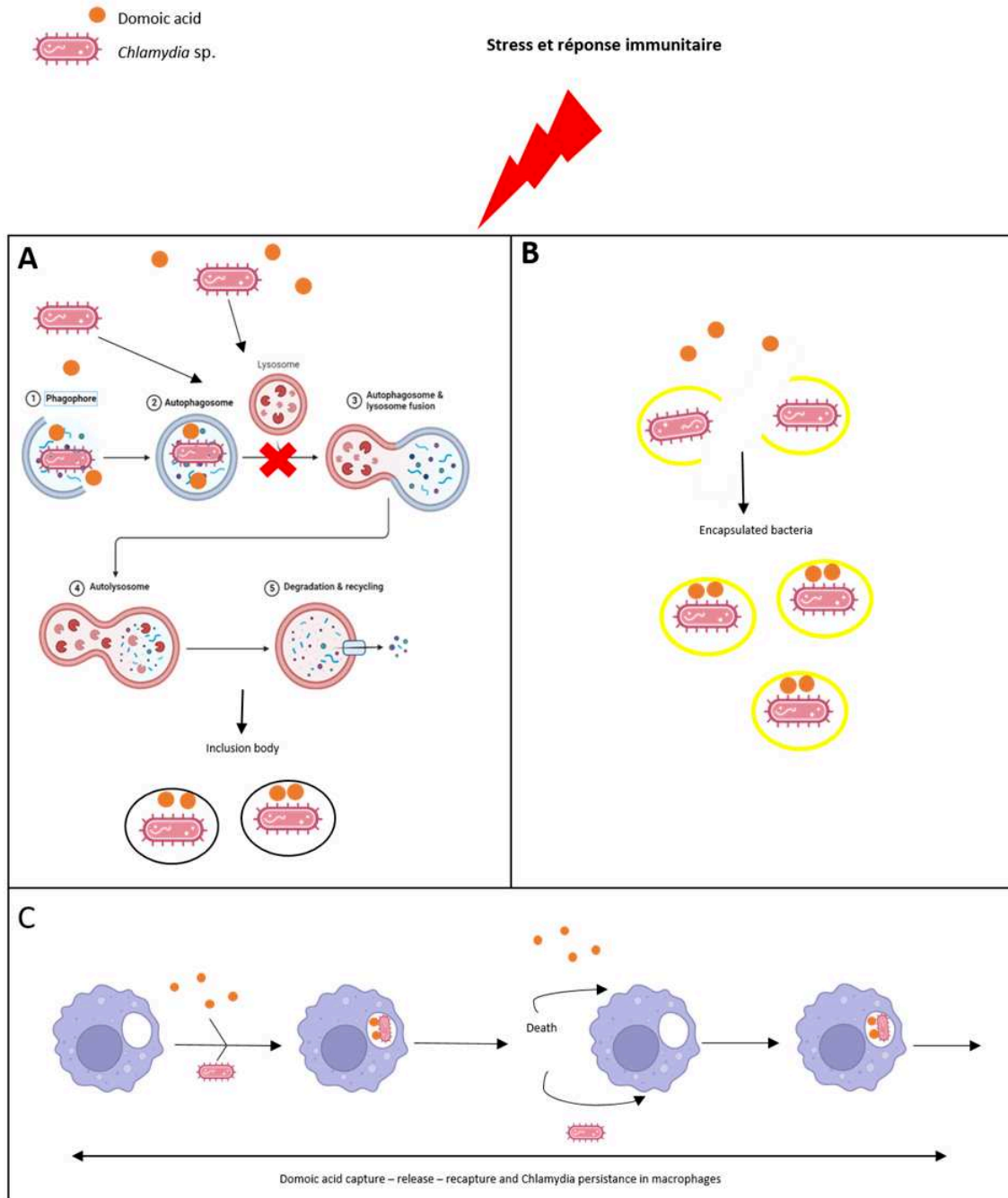


Figure 8 : Représentation schématique des trois hypothèses pouvant conduire à la longue rétention de l'acide domoïque par un mécanisme impliquant la bactérie du genre *Chlamydia*. La réponse de la bactérie face au système immunitaire peut être de bloquer la fusion entre l'autophagosome et le lysosome (entre l'étape 2 et 3) induisant ainsi des corps d'inclusion piégeant l'acide domoïque présent (A). Le genre *Chlamydia* peut également, pour déjouer le système immunitaire ou face à des conditions de stress telles qu'une carence en fer ou en acides aminés tels que le tryptophane, s'encapsuler et entrer dans une phase de persistance/dormance. L'encapsulation englobe l'acide domoïque, acide aminé qui se trouve dans l'environnement (B). Enfin, la persistance des *Chlamydia* sp. se divisant au sein des macrophages entraîne un phénomène de capture-sécrétion-recapture entraînant l'acide domoïque présent (C).

Une autre hypothèse qu'il est possible d'avancer concernant la longue rétention de l'acide domoïque qui impliquerait le rôle des *Chlamydia* sp. concerne une autre méthode pour contourner la réponse immunitaire. Ce genre bactérien est en effet capable de bloquer la fusion entre les autophagosomes et les lysosomes (Huang and Brumell, 2014) (**Fig. 8A**). Il a également été démontré que *C. trachomatis* était capable de s'accumuler dans des autophagosomes non dégradés (Al-Younes *et al.*, 2004) et de persister pour un certain temps dans les macrophages (Gracey *et al.*, 2013; Herweg and Rudel, 2016). Dans plusieurs études, à l'aide de coupes et de marquages immunohistologiques, les *Chlamydia* sont souvent décrites comme des corps d'inclusions cytoplasmiques (Renault and Cochenec, 1995). Plus récemment, des études portant sur la localisation de l'acide domoïque dans la glande digestive de bivalves ont mis en évidence que 90% de la charge en toxine était localisée dans le cytoplasme des cellules digestives (Mauriz and Blanco, 2010). Plus précisément, ce signal n'est pas totalement libre dans le cytosol mais une partie est retrouvée au sein de structures autophagiques décrites comme des corps d'inclusion par l'étude de García-Corona et collaborateurs (García-Corona *et al.*, 2022). L'origine de la longue rétention de l'acide domoïque dans la glande digestive de la coquille Saint-Jacques n'est pas connue, mais García-Corona et collaborateurs (García-Corona *et al.*, 2024a) ont proposé une comparaison intéressante avec l'encre lors du tatouage. Ils décrivent le phénomène de capture et relargage de l'encre par les macrophages (Baranska *et al.*, 2018) à l'origine de la tenue dans le temps des tatouages, comme pouvant être similaire au mécanisme sous-jacent à la dépuraison lente de l'acide domoïque, qui serait également capturé par les macrophages puis relâché sans avoir pu être excrété puis à nouveau capturé. L'autophagie et la macrophagie sont des processus analogues qui semblent impliqués dans la longue rétention de l'acide domoïque (García-Corona *et al.*, 2024b). Ces deux mécanismes seraient également impliqués dans la persistance des *Chlamydia* au sein d'un hôte (Al-Younes *et al.*, 2004; Gracey *et al.*, 2013; Herweg and Rudel, 2016). Avec ces connaissances, il est pertinent de se demander si au cours de ce processus de formation de corps d'inclusion autour des bactéries, l'acide domoïque présent dans les mêmes tissus pourrait se retrouver piégé au sein de ces structures (**Fig. 8C**). Une fois que le système immunitaire est dupé et que les bactéries sortent de cette phase, l'acide domoïque pourrait être à nouveau accessible au système de dépuraison qui reprendrait son cours. En effet, bien que la dépuraison de la toxine soit longue, elle n'est pas totalement bloquée, puisqu'une diminution est tout de même observée avec le temps, même si elle peut être masquée par un épisode de recontamination.

Pour savoir si les *Chlamydia* sp. sont impliquées dans la longue rétention de l'acide domoïque par l'un ou l'autre de ces mécanismes ou la synergie des deux, il serait très intéressant d'arriver à combiner la localisation de ces corps d'inclusion portant un marquage à l'acide domoïque avec la présence des bactéries *Chlamydia* sp. Des cryocoupes de glandes digestives de coquilles Saint-Jacques contaminées ont été envoyées à la plateforme d'histopathologie de l'Université de Rennes (H2P2) afin d'effectuer un double marquage par immunohistochimie, avec des anticorps ciblant d'une part l'acide domoïque et d'autre part les bactéries *Chlamydia*. Cette technique permettrait, par rapport au marquage immunohistochimique de l'acide domoïque réalisé sur coupe histologique classique (García-Corona *et al.*, 2022), d'une part une meilleure conservation de l'acide domoïque qui est en grande partie perdue par l'utilisation des solvants utilisés classiquement en histologie et également d'appliquer un marquage à l'aide d'anticorps dirigés contre *C. trachomatis*. Malheureusement, pour le moment cette expérience de colocalisation n'a pu être menée à bien du fait de la perte de la majorité des tissus sur les coupes lors du processus de cryo-histologie. De plus, la détection de *Chlamydia* sp. a été réalisée grâce à la technique du metabarcoding, qui permet une identification non ciblée, généralement jusqu'au niveau du genre, de toutes les bactéries présentes dans un échantillon. Ainsi, les espèces spécifiques ne sont pas nécessairement connues ou référencées, ce qui pourrait signifier que l'anticorps utilisé ne cible pas les espèces de *Chlamydia* présentes chez *P. maximus*. Étant donné que les *Chlamydia* sp. exigent un milieu spécifique pour être cultivées (Herbrink *et al.*, 1991), il n'a pas été possible d'isoler ces bactéries à partir des échantillons de glandes digestives prélevés pour l'étude sur la partie cultivable du microbiote de cet organe chez plusieurs espèces de bivalves exposés à l'acide domoïque (voir chapitre 3). L'isolement de glandes digestives de coquilles Saint-Jacques naturellement contaminées dans un milieu favorable aux *Chlamydia* pourrait permettre d'identifier précisément l'espèce en question, facilitant ainsi la corrélation avec la présence d'acide domoïque dans les tissus.

Une étude portant sur l'utilisation de souches de *Lactobacillus crispatus* pour inhiber les premières étapes de l'infection par *Chlamydia trachomatis* (Nardini *et al.*, 2016) a permis de mettre en évidence que ces bactéries *L. crispatus* semblent inactiver le premier stade de colonisation de *Chlamydia*. Comme représenté sur la figure 7, c'est lors de sa phase de corps élémentaire (elementary body en anglais, EB) qu'agit *L. crispatus* et limite ainsi l'entrée de *Chlamydia* sp. et sa colonisation dans les cellules épithéliales de l'hôte. Ceci est possible grâce à la production de métabolites extracellulaires par *L. crispatus*, notamment l'acide lactique, capables d'inactiver des pathogènes.

Si l'hypothèse d'une colocalisation entre l'acide domoïque et une ou des bactéries (Chapitre 3) se confirmait, il serait intéressant de chercher des bactéries pouvant entrer en compétition avec ces dernières afin de, peut-être, limiter leur implantation ou leur action.

### **Les bactéries comme piste pour limiter la contamination ou accélérer la dépuraction de l'acide domoïque**

L'utilisation de l'acide domoïque par certaines souches bactériennes en fait d'excellentes candidates pour améliorer/accélérer sa dépuraction par la coquille Saint-Jacques. Toutes les bactéries n'ont pas la capacité de croître en présence d'acide domoïque mais ce serait le cas de souches de *Moraxella* sp., *Alteromonas* sp. et *Pseudomonas* sp. (Stewart *et al.*, 1998). Ces souches ont été retrouvées dans les glandes digestives d'espèces de bivalves (*M. arenaria* et *M. edulis*) dépurant rapidement l'acide domoïque (Stewart *et al.*, 1998) (Chapitre 3). Il est donc possible que la coquille Saint-Jacques puisse manquer de certaines souches qui pourraient lui permettre de limiter l'accumulation de la toxine. Supplémenter *P. maximus* en bactéries métabolisent l'acide domoïque et qui coloniseraient son tractus digestif ainsi que sa glande digestive pourrait limiter la contamination de la coquille Saint-Jacques dû à l'utilisation de la toxine par les bactéries au moment de son passage dans le tractus digestif. La concentration finale dans la glande digestive de l'animal serait moins importante et la décontamination sous le seuil de sécurité sanitaire serait plus rapide et réduirait le temps de fermeture des campagnes de pêche. Mes travaux de thèse ont été menés avec pour objectif de simplifier au maximum les scénarios de dépuraction envisagés pour qu'ils nécessitent le moins de logistique et de frais possibles. Nos résultats de transfert de microbiote de la moule vers la coquille Saint-Jacques par simple exposition sont les premiers à valider l'efficacité, au moins temporaire, de cette méthode pour transmettre des bactéries exogènes à *P. maximus* (Chapitre 3). Bien qu'un système de coculture entre ces deux espèces de bivalves pourrait être mis en place de façon expérimentale, l'utilisation de probiotiques directement en écloserie apparaît comme une solution plus réaliste. Ceci est possible grâce à la présence en Bretagne de l'Écloserie du Tinduff qui produit des naissains en soutien aux stocks de coquilles Saint-Jacques. En exposant directement et régulièrement les larves de coquilles Saint-Jacques par balnéation à un probiotique, l'action des bactéries ciblées pourrait être observée que ces dernières s'implantent ou non (Dantan *et al.*, 2024). La conception de ce probiotique pourrait également comprendre des souches bactériennes ayant un effet bénéfique sur la survie des larves face à d'autres problématiques comme les infections par les bactéries du genre *Vibrio* qui menacent parfois les

écloseries (Kesarcodi-Watson *et al.*, 2016; Stevick *et al.*, 2019). L'effets des probiotiques peut également se transmettre à la génération suivante notamment chez les huîtres *C. gigas* (Fallet *et al.*, 2022). Si cet effet était également présent avec l'exposition à un cocktail probiotique établi pour la coquille Saint-Jacques cela représenterait un atout supplémentaire pour cette aide à la dépuraison.

Nos travaux ont spécifiquement identifié deux genres, *Pseudoalteromonas* et *Cellulophaga*, partagés par trois dépurateurs rapides (*M. edulis*, *D. trunculus* et *M. varia*) (Chapitre 3). Le genre *Pseudoalteromonas* en raison de sa capacité à dégrader et à réduire une autre phycotoxine, la PST produite par *Alexandrium tamarense*, offre une perspective prometteuse pour le développement de méthodes de dépuraison de l'acide domoïque. Sept souches du genre bactérien *Pseudoalteromonas* ont pu être identifiées comme ayant cette action (Donovan *et al.*, 2009, 2008). Le genre *Cellulophaga* se retrouve associé à certaines *Pseudo-nitzschia pungens* et semble influencer positivement leur production d'acide domoïque. Ces bactéries ont également causé le déclin d'une culture de *Pseudo-nitzschia australis* toxique (Sison-Mangus *et al.*, 2014), on peut donc émettre l'hypothèse que le genre *Cellulophaga* est capable de métaboliser l'acide domoïque libéré dans l'environnement.

Deux souches testées au cours de ma thèse n'ont pas donné de résultats sur la dégradation d'acide domoïque en solution, mais 12 échantillons de *Pseudoalteromonas* ont été répertoriés dans notre souchothèque et peuvent représenter autant d'espèce de *Pseudoalteromonas* différentes avec des activités sur l'acide domoïque pouvant être différentes. Notre souchothèque comporte également 8 échantillons différents identifiés comme appartenant au *Cellulophaga* et comme pour celles de *Pseudoalteromonas*, toutes devraient être séquencées pour être identifiées et chaque souche différente devrait être testée. De plus, l'effet de *Cellulophaga* peut également être dépendant de la présence de cellules de *Pseudo-nitzschia* sp. et non directement de l'acide domoïque. Un test d'exposition des coquilles Saint-Jacques aux bactéries en présence de cellules *Pseudo-nitzschia australis* toxiques pourrait s'avérer utile pour tester le potentiel probiotique de ce genre bactérien.

La collection de bactéries produite dans le cadre de mes travaux de thèse compte 179 souches bactériennes pouvant représenter de nombreuses possibilités et de combinaisons bactériennes à tester sur de l'acide domoïque en solution.

### Conclusion

L'ensemble de ces travaux a permis d'approfondir les connaissances sur les différentes sources de contamination de la coquille Saint-Jacques, *P. maximus*, en rade de Brest en démontrant l'implication des cellules de *Pseudo-nitzschia* sp. et de l'acide domoïque présents à la fois en surface, mais aussi et surtout au fond de la colonne d'eau, à l'interface eau/sédiment où vit la coquille Saint-Jacques (**Figure 9**). L'espèce *Pseudo-nitzschia australis* est également confirmée comme étant l'espèce toxique responsable des plus grands épisodes de contamination des coquilles et de la fermeture des campagnes de pêche. Ceci permet d'orienter les prochaines études d'aide à l'accélération de la dépuraison de l'acide domoïque chez *P. maximus* vers d'autres pistes. Ces résultats serviront également dans un futur proche à mettre en place un modèle de croissance et de contamination/décontamination de *P. maximus* prenant en compte les sources de contamination ainsi que la réponse écophysologique de la coquille (travaux de thèse d'Eline Le Moan) afin, *in fine*, d'estimer entre autres le temps de dépuraison nécessaire aux stocks de coquilles après un bloom. De précieuses informations qui aideront à la prise de décision pour les pêcheurs.

Cette thèse a par ailleurs fait progresser la recherche de propositions d'aide à la dépuraison de l'acide domoïque chez *P. maximus* en émettant l'hypothèse qu'une différence de microbiote pouvait être en partie à l'origine des différences de contamination/dépuraison rencontrées entre différentes espèces de bivalves. En mettant à disposition une collection de bactéries cultivables pouvant être testées sur de l'acide domoïque en solution puis sur des larves de *P. maximus* en éclosion, ce travail est une avancée non négligeable vers le développement d'un probiotique agissant soit sur la contamination et/ou la décontamination de l'acide domoïque.

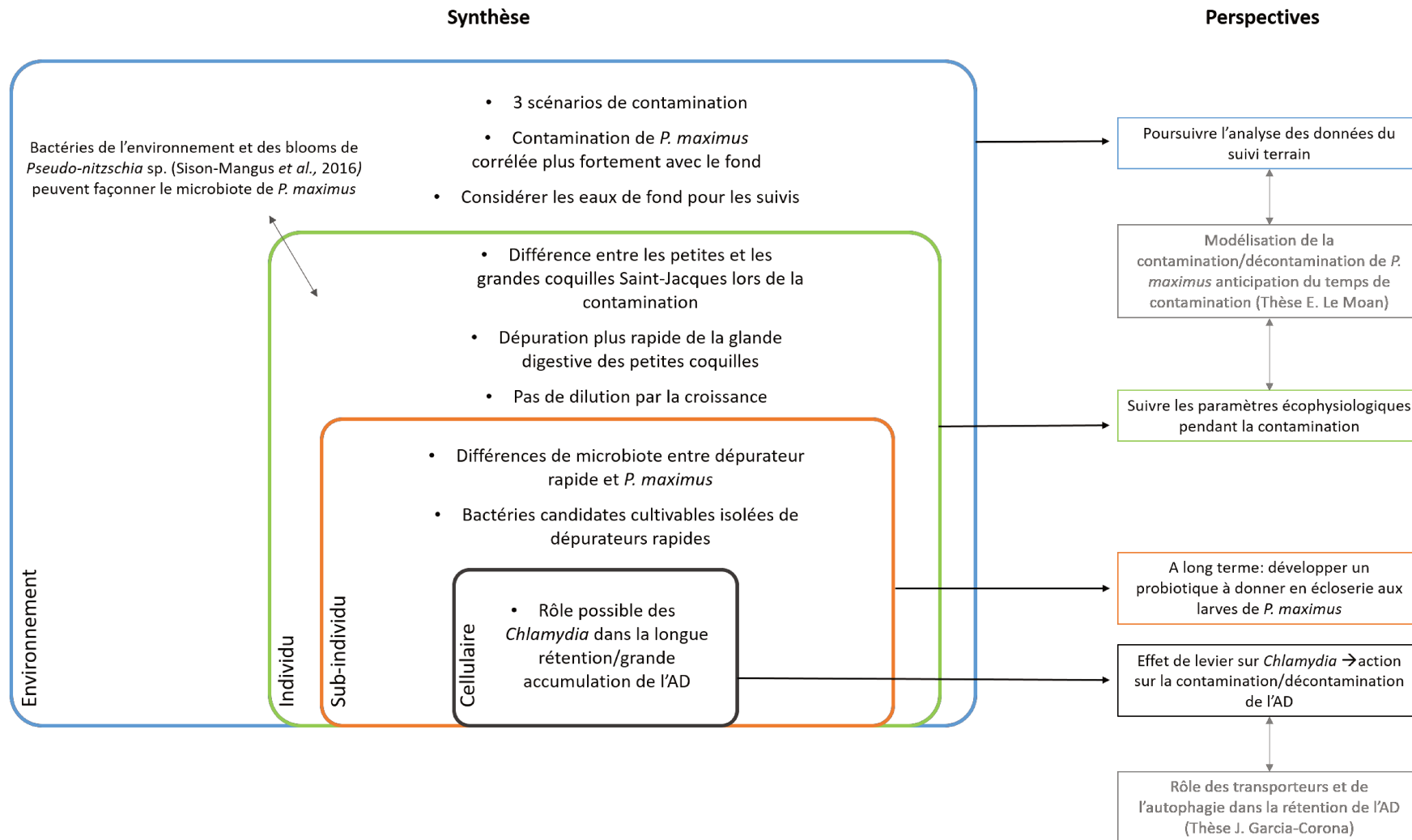


Figure 9 : Schéma de synthèse des résultats mis en avant dans cette thèse ainsi que des perspectives qui en résultent et des liens avec les autres thèses du projet MASCOET.





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# ANNEXES

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## Annexe I – Bilan de thèse

### Articles en préparation (4)

Seven years monitoring of *Pseudo-nitzschia sp.* and domoic acid in surface and bottom waters of the bay of Brest and associated King scallop (*Pecten maximus*) contamination.

**Margot Deleglise**, Tania Hernandez-Farinas, Aude Leynaert, Gaspard Delebecq, Amélie Derrien, Aouregan Terre-Terillon, Béatriz Beker, Malwenn Lassudrie, Justine Castrec, Marc Long, Erwan Amice, Thierry Le Bec, Julien Thébault, Laurent Chauvaud, Caroline Fabioux, Hélène Hegaret

Exploring microbiota transfer as a potential to mitigate the long retention of domoic acid in King scallop (*Pecten maximus*).

**Margot Deleglise**, Cyril Noël, Hélène Hegaret, Guillaume Barnouin, Adeline Bidault, Clément Offret, Malwenn Lassudrie, Caroline Fabioux.

Isolation and functional testing of three cultivable bacteria for domoic acid depuration.

**Margot Deléglise**, Hélène Hegaret, Adeline Bidault, Alix Drouart, Caroline Fabioux.

Domoic acid decontamination in adult King Scallops of two different size group: Insights from a two-month study.

**Margot Deléglise**, Jean Vanmaldergem, Amélie Derien, Eline Le Moan, Florian Breton, Caroline Fabioux, Hélène Hegaret & Jonathan Flye-Sainte-Marie.

### Articles annexes à la thèse (2)

García-Corona, J.L., Hegaret, H., **Deléglise, M.**, Marzari, A., Rodríguez-Jaramillo, C., Foulon, V., Fabioux, C. (2022). First subcellular localization of the amnesic shellfish toxin, domoic acid, in bivalve tissues: Deciphering the physiological mechanisms involved in its long-retention in the king scallop *Pecten maximus*. *Harmful Algae*, 116, 102251. <https://doi.org/10.1016/j.hal.2022.102251>.

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Vanmaldergem, J., García-Corona, J. L., **Deléglise, M.**, Fabioux, C., Hegaret, H. (2023). 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*. *Aquatic Living Resources*, 36, 14. <https://doi.org/10.1051/alr/2023011>.

### **Présentations orales en conférences nationales et internationales (6)**

How to contribute to scallop (*Pecten maximus*) management after *Pseudo-nitzschia australis* blooms ? A study of domoic acid contamination and decontamination kinetics.

**Margot Deléglise**, Caroline Fabioux, Malwenn Lassudrie, Elodie Fleury, Helene Hegaret. Conférence annuelle du GdR Phycotox, Novembre 2020 (Distanciel).

Scallop contamination by domoic acid: is surface water monitoring for *Pseudo-nitzschia* sufficient?

**Margot Deléglise**, Aude Leynaert, Justine Castrec, Marc Long, Amélie Derrien, Aouregan Terre-Terillon, Erwan Amice, Thierry Le Bec, Julien Thébault, Laurent Chauvaud, Caroline Fabioux, Hélène Hegaret. 19th International Conference on Harmful Algae (ICHA), Octobre 2021 (Distanciel)

Investigating leads to understand the long contamination of King Scallop (*Pecten maximus*) and the importance of considering bottom water in monitoring.

**Margot Deléglise**, Aude Leynaert, Gaspard Delebecq, Justine Castrec, Marc Long, Malwenn Lassudrie, Amélie Derrien, Aouregan Terre-Terillon, Erwan Amice, Thierry Le Bec, Julien Thébault, Laurent Chauvaud, Caroline Fabioux, Hélène Hegaret. Conférence annuelle du GdR Phycotox, Mai 2022 (Brest).

Etude du transfert de microbiote digestif et fécal de la moule (*M. edulis*) vers la coquille Saint-Jacques (*P. maximus*).

**Margot Deléglise**, Cyril Noël, Guillaume Barnouin, Clément Offret, Malwenn Lassudrie, Hélène Hegaret, Adeline Bidault, Caroline Fabioux. Journée de la ZABRI, Novembre 2022 (Brest).

Testing the transfer of fecal and digestive gland microbiotas between mussels (*Mytilus edulis*) and the King scallop (*Pecten maximus*) for accelerated depuration of domoic acid.

**Margot Deléglise**, Cyril Noël, Guillaume Barnouin, Clément Offret, Malwenn Lassudrie, Hélène Hegaret, Adeline Bidault, Caroline Fabioux. 115th National Shellfisheries Association (NSA), Mars 2023 (Batlimore, USA)

Investigating the potential for rapid domoic acid depuration through the transfer of fecal and digestive gland microbiotas between the Blue mussel (*Mytilus edulis*) and the King scallop (*Pecten maximus*).

**Margot Deléglise**, Cyril Noël, Guillaume Barnouin, Clément Offret, Malwenn Lassudrie, Hélène Hegaret, Adeline Bidault, Caroline Fabioux. Conférence annuelle du GdR Phycotox & GIS Cyanobacteries, Mai 2023 (Nantes).

### **Présentation de poster (2)**

Etude des cinétiques de contamination et de décontamination de l'acide domoïque chez la coquille Saint-Jacques *Pecten maximus*.

**Margot Deléglise**, Hélène Hégaret, Caroline Fabioux, Malwenn Lassudrie, Jonathan Flye Sainte Marie. Journées de l'école doctorale 2022 (Brest).

Accelerating domoic acid depuration in King scallop *Pecten maximus* through the use of probiotics?

**Margot Deléglise**, Cyril Noël, Guillaume Barnouin, Clément Offret, Malwenn Lassudrie, Hélène Hegaret, Adeline Bidault, Caroline Fabioux. 20th International Conference on Harmful Algae (ICHA), Novembre 2023 (Hiroshima, JP)

### **Participations dans le cadre du projet MASCOET (5)**

Étude des cinétiques de contamination et de décontamination de l'acide domoïque chez *Pecten maximus*.

**Margot Deléglise**, Caroline Fabioux, Malwenn Lassudrie, Elodie Fleury, Helene Hégaret. Réunion annuelle du projet MASCOET, Novembre 2020 (Distanciel).

Étude des cinétiques de contamination et de décontamination de l'acide domoïque chez *Pecten maximus*.

**Margot Deléglise**, Caroline Fabioux, Malwenn Lassudrie, Elodie Fleury, Helene Hégaret. Réunion annuelle du projet MASCOET, Novembre 2021 (Brest).

Étude des cinétiques de contamination et de décontamination de l'acide domoïque chez *Pecten maximus*.

**Margot Deléglise**, Caroline Fabioux, Malwenn Lassudrie, Elodie Fleury, Helene Hégaret. Réunion annuelle du projet MASCOET, Février 2023 (Nantes).

Le Fur Ines, Bruneau Audrey, Blanchet Aline, Cugier Philippe, **Deleglise Margot**, Derrien Amelie, Fabioux Caroline, Guesdon Stephane, Gueux Aurore, Hegaret Helene, Hess Philipp, Lassudrie Malwenn, Lemoine Maud, Neaud-Masson Nadine, Piraud Aude, Terre Terrillon Aouregan (2022). Expertise sur le retour d'expérience de la contamination ASP dans les Pertuis Charentais fin 2021. DDTM 17 - Direction Départementale des Territoires et de la Mer de Charente-Maritime, La Rochelle, Ref. ODE/LITTORAL/LER/PC-22.007 Expertise Ifremer N° 22-010, 34p.<https://archimer.ifremer.fr/doc/00797/90883/>

Réunions comité des pêches (Novembre 2020 ; Mai 2023)

### **Organisation (1)**

Comité d'organisation local de la conférence annuelle du GdR Phycotox 2022 (Brest).

### **Encadrement de stagiaires (4)**

Pierre Gouasdoué, Master 1 Microbiologie fondamentale et appliquée, UBO (Janvier-Février 2021).

Jean Vanmaldergem, Reprise d'étude (Janvier-Juin 2021).

Guillaume Barnouin, Deuxième année du cursus ingénieur AgroParisTech (Mai-Juillet 2021).

Alix Drouart, Master 1 Sciences de la Mer et du Littoral, Mention Biologie, Parcours Biologie des Organismes et des Populations, UBO (Janvier-Février 2022).

**Enseignements (2)**

64H TP/TD (Biologie Animale L1 + Analyse fonctionnelle du génome L3). Université de Bretagne Occidentale. Année universitaire 2021-2022

64H TP/TD (Analyse fonctionnelle du génome L3). Université de Bretagne Occidentale. Année universitaire 2022-2023

**Formations (9)**

Cytométrie en flux (2020, Brest).

Techniques de Microscopie électronique (2020, Brest).

Introduction à l'intégrité scientifique (2020, Distanciel).

Formation à la Publication scientifique (2020, MOOC).

DocuSciences - Médiation de problématiques scientifiques, sociales & environnementales par l'usage des techniques du cinéma documentaire (2021, Brest).

Entrepreneuriat - et table ronde avec des docteurs dirigeants d'entreprise ou en entreprise (2021, Brest).

Hygiène et Sécurité - Formation Hygiène et sécurité (NEO) (2021, Distanciel).

Formation métabarcoding, plateforme bio-informatique de Roscoff ABiMS et Ifremer SeBIMER (2021, Roscoff).

Utilisation de l'HPLC (2021, Ifremer Concarneau et LEMAR Brest)

Habilitation à conduire un autoclave (2023, LEMAR Brest).

**Financements obtenus (5)**

Bourse GdR Phycotox participation ICHA 2021 (370€)

Financement IUEM pour un stagiaire de M2 pour 2023 (financement rendu car analyses métabarcoding réalisées en collaboration avec le SeBIMER, Ifremer)

Projet ZABrI 2022 – Traitement des échantillons métabarcoding par la plateforme Genome Quebec (2200€)

Projet AAP blanc IUEM 2022 – Traitement des échantillons métabarcoding par la plateforme plateforme Génome Quebec (3000€)

Bourse mobilité sortante Isblue NSA 2023 (1782€)

Bourse GdR Phycotox inscription ICHA 2023 (370€)



# Annexe II – Article Vanmaldergem *et al.* (2023)

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PERSPECTIVE NOTE

OPEN ACCESS

## 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-nitzschia* (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<sup>-1</sup>) for 6 days. HPLC toxin quantification analyses did not reveal 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; LaBarre 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  $20 \text{ mg kg}^{-1}$  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* (Novacek 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 re-intoxication 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  $\text{mg DA kg}^{-1}$ ) 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* ( $10.7 \pm 0.7 \text{ cm}$ ;  $231 \pm 46 \text{ g}$ ) were collected from a natural bed by dredging at Roscanvel ( $48^\circ 18' 3.408'' \text{ N}$ ,  $4^\circ 32' 23.108'' \text{ O}$ ) at the entrance to the Bay of Brest, France, on the 8th of April 2021, during a bloom of *Pseudo-nitzschia australis* ( $1 \times 10^5 \text{ cells L}^{-1}$ ) 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 *Isochrysis lutea* (clone *T.iso*) (Bendif et al., 2013) at a concentration of  $5 \times 10^8 \text{ cells scallop}^{-1} \text{ day}^{-1}$ .

### 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  $1\text{-}\mu\text{m}$ -filtered seawater. The culture was performed in 6-L glass bottles before being inoculated into 300-L acrylic cylinders maintained at  $18^\circ \text{C}$  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<sup>®</sup>, Darmstadt, Germany) into 1 L of Milli-Q water and stored at 5 °C at a final concentration of 100 g L<sup>-1</sup>.

### 2.4 Experimental design

After acclimatization, the 24 scallops were distributed to six 20 L plastic tanks ( $n=4$  scallops tank<sup>-1</sup>) 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<sup>-1</sup>) for 6 days with *Tiso* at the same concentration of 10<sup>5</sup> cells mL<sup>-1</sup> using a peristaltic pump, thus reaching 5 × 10<sup>6</sup> cells scallop<sup>-1</sup> day<sup>-1</sup>. Treated scallops were all fed similarly, i.e. continuously (flow rate of 14 mL min<sup>-1</sup>) for 6 days with *Tiso* at the same concentration of 10<sup>5</sup> cells mL<sup>-1</sup> with an addition of NAC at 250 mg L<sup>-1</sup> in their ration using a peristaltic pump, thus reaching respectively 5 × 10<sup>8</sup> cells scallop<sup>-1</sup> day<sup>-1</sup> and 1.25 g NAC scallop<sup>-1</sup> day<sup>-1</sup>. NAC treated scallops were thus supplied with NAC, incorporated in their algal ration, at a concentration of 250 mg L<sup>-1</sup>, 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<sup>-1</sup>. 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<sup>-1</sup>. The LODs of this HPLC-UV method ranged from 0.2 to 1 mg DA kg<sup>-1</sup> 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).

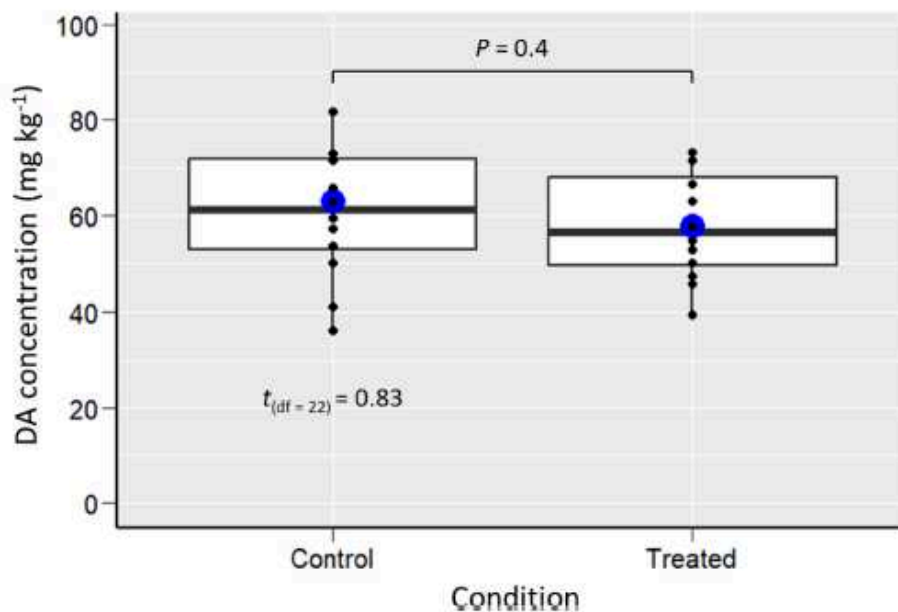
## 3 Results

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<sup>-1</sup>) compared to those of the control group (63.03 ± 18.20 mg DA kg<sup>-1</sup>) 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<sup>-1</sup> 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<sup>-1</sup>). 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<sup>-1</sup>) 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 ( $250 \text{ mg L}^{-1}$ ) (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 ( $4.6 \text{ mg DA kg}^{-1}$ ). 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  $7.6 \text{ mg kg}^{-1}$  in the DG and  $1 \text{ mg kg}^{-1}$  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  $250 \text{ mg L}^{-1}$ ). Depuration was only enhanced with the highest concentration of NAC tested,  $250 \text{ mg L}^{-1}$ . After 2 days, the estimated DA concentration in the adductor muscle was twice as low ( $0.327 \text{ mg kg}^{-1}$ ) as in the control group ( $0.771 \text{ mg kg}^{-1}$ ) or the  $50 \text{ mg L}^{-1}$  group ( $0.789 \text{ mg kg}^{-1}$ ). After 7 days, DA concentration in the adductor muscle was twenty times lower ( $0.02 \text{ mg kg}^{-1}$ ) than in the control group ( $0.402 \text{ mg kg}^{-1}$ ) or in the  $50 \text{ mg L}^{-1}$  group ( $0.436 \text{ mg kg}^{-1}$ ). 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  $\sim 90\%$ , 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 ( $4.6 \text{ mg DA kg}^{-1}$ ) 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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Annexe III – Article Garcia-Corona *et al.* (2023)

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Original Article

First subcellular localization of the amnesic shellfish toxin, domoic acid, in bivalve tissues: Deciphering the physiological mechanisms involved in its long-retention in the king scallop *Pecten maximus*

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## ABSTRACT

Domoic acid (DA), the phycotoxin responsible for amnesic shellfish poisoning (ASP), is an excitatory amino acid naturally produced by at least twenty-eight species of the bloom-forming marine diatoms *Pseudo-nitzschia* spp. Suspension feeders, such as bivalve mollusks, can accumulate and lengthy retain high amounts of DA in their tissues, threatening human health and leading to extensive-prolonged fishery closures, and severe economic losses. This is particularly problematic for the king scallop *Pecten maximus*, which retains high burdens of DA from months to years compared to other fast-depurator bivalves. Nonetheless, the physiological and cellular processes responsible for this retention are still unknown. In this work, for the first time, a novel immunohistochemical techniques based on the use of an anti-DA antibody was successfully developed and applied for DA-detection in bivalve tissues at a subcellular level. Our results show that in naturally contaminated *P. maximus* following a *Pseudo-nitzschia australis* outbreak, DA is visualized mainly within small membrane-bounded vesicles (1 – 2.5 µm) within the digestive gland cells, identified as autophagosomic structures by means of immunoelectron microscopy, as well as in the mucus-producing cells, particularly those from gonad ducts and digestive tract. Trapping of DA in autophagosomes may be a key mechanism in the long retention of DA in scallops. These results and the development of DA-immunodetection are essential to provide a better understanding of the fate of DA, and further characterize DA contamination-decontamination kinetics in marine bivalves, as well as the main mechanisms involved in the long retention of this toxin in *P. maximus*.

## 1. Introduction

Up to date, fifty-two bloom-forming species of diatoms of the genus *Pseudo-nitzschia* have been identified in all the oceans around the world (Lelong *et al.*, 2012; Bates *et al.*, 2013), and at least twenty-eight of these are capable of synthesizing domoic acid (DA), an extremely dangerous amnesic phycotoxin responsible for amnesic shellfish poisoning (ASP) in humans (Lundholm *et al.*, 2009; Trainer *et al.*, 2012; Zabaglio *et al.*, 2016; Basti *et al.*, 2018). This toxin is a water-soluble amino acid, which acts as a potent neurotransmitter binding to the N-methyl-D-aspartate receptors in neurons of the hippocampus. DA is a structural analog of glutamic acid, proline, and glycine, three neurotransmitters targeting the

NMDA-receptors essential to memory and synaptic plasticity, exhibiting respectively a three-fold to 100 fold higher affinity (Zaman *et al.*, 1997; Lefebvre and Robertson, 2010; Zabaglio *et al.*, 2016).

In the last two decades, *Pseudo-nitzschia* blooms have become more intense and frequent worldwide (Lelong *et al.*, 2012; Delegrange *et al.*, 2013), affecting large exploitable populations of suspension-feeding fish and molluscs, which are the main vector of ASP toxin to higher levels of the food chain, since they can accumulate large amounts of DA in their tissues through their filter-feeding activity (Trainer *et al.*, 2012; Hallegraeff, 2017; Basti *et al.*, 2018). Given the toxicity of DA, and as its presence in seafood represents a potential risk for human health, several countries have successfully established monitoring programs in places

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where *Pseudo-nitzschia* blooms are recurrent and intense (Lelong et al., 2012), and also an international sanitary threshold of 20 mg DA kg<sup>-1</sup> to regulate the maximum allowable amount of this toxin in bivalves (EFSA Panel on Contaminants in the Food Chain, 2009).

The rates of accumulation and depuration of DA in bivalves are species-specific and highly variable (Blanco et al., 2006; Bogan et al., 2007). Therefore, the incidence of toxigenic *Pseudo-nitzschia* blooms on the harvest of natural beds depends on the balance between the kinetics of assimilation and elimination of the toxin (Álvarez et al., 2020; Blanco et al., 2020). In this sense, bivalves have been broadly classified into two wide categories, rapid and slow DA detoxifiers. The former depurate the toxin within days to weeks and includes some species of mussels as *Mytilus galloprovincialis* (Blanco et al., 2002), *M. edulis* (Nováček et al., 1992; Mafra et al., 2010; Brennan et al., 2017), and *Perna conchalis* (MacKinnon et al., 1993), oysters such as *Crassostrea virginica* (Mafra et al., 2010) and *C. gigas* (Jones et al., 1995), and pectinids like *Argopecten purpuratus* (Álvarez et al., 2020). The slow depurators can take months to years to depurate the DA. The main examples are some commercially important bivalves like *Pecten maximus* (Blanco et al., 2002; Blanco et al., 2006; Brennan et al., 2017), *Placopecten magellanicus* (Wohlgemuth et al., 1992; Douglas et al., 1997), *Siliqua patula* (Homer et al., 1993), and *Spondylus cruentus* (Ha et al., 2006).

The king scallop *P. maximus* is a high-valuable resource in Europe, and the third most important fishery species in France, with annual catches above 60,000 tons yielding a total of 87 million euros in 2017 (FAO, 2020). Nonetheless, the exploitation of this species is particularly problematic since during blooms of toxigenic *Pseudo-nitzschia* species, scallops can accumulate amounts up to ~3,000 mg DA kg<sup>-1</sup> in the digestive gland (Blanco et al., 2006), and lengthy retain them, even for years, due to its extremely low depuration rates, from 0.025 to 0.007 d<sup>-1</sup> (Blanco et al., 2002; Blanco et al., 2006). Considering the slow depuration and the risk for human health, these contamination episodes lead to extensive-prolonged fishery closures, and consequently severe economic losses.

More than 90% of the DA burdens are accumulated in the non-edible tissues of the scallops (Blanco et al., 2006). It has been proposed that DA is mainly in “free-soluble” form in the cytoplasm of the digestive gland cells (Mauris and Blanco, 2010), and especially in the large digestive (absorptive) cells, responsible of the intracellular digestion of the pinocytized particulate matter using a complex enzymatic equipment in *P. maximus* (Beninger and Le Pennec, 2016). Hence, the digestive cells could have a particular contribution to the high accumulation of DA in the digestive gland (Blanco et al., 2020). The long retention time of the toxin has been hypothesized to be due to the lack of some efficient membrane transporters in *P. maximus* (Mauris and Blanco, 2010), or the presence of some high and low-affinity glutamate receptor as the present in the razor clam *Siliqua patula* (Trainer and Bill, 2004). Nevertheless, these hypotheses has not been confirmed yet. Despite the ecological and economic consequences associated with high accumulation of DA in scallops, the mechanisms underlying such a long retention of DA in *P. maximus* are still poorly understood. Hence, the aim of this work was to develop an immunohistochemical method to detect DA at the sub-cellular level in contaminated *P. maximus* tissues and thus decipher the subcellular mechanisms involved in its accumulation and long-retention.

## 2. Materials and methods

### 2.1. Biological material and sampling

Twenty adult *Pecten maximus* scallops (9.8 ± 0.1 cm shell length; 171.5 ± 5 g total weight) were collected by dredging from natural beds at three different sites in the west coast of Brittany, France. Six animals were obtained from the Bay of Concarneau (CN) in November 2019 (47° 52' 30.07" N, 3° 55' 20.82" W), and seven more from Camaret-sur-Mer (CM; 48° 26' 33.0096" N, 4° 35' 49.6104" W) in May 2021, after

toxigenic *Pseudo-nitzschia* blooms. Additionally, seven scallops were collected from the Bay of Brest (BB) in December 2020 (48° 19' 11" N, 4° 26' 33" W) and used as negative controls since no ASP outbreaks had recently been documented in this area.

Whole soft-bodies were carefully excised from the shells. The organs were then dissected in two groups: a) digestive gland (DG), and b) rest of tissues (RT) which included the gonad, the muscle, the heart, the kidney, the foot, gills and the mantle. As mentioned above, the digestive gland accumulates up to 90% of total domoic acid (DA) burdens (Blanco et al., 2020); for this reason, this organ was first carefully dissected and separated from the RT to avoid any transfer of toxin between organs. Consequently, the DG was separated into three pieces for subsequent histology, toxin quantification, and transmission electron microscopy analysis, as described below. The RT section was used for histology.

### 2.2. Toxin extraction and quantification by high performance liquid chromatography (HPLC)

Since the digestive gland accumulates most of DA, only this tissue was used for DA quantification in this work. For all 20 individuals, DA was extracted from scallop digestive gland following the procedure described by Quilliam et al. (1995). Frozen samples (-20°C) were homogenised from 200 ± 5 mg of tissue in 1 mL of MeOH:MQ water (1:1, v/v) using a Laboratory Mixer Mill MM 400 system (Retsch® Fisher Scientific, Illkirch-Graffenstaden, FR) at 30 Hz/s for 10 min maintaining them in an ice bath. The extract was 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. An aliquot of 200 µL was filtered through a 0.2 µm nylon centrifugal filter (VWR International, Radnor, PA, USA) at 10,000 g for 5 min, at 4°C. Since there may be substantial DA degradation in aqueous solutions stored in regular freezer (Thomas et al., 1990), the filtered extracts were stored in amber-glass autosampler vials (Thermo Scientific, Rockwood, TN, USA) at -20 °C for two days and analysed all at the same time.

All fractions obtained were analysed using a Thermo Scientific (Sunnyvale, CA, USA) HPLC System with an UV spectrophotometer Waters 996 PDA-UV detector, using a C18 reverse phase column (5 µm, 250 × 4.6 mm, Phenomenex). The separation was carried out using a mobile phase consisting of eluent A (Distilled water + 0.1 % TFA) and eluent B (ACN + 0.1 % TFA) with gradient conditions from 5 to 20% ACN in 20 min at a flow rate of 1 mL min<sup>-1</sup>, with an injection volume of 20 µL. The column temperature was maintained at 40°C. A calibration curve was generated by serial dilutions in MeOH:H<sub>2</sub>O (1:1, v/v) until concentrations of 0.2, 0.5, 1.0, 2.0, 4.0 and 8 µg DA mL<sup>-1</sup> ( $r = 0.99$ ) of certified DACS-1C DA standards obtained from National Research Council (Halifax, Canada). Thereupon, DA concentration was computed by comparing the absorbance at 242 nm of the chromatographic peaks of the samples with those of the reference solutions once it was checked that the retention time and the absorbance spectrum were the same. The LODs of this HPLC-UV method ranged from 0.2 to 1 mg DA kg<sup>-1</sup> tissue.

### 2.3. Histology and immunohistochemical staining of domoic acid

For all 20 scallops, the piece of digestive gland dedicated to histology (DG) and the rest of the tissues (RT) were separately fixed in Davidson solution for 24 h (Kim et al., 2006), and preserved in Ethanol 70 % at 4°C until processing. Then, tissue samples were dehydrated in ethanol series, cleared in xylene, embedded in paraffin (Paraplast Plus, Leica Biosystems, Richmond, IL, USA), thin-sectioned (4 µm), mounted in poly-sine coated glass-slides (Sigma-Aldrich, St. Louis, MO, USA) and dried overnight at 37°C (Couta & Couta, 2012), as detailed in Table 1. A series of 4 consecutive sections was performed for each samples, which were used for (i) immunohistochemical detection of DA (test and negative control), (ii) multichromatic staining and (iii) Hematoxyline/eosin staining.

Sections were deparaffinized and rehydrated in regressive series of



**Table 1**  
Full stepwise sequence of the immunohistochemical staining method.

Step	Reagent/Solution	Duration	Temperature
<i>Tissue processing</i>			
Fixation	Davidson solution	24 to 48 h	~ 4°C
Preservation	Ethanol 70%	Days/ months	~ 4°C
Dehydration	Ethanol 80%, 95% and 100%	8 × 1 h	~ 20°C
Clarifying	Clarlal	2 × 1 h	~ 20°C
Impregnation	Paraffin	Overnight	~ 60°C
<i>Staining</i>			
Deparaffinization	Clarlal	2 × 3 min	~ 20°C
Hydration	Ethanol 100%, 95% and 80%	5 × 3 min	~ 20°C
Antigen retrieval	Universal HIER reagent 1 ×	3 min	~ 120°C
Wash	Washing buffer <sup>a</sup>	3 × 5 min	~ 20°C
Peroxidase quenching	Blocking peroxidase solution	2 h	~ 20°C
Wash	Washing buffer	2 × 5 min	~ 20°C
1st immune-staining	Primary Ab anti-DA	Overnight	~ 4°C
Wash	Washing buffer	2 × 5 min	~ 20°C
2nd immune-staining	Secondary Ab HRP conjugated	1 h	~ 37°C
Wash	Washing buffer	2 × 5 min	~ 20°C
Revelation	DAB+ substrate	10 min	~ 20°C
Wash	Washing buffer	2 × 5 min	~ 20°C
Counterstaining	Hematoxylin	1 min	~ 20°C
Rinse	Tap water	A few dips	~ 20°C

<sup>a</sup> TBS is recommended over PBS in washing buffer to get a cleaner background. 0.025% Triton X-100 in the TBS reduces surface tension, allowing reagents to cover the tissue section easily.

Ab = antibody

ethanol before immunohistochemical staining (Table 1). Following preliminary trials, the final procedure employed for immunostaining was performed as described below. An antigen retrieval step was applied in order to break potential methylene bridges formed during formalin-fixation and expose antigenic sites to allow the antibodies epitope to bind. For this, sections were placed in the Universal HIER Antigen Retrieval Reagent (abcam®, Cambridge, UK) diluted in MQ water in a ratio 1:10 (v/v), heated using a pressure cooker until full pressure for 3 min, and subsequently rinsed in washing buffer (TBS 20 mM, NaCl 150 mM, pH 7.6, with 0.025% Triton<sup>TM</sup> X-100). In order to quench endogenous peroxidase activity, samples were treated with a Hydrogen Peroxide Blocking Solution (abcam®, Cambridge, UK) at room temperature, and washed in washing buffer.

A polyclonal primary antibody anti-DA (abcam®, Cambridge, UK) was diluted (1:1,000) in TBS 1 × with 1% BSA, applied on slides, and incubated in the dark overnight. Sections were rinsed in washing buffer and then incubated in the dark for 1h with the HRP sharped IgG Goat anti-Rabbit secondary antibody (abcam®, Cambridge, UK) diluted (1:10,000) in TBS 1 × with 1% BSA. Immunohistochemistry experimental conditions, as well as antibody optimization-dilutions are detailed in Table 2.

Samples were then washed and revealed with diaminobenzidine (DAB+ Chromogen Substrate Kit, abcam®, Cambridge, UK) for 10 min in the dark. Finally, slides were rinsed in washing buffer, counterstained with hematoxylin, and mounted in Faramount Aqueous Medium (Dako®, Carpinteria, CA, USA). The complete version of the suggested immunohistochemical procedure is presented in Table 1.

Additionally, a series of slides from the same samples were stained with a multichromic procedure according to Costa and Costa (2012). This technique consists in a combination of Alcian Blue and Periodic Acid-Schiff's for the demonstration of acid mucopolysaccharides and neutral glycoconjugates, in blue and magenta tones, respectively, Hematoxylin blueing for nuclear materials, and Picric Acid to identify proteins in yellow hues.

A last set of sections for both DG and RT was stained with

**Table 2**  
Antibody (Ab) optimization and immunohistochemical experimental conditions.

Conditions	Concentrations Primary AbAnti-DA	Secondary AbHRP/nanogold conjugated	Antigen retrieval <sup>a</sup>	Peroxidase Quenching <sup>b</sup>
<i>IHC</i>				
Negative control	Without	1: 10,000	Yes	Yes
Treated	1:1,000 Immunogold	1: 10,000	Yes	Yes
Negative control	Without	1: 500	No	No
Treated	1:200	1: 500	No	No

<sup>a</sup> Antigen retrieval allows to break potential methylene bridges formed during formalin-fixation and expose antigenic sites to allow the antibodies epitope to bind.

<sup>b</sup> Endogenous peroxidase blocking is necessary to avoid non-specific staining.

Hematoxylin-Eosin as reference (Kim et al., 2006), and mounted in DPX resin. The slides were examined under a Zeiss Axio Observer Z1 light-microscope. The digestive stages of the diverticula in the DG were classified as holding, absorptive, digestion, advanced digestion, and undergoing breakdown or regeneration, according to Mathers (1976) and Beninger and Le Pennec (2016).

A six-level semi-quantitative scale from 0 (absent) to 2.5 (very high) was established to assess the intensity of the chromogenic anti-DA signal present in the mucus/globose cells of different tissues, the digestive gland, and the small inclusion bodies (IBs) in the digestive cells of the scallops (Table 3).

#### 2.4. Transmission electron microscopy and immunogold labeling

Transmission electron microscopy (TEM) studies were necessary in order to identify the small IBs with chromogenic anti-DA signal within the cells of the digestive gland. For this purposes, three small pieces of DG (~ 1 mm<sup>3</sup>) were carefully dissected from some of the non-contaminated scallops collected in the Bay of Brest (n = 5), used as negative controls, and some of the contaminated-scallops from Camaret-sur-Mer (n = 5) with strongest IHC signal in the IBs within the digestive cells. Samples from scallops collected at Concarneau in 2019 were not considered for these analyses since the digestive glands were not processed for TEM purposes.

Samples were pre-fixed in glutaraldehyde 3 % (v/v) with 0.2 M cacodylate buffer (pH 7.4) supplemented with NaCl (21 mg mL<sup>-1</sup>) for 3 h at 4°C, rinsed in the same buffer (3 × 5 min), and subsequently post-fixed in 1% (w/v) osmium tetroxide in 0.2 M cacodylate buffer (pH 7.4) for 1 h in an ice bath in the dark. Fixed specimens were rinsed in Milli-Q water (3 × 5 min) and dehydrated through successive baths of ethanol. Finally, samples were embedded into Spurr's resin (Science Services, Munich, Germany). After polymerization at 60°C for 24h, semi-thin sections were cut to 300 nm thickness for quality control and then ultra-thin (ca. 70-80 nm) sections were cut for examination on a Leica EM UC6 ultramicrotome (Leica Microsystems, Germany) equipped with a 45° DIATOME diamond knife and floated on nickel grids (200 mesh).

Immunogold labeling was performed according to Skepper and Powell (2003) with minor modifications. Briefly, the grids were etched with drops of 4% sodium metaperiodate for 10 min to unmask antigenic sites on the surface of the section, rinsed three times on successive drops of MQ water, and placed on drops of 1% aqueous periodic acid for 10 min to remove eventual osmium tetroxide residue. Sections were then placed on a drop of blocking solution consisting of PBS 0.01 M, 0.01% Triton X-100, Glycine 20 mM, and 1% BSA for 10 min to reduce nonspecific binding of antibodies. The anti-DA antibody (abcam) was diluted 1:200 in blocking solution, and the sections were incubated with the primary antibody solution overnight at 4°C in a moist chamber. After washing with blocking solution (6 × 5 min), the sections were incubated

with the Goat anti-Rabbit IgG secondary antibody conjugated with 6-nm gold particles (abcam/ab41498) diluted 1:500 in blocking solution for 2 h at 23°C, and consecutively rinsed in blocking solution and MQ water. Contrast reagents (e.g. uranyl acetate and lead citrate) were not applied to avoid masking the nanogold particles. Immunogold labeling experimental conditions, as well as antibody optimization-dilutions are shown in Table 2. Finally, the samples were examined under a transmission electron microscope JEOL JEM 1400 operated at 120 kV on the imaging platform of Brest University. The autophagosomal structures identified in this work by means of MET were classified according to their morphology and stage of development in marine bivalve cells (Owen, 1972; Yurchenko & Kalachev, 2019; Picot et al., 2019).

### 2.5. Statistical analysis

To determine significant differences in toxin burdens in the digestive gland of scallops collected in the different sampling sites, *a priori* Pligner-Killeen's and Shapiro-Wilk test were used to evaluate the heterogeneity of variances and normality of frequencies of the data, respectively (Hector, 2015); the assumptions were not met. Values of DA concentrations were analyzed using a Kruskal-Wallis Test, where "the sampling site" was fixed as factor. In case of significant differences, a *post hoc* pairwise Wilcoxon rank test with Benjamini & Hochberg (BH) p-value adjustment was used to detect differences among means. For IHC results, Chi-square test ( $\chi^2$ ) were applied to assess statistically significant differences in the chromogenic anti-DA signal present in each tissue of the scallops. When needed, *a posteriori* Tukey HSD test were used to identify

differences between means. All the statistical analyses were performed using command lines in the R language (R v. 4.0.2, R Core Team, 2020), and graphics were generated with the R package ggplot2 on the Rstudio programming interface. All values are expressed as mean  $\pm$  standard error (SE). Differences were considered statistically significant at  $\alpha = 0.05$  for all analyses (Hector, 2015).

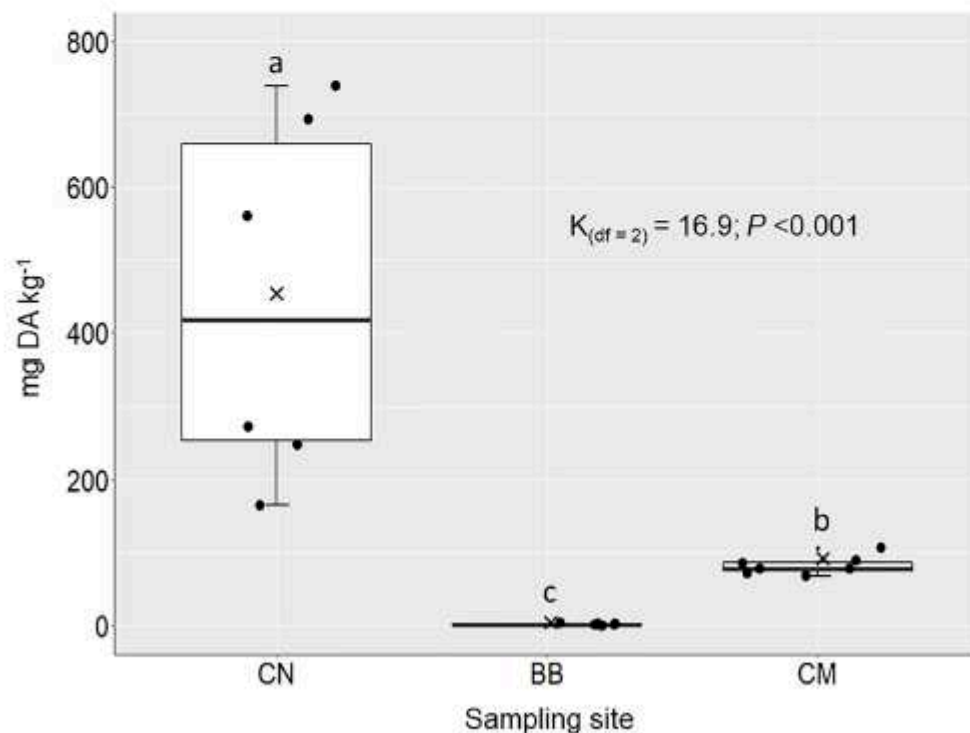
## 3. Results

### 3.1. Domoic acid (DA) quantification

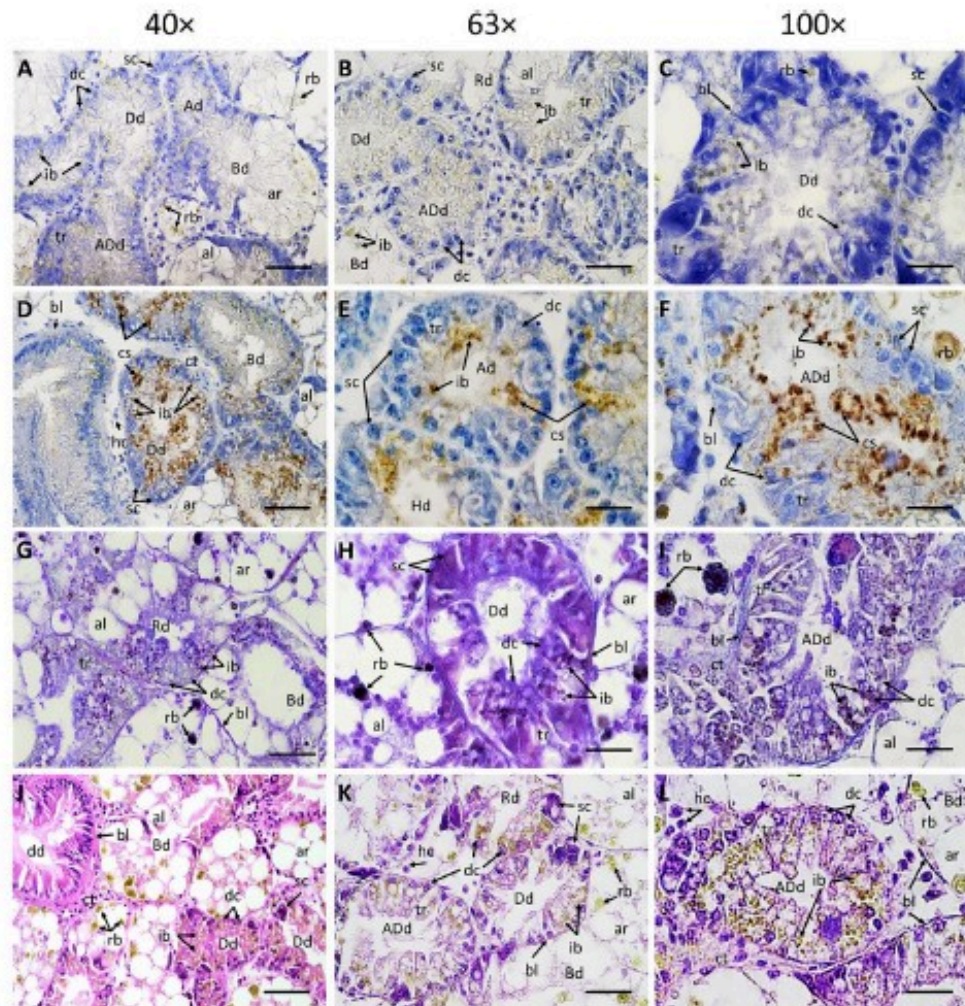
Significant differences in the amount of DA accumulated in the digestive gland (DG) of the scallops from the three sampling sites were found after toxin quantification analysis by HPLC-UV (Fig. 1). Highest burdens ( $P < 0.05$ ) of toxin were recorded in animals from Concarneau (CN) ( $446.6 \pm 101.3$  mg DA kg<sup>-1</sup>) followed by those from Camaret-sur-Mer (CM) ( $82.5 \pm 4.9$  mg DA kg<sup>-1</sup>), while the significant lowest values were detected in the scallops from the Bay of Brest (BB) ( $1.6 \pm 0.4$  mg DA kg<sup>-1</sup>).

### 3.2. Histology and immunohistochemistry (IHC)

The presence of DA was detected by IHC, as brown chromogenic signal, within the tissues of all contaminated scallops (Figs. 2–4). The absence of non-specific background staining during IHC process was confirmed in control slides incubated with the secondary antibody but without the primary anti-DA antibody (Figs. 2 A-C, 3 C, D and 4 E-H).



**Fig. 1.** DA concentrations in the digestive gland of the scallops *P. maximus* naturally contaminated during outbreaks of the toxic *Pseudo-nitzschia* spp. and collected at three sites (CN = Concarneau [n = 6], BB = Bay of Brest [n = 7], and CM = Camaret-sur-Mer [n = 7]) of the northwest coast of France between 2019 and 2021. 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 crosses are the means. Data were analyzed using the sampling sites (three levels) as independent variables in a Kruskal-Wallis Test. The K-test statistic and degrees of freedom (df) are reported. Different superscript letters denote statistically significant differences between groups of scallops. The level of statistical significance was set at  $\alpha = 0.05$ .



**Fig. 2.** Microphotographs of digestive glands of scallops *P. maximus* naturally highly contaminated ( $\sim 60$  to  $750 \text{ mg DA kg}^{-1}$ ) collected at Camaret-sur-mer ( $n = 7$ ) and Concarneau ( $n = 6$ ) in the northwest coast of France between 2019 and 2021 during outbreaks of the toxic *Pseudo-nitzschia* spp. (A–C) negative controls of the IHC staining incubated with the secondary antibody but without the primary anti-DA antibody (1: 10,000 and 1: 0, respectively); (D–F) specific anti-DA immunohistochemical (IHC) staining incubated with the primary and secondary antibodies (1: 1,000 and 1: 10,000, respectively); (G–I) multichromic histochemical staining for the demonstration of neutral carbohydrates (violet-magenta dyes), acid glycoconjugates (blue hues), and proteins (yellowish tones); (J–L) conventional histological Hematoxylin-Eosin staining. Ad = digestive diverticulum in absorptive condition, ADd = digestive diverticulum in advanced digestive condition, al = adipocyte-like digestive cell, ar = acinar region, Bd = digestive diverticulum undergoing breakdown, bl = basal lamina, cs = positive anti-DA chromogenic signal, ct = connective tissue, dc = digestive cells, Dd = digestive diverticulum in digestive condition, dd = digestive duct, hc = hemocytes, Hd = digestive diverticulum in holding condition, ib = inclusion bodies, rb = residual bodies, Rd = diverticulum showing regeneration, sc = secretory cells, tr = tubular region. Scale bar:  $40 \times = 50 \mu\text{m}$ ,  $63 \times = 30 \mu\text{m}$ ,  $100 \times = 10 \mu\text{m}$ .

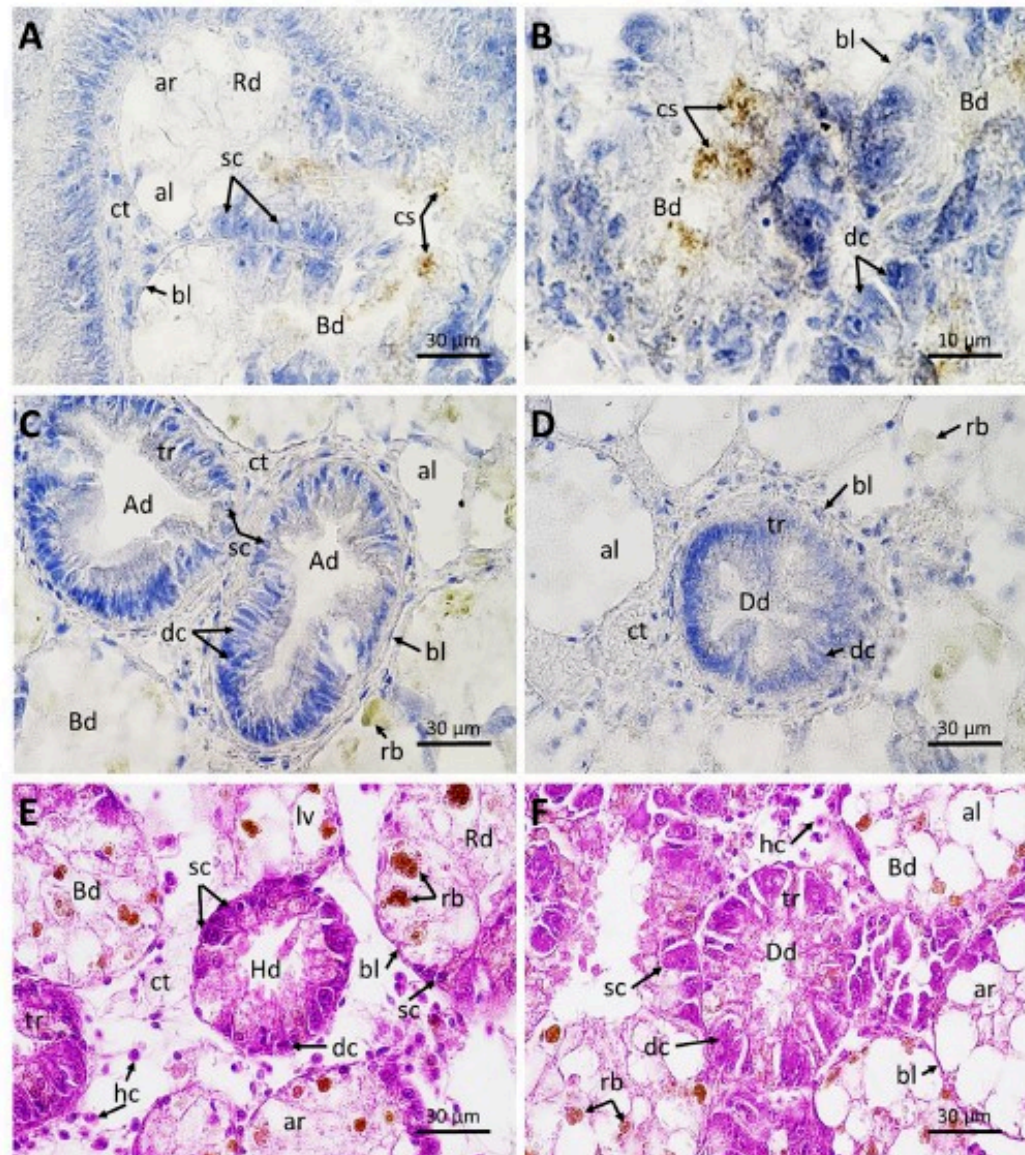
The DA brown chromogenic signal was observed mainly throughout the DG, and readily detected in highly contaminated scallops from CN and CM. The typical DA immunostaining observed in the DG of scallops sampled at CN and CM is illustrated in Fig. 2D–F. As shown in Fig. 2D, within the DG, the strongest immunoreactivity was observed in small ( $\sim 1$ – $2.5 \mu\text{m}$ ) spherical inclusion bodies (IBs) distributed exclusively throughout the cytoplasm of the digestive (absorptive) cells of the digestive diverticula, which trapped an intense chromogenic staining (Fig. 2E, F). The anti-DA chromogenic signal detected in the DG of scallops from CN and CM has the same sub-cellular localization although DA burdens were significantly different between scallops from the two locations.

The multichromic staining allowed to clearly identifying these IBs within the cytoplasm of the digestive cells (Fig. 2G). As observed in Fig. 2H and 2I, the IBs had a dark violet-magenta dye, indicating the

presence of neutral carbohydrates and neutral glycoconjugates on their surface. The IBs did not acquire any coloration with the conventional H&E staining (Fig. 2L). No histopathological patterns were observed in the DG of the scallops, even for the highest toxin burdens (Fig. 2J, K). The overall histological evidence allowed to observe that the IBs with DA-immunoreactivity were found mainly in the digestive cells of the diverticula in stages of active digestion (Fig. 2F, H, I, K, L).

In the samples from significantly weakly-contaminated scallops from BB, a slight-blurred and not well-located DA-chromogenic signal was observed in the “breakdown” and “regenerating” digestive diverticula of the DG (Fig. 3A, B). Nonetheless, it was possible to localize a few IBs with immunoreactivity in the cytoplasm of the remaining digestive cells (Fig. 3B). The H&E staining also allowed corroborating the absence of histopathologies in the DG due to DA accumulation (Fig. 3E, F).

The DA-localization in the rest of the tissues was similar in all the

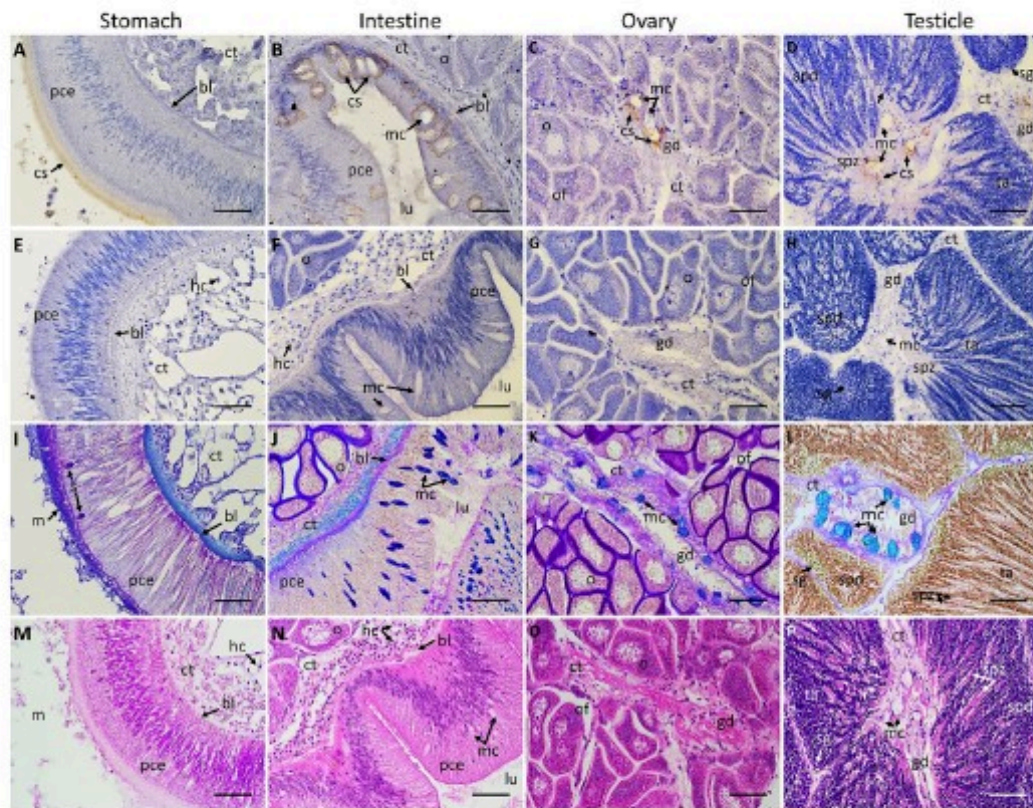


**Fig. 3.** Microphotographs of digestive glands of scallops *P. maximus* naturally low contaminated with  $-2 \text{ mg DA kg}^{-1}$  collected at the Bay of Brest ( $n = 7$ ) on the northwest coast of France in December 2020. (A-B) specific anti-DA immunohistochemical (IHC) staining incubated with the primary and secondary antibodies (1: 1,000 and 1: 10,000, respectively); (C-D) negative controls of the IHC staining incubated with the secondary antibody but without the primary anti-DA antibody (1: 10,000 and 1: 0, respectively); (E-F) conventional histological Hematoxylin-Eosin staining. Ad = digestive diverticulum in absorptive condition, al = adipocyte-like digestive cell, ar = ascinar region, Bd = digestive diverticulum undergoing breakdown, bl = basal lamina, cs = positive anti-DA chromogenic signal, ct = connective tissue, dc = digestive cells, Dd = digestive diverticulum in digestive condition, hc = hemocytes, Hd = digestive diverticulum in holding condition, rb = residual bodies, Rd = diverticulum showing regeneration, sc = secretory cells, tr = tubular region.

scallops contaminated from  $-2$  up to  $-750 \text{ mg DA kg}^{-1}$  (Fig. 4). The DA-labeling was detected only in the mucus of the epithelia that lines the outer part of the stomach (Fig. 4A), in the globose cells embedded in the epithelium of the intestine (Fig. 4B), and in the globose cells of the spawning channels or gonadic ducts in the female (Fig. 4C) and male (Fig. 4D) gonads. No DA signal was found in any other tissues such as gills, mantle, labial palps, kidneys or adductor muscle. With the multi-chromatic staining, it was possible to corroborate the presence of a light-blue coloration corresponding to acid glycoconjugates in the globose cells with immunolabeling (Fig. 4I-L). As seen in Fig. 4M-P, no histopathologies were observed in any of the additional tissues analyzed in

this work.

As shown in Table 4, DA staining coverage in the DG was the same ( $P > 0.05$ ) for scallops from CN and CM, while the anti-DA chromogenic signal detected in the DG of the scallops collected at BB was significantly lower. On the other hand, the chromogenic signal detected in the rest of the tissues (stomach, intestine, ovary, and testicle) was not different ( $P > 0.05$ ) between the strongly (CN and CM) and weakly (BB) contaminated scallops.



**Fig. 4.** Microphotographs of the rest of the tissues of scallops *P. maximus* naturally contaminated between  $-2$  and  $750 \text{ mg DA kg}^{-1}$  collected at three sites (Concarneau [n = 6], Bay of Brest [n = 7], and Camaret-sur-mer [n = 7]) of the northwest coast of France between 2019 and 2021. (A-D) Specific anti-DA immunohistochemical (IHC) staining incubated with the primary and secondary antibodies (1: 1,000 and 1: 10,000, respectively); (E-H) negative controls of the IHC staining incubated with the secondary antibody but without the primary anti-DA antibody (1: 10,000 and 1: 0, respectively); (I-L) multichromic histochemical staining for the demonstration of neutral carbohydrates (violet-magenta dyes), acid glycoconjugates (blue hues), and proteins (yellowish tones); (M-P) conventional histological Hematoxylin-Eosin staining. bl = basal lamina, cs = positive anti-DA chromogenic signal, ct = connective tissue, gd = gonadic duct, hc = hemocytes, lu = lumen, m = mucus, mc = mucocyte, o = oocyte, of = ovarian follicle, pce = pseudostratified columnar epithelium, sg = spermatogonia, spd = spermatids, spz = spermatozoa, ta = testicular acinus. Scale bar:  $40 \times = 50 \mu\text{m}$ .

**Table 3**  
Semi-quantitative scale categorizing the intensity of chromogenic anti-DA signal observed on the IHC slides.

Level intensity	Occurrence of the chromogenic anti-DA staining in the examined tissue area
0	Absence
0.5	Very low (<5 occurrence/presence in all fields at magnification $10 \times$ )
1	Low (>5 occurrence/presence in all fields at magnification $10 \times$ )
1.5	Moderate (presence in all fields at magnification $20 \times$ / covering about one tenth of the tissue area)
2	High (presence in all fields at magnification $40 \times$ / covering about one fifth of the tissue area)
2.5	Very high (presence in all fields at magnification $60 \times$ / covering about one-third or above of the tissue area)

**3.3. Immunoelectron microscopy**

The IBs observed in the cytoplasm of the digestive cells in the diverticula of scallops with a dark-violet coloration by means of multichromic staining, and presenting a strong DA-immunostaining were analyzed by transmission electron microscopy (TEM) in order to decipher their cellular nature (Fig. 5). The diameter of these IBs ranged between 1 and  $2.5 \mu\text{m}$ . Early single-membrane-bound IBs structures

(Fig. 5A) were observed frequently in the apical and sub-apical regions of the digestive cells. Meanwhile late-developed structures with a double-membrane-bound and a halo (Fig. 5B) were observed mainly in the mid-basal region of the cytoplasm, and often clustered into groups of 3-6 vesicles that may be or not surrounded by a single-membrane (Fig. 5C) and fusing with the lysosomes of the cell (Fig. 5D). The morphological observations by TEM described above allowed identifying these IBs as autophagic vesicles.

On a second hand, we coupled the use of the specific anti-DA antibody and a secondary antibody conjugated with gold nanoparticles to the TEM analyzes (immunogold labeling). As seen in Fig. 5A-D, no anti-DA signal was observed in any subcellular structure of the GD in the slide incubated without anti-DA primary antibody. By means of the immunogold labeling, DA-signal was found mostly in the undigested material attached to the inner side of the membranes within early (Fig. 5E, F) and late-autophagosomes (Fig. 5G, H), while a slight signal of gold-nanoparticles corresponding to the toxin was observed in the halo of autophagosomes and in the cytoplasm of the digestive cells (Fig. 5E-H).

**4. Discussion**

In this work, for the first time, immunolabeling by IHC using photonic microscopy and immunogold using TEM has been successfully

**Table 4**

Comparison of IHC staining intensity of DA in the tissues of the scallops *P. maximus* naturally contaminated and collected at three sites (CN = Concarneau [n = 6], BB = Bay of Brest [n = 7], and CM = Camaret-sur-mer [n = 7]) of the northwest coast of France between 2019 and 2021. NA: not available (not enough data), “—”: no chromogenic anti-DA staining.

Tissue	Sampling site			Statistical analysis	
	CN	BB	CM	$\chi^2$ , n	P
Digestive gland	2.4 ± 0.08 <sup>a</sup>	0.57 ± 0.13 <sup>b</sup>	2.4 ± 0.09 <sup>a</sup>	20.4, 20	<0.05
Stomach	1.2 ± 0.11 <sup>a</sup>	0.93 ± 0.14 <sup>a</sup>	1.3 ± 0.1 <sup>a</sup>	5.9, 20	>0.05
Intestine	1.3 ± 0.1 <sup>a</sup>	1.07 ± 0.17 <sup>a</sup>	1.3 ± 0.1 <sup>a</sup>	4.3, 20	>0.05
Ovary	1.2 ± 0.1 <sup>a</sup>	0.93 ± 0.13 <sup>a</sup>	1.3 ± 0.1 <sup>a</sup>	5.8, 20	>0.05
Testicle	1.1 ± 0.09 <sup>a</sup>	1 ± 0.15 <sup>a</sup>	1.4 ± 0.09 <sup>a</sup>	4.2, 20	>0.05
Gills	—	—	—	NA	
Adductor muscle	—	—	—	NA	
Mantle	—	—	—	NA	
Labial palps	—	—	—	NA	

Data (mean ± SE) were analyzed according to the sampling sites (three levels) in a Chi-square test ( $\chi^2$ ). The  $\chi^2$  test statistic and sample size (n) are reported. Different superscript letters denote statistically significant differences at  $p < 0.05$ .

used for the localization of DA at the subcellular level in naturally contaminated marine mollusc tissues. The technique set up in the present paper has been shown to work for the immunostaining of DA with high precision, either in heavily contaminated (up to 750 mg DA kg<sup>-1</sup> GD) or in weakly-contaminated scallops (~1 mg DA kg<sup>-1</sup> DG) without nonspecific labeling. Although other methods, such as HPLC-UV/MS (Quilliam et al., 1999) and ELISA (Litaker et al., 2008), have been widely used to quantify DA content in contaminated shellfish with a high-resolution power (0.1–1 µg DA g<sup>-1</sup>), they do not allow the subcellular visualization of DA in the tissues, as opposed to the immunolabeling methods developed in this study. Furthermore, this immunostaining method has proven to be suitable to be coupled with TEM, allowing to pinpoint DA localization.

Using a subcellular fractionation analysis on homogenized DA-contaminated digestive glands of *P. maximus*, Maurin and Blanco (2010) found that almost 90% of the toxin accumulated in this organ was in soluble form in the cytoplasm of the cells, with a mostly homogeneous distribution within the DG (Blanco et al. 2020). One mechanism that could influence high accumulation and long retention of DA in this species could be its binding to high affinity receptors, as those found in the razor clam *S. patula* (Trainer and Bill, 2004). Moreover, Maurin and Blanco (2010) concluded that the cause of the long DA-retention was not the binding of the toxin to some cellular component, but the lack of some efficient membrane transporters in the scallops. Our results cope with these findings, since most of the DA immune-signal was localized in the cytoplasm of the digestive cells of the digestive diverticula. Several digestive stages (holding, absorptive, digestion, advanced digestion, breakdown, and regeneration) have been described for the digestive diverticula of *P. maximus* (Mathers, 1976). In this work, the inclusion bodies (IBs) with anti-DA signal were observed mostly in the digestive cells of the diverticula in states of active digestion (absorption, digestion and advanced digestion). This is probably due to digestive cells predominate in these digestion stages and are responsible for the intracellular enzymatic digestion of the material ingested by pinocytosis (Beninger and Le Pennec, 2016). Free domoic acid in the cytoplasm was visualized by immunogold. Nonetheless, the evidence of this work suggests that a significant proportion of the toxin is not simply ‘free-dissolved’ in the cytoplasm, but is enclosed in small (1–2.5 µm) membrane-bound vesicles, identified as autophagosomal structures by means of TEM, distributed throughout the cytoplasm of digestive cells in

digestive condition.

Autophagy is a well-developed, highly regulated, and complex-dynamic system related to ingestion, storage and catabolic processes of intracellular digestion (Balbi et al., 2018; Wang et al., 2019; Zhao et al., 2021). In bivalves, autophagy plays a key role in maintaining cell homeostasis (Carella et al., 2015). This mechanism has been used as an indicator of cell injury in response to different stressors (Moore, 2004; Picot et al., 2019), such as environmental changes (Moore, 2008), and the innate-immune response to pathogens (Canesi et al., 2002; Moreau et al., 2015; Canesi et al., 2016; Balbi et al., 2018). However, nothing is still known on the role of autophagy in ingestion, mobilization and excretion of phycotoxins in these organisms.

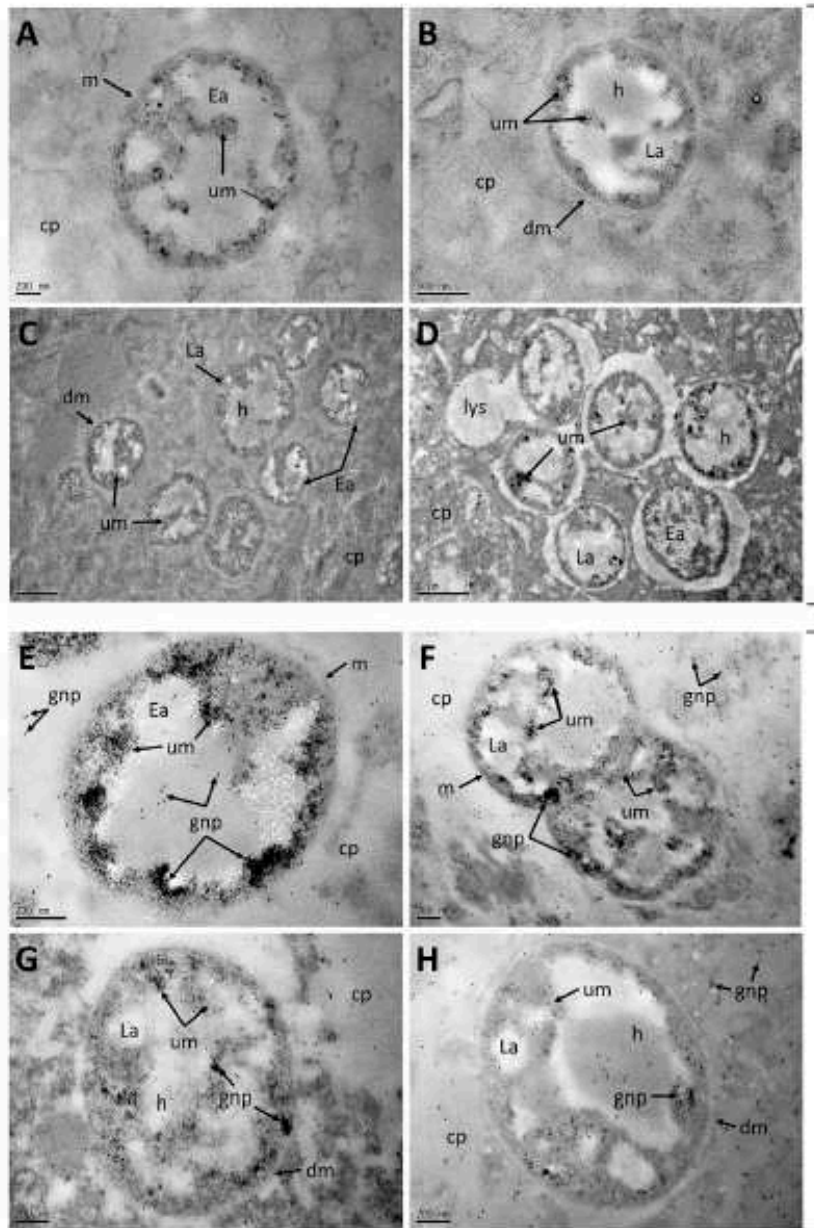
During autophagy, cytoplasmic components, either of exogenous (e. g. contaminants, and pathogens), or endogenous (macromolecules and organelles) origin are sequestered into spherical-shaped vesicles with double membrane layers called autophagosomes. Subsequently, they are delivered to lysosomes for degradation, where the outer membrane of the autophagosome fuses with a lysosome to form an autolysosome (Cuervo, 2004; Wang et al., 2019). Finally, the hydrolases of the lysosome degrade the autophagosome-delivered contents and its inner membrane (Zhao et al., 2021).

In samples of DA-contaminated scallops, mostly two types of membrane-bound autophagosomal vesicles were identified by transmission electron microscopy as part of this dynamic system. Early autophagosomes, which are usually involved in the ingestion and accumulation of exogenous materials, were present mainly in the apical region of the digestive cells; whereas in the mid- and basal regions of the cytoplasm we observed late-autophagosomes. These autophagosomes are involved in digestion and accumulation of undigested and indigestible residues, which may then be stored within the cell or eliminated (Owen, 1972; Zhao et al., 2021). The transformation rate from early to late-autophagosomes is presumably dependent on the nature of the ingested material, and variations of this basic but highly-complex cycle probably depends on feeding rates, nature of the ingested food/substances, and the mode of release/excretion of the autophagosomal vesicles (Owen, 1972; Cuervo, 2004). The processing of autophagosomes by intracellular digestion could be a key to explain the long retention time of DA in the digestive cells of *P. maximus*. The toxin is probably normally ingested and accumulated in early autophagosomes, but cannot then be digested by the lysosomal machinery, thus remaining stored within autophagosomes as indigestible material in the cytoplasm of the cells. Moreover, it is difficult to know exactly how long it may take for the material present within autophagosomes to be excreted; since some experiments suggest that it can go from a few minutes to indefinite periods of time (Owen, 1972; Cuervo, 2004).

After DA injection in the adductor muscle, and subsequent transcriptomic analysis of the digestive gland of *P. maximus*, Ventoso et al. (2021) found as well as an upregulation of genes related to autophagy and vesicle-mediated transport. Even though these results were not obtained under conditions of ingestion of the toxin through the filtration of toxic *Pseudo-nitzschia* cells, these findings could also indicate that the formation of autophagosomal structures could be part of explanation for DA long retention, blocking its digestion and excretion.

In order to corroborate whether autophagy is the subcellular mechanism involved in the long retention of DA in the DG of *P. maximus*, the next step would be to follow, by means of digital image analysis, the evolution of the anti-DA chromogenic signal in the tissues in parallel to the formation of autophagosomes with strong DA-immunoreactivity within the digestive cells during the contamination and decontamination processes.

There is evidence of the profound interspecific differences in the retention and depuration of DA in bivalves, even between pectinid species, like for example *P. maximus* and *A. purpuranus*. While the former is capable of accumulating up to 3,000 mg DA kg<sup>-1</sup> and retain it for months or even years (Blanco et al., 2006), *A. purpuranus* transfers almost all the DA accumulated in the digestive gland to other organs (mainly



**Fig. 5.** Electronmicrographs of ultrathin sections (70–80 nm) across the digestive glands of scallops *P. maximus* naturally contaminated ( $\sim 75 \text{ mg DA kg}^{-1}$ ) during outbreaks of the toxic *Pseudo-nitzschia* spp. and collected in Camaret-sur-mer ( $n = 7$ ) on the northwest coast of France in 2021. Detection of autophagic structures with positive DA immune-signal within digestive cells was possible by means of transmission electron microscopy (TEM). (1A–D) Negative controls of the immunogold labeling incubated with the secondary antibody but without the primary anti-DA antibody (1: 200 and 1: 0, respectively); (2E–H) Specific anti-DA immunogold labeling incubated with the primary anti-DA antibody and the secondary antibodies conjugated with 6-nm gold nanoparticles (1: 200 and 1: 500, respectively). cp = cytoplasm, dm = double-membrane-bound, Ea = early autophagosomes, gnp = gold nanoparticles, h = halo, La = late autophagosomes, lys = lysosomes, m = single-membrane-bound, um = undigested material.

the intestine and the gonad) within a few days and then excrete the toxin into the environment (Alvarez et al., 2020). Although the physiological mechanisms enabling *A. purpuratus* to quickly depurate the DA are unknown, Alvarez et al. (2020) hypothesized a two-compartment model, where the toxin acquired by the DG is quickly transported to other organs. In *P. maximus*, we could hypothesize that a significant part of DA accumulated stay in DG due to the absence of specific transporter as proposed by Mauriz and Blanco (2020), and that, secondly, its detoxification be slower due to the formation of autophagosomes that retain the DA. Further analyses, comparing these species, using histological, immunohistochemistry, as well as molecular biology techniques appear necessary to confirm this hypothesis and to determine whether autophagy appears in other slow-depurator shellfish species.

In the rest of the tissues of *P. maximus*, the IHC technique developed in this work revealed specific toxin-immunoreactivity and thus DA-

localization within the mucus, particularly in the mucocytes of some epithelia such as the stomach and intestine, and in the mucocytes of the gonad spawning-ducts. Mucus is composed of water, glycoproteins and mineral salts (Davies and Hawkins, 1998), and is produced by almost all the epithelia of mollusks, playing an essential role in several functions such as lubrication, nutrition, the first barrier against environmental stress, and as an innate-immune barrier against pathogenic infections (Allam and Pales Espinosa, 2016). Hence, complementary studies are necessary to determine if DA has an affinity or is chemically-bound to any of the components of mucus, and if the latter may be involved in DA-depuration or retention in the scallops. This hypothesis is totally new, since DA detection techniques in contaminated bivalve tissues had never allowed to localize the toxin at the level of mucus or mucus-producing cells during a contamination and decontamination scenario.

## 5. Conclusions

The DA-immunodetection methods proposed in this work by immunohistochemistry and immunogold are innovative ways to visualize the phycotoxin DA in the tissues of the king scallop *P. maximus*, and to decipher the subcellular mechanism involved in the retention of this toxin in a marine bivalve. The results of this work show that, most of the DA is found in the cytoplasm of digestive cells of *P. maximus*, as previously mentioned by Mauric and Blanco (2010). Notwithstanding, most part of DA-signal does not appear free in the cytoplasm, but mainly within autophagic structures as revealed by DA-immunostaining, suggesting that autophagic subcellular mechanisms could play a crucial role in the retention of the ASP toxin in the digestive cells of scallops. Furthermore, the role of mucus in the retention-depuration of DA in *P. maximus* must be investigated, since the toxin was only immunolocalized in the mucus of specific remaining tissues.

DA-immunodetection also provides a great tool to compare DA-localization within species depurating at different speed over a contamination and decontamination period. The findings of this work constitute an important step forward in explaining the slow depuration of DA in *P. maximus*, and provide basic knowledge for the proposal of procedures to accelerate the depuration of the toxin in this species.

## 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, 20 scallops were considered as the minimum number of organisms needed for this work.

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## Declaration of Competing Interest

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

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**Titre :** Suivi de la contamination des coquilles Saint-Jacques (*Pecten maximus*) par l'acide domoïque et exploration du rôle du microbiote dans sa décontamination

**Mots clés :** *Pecten maximus*, acide domoïque, *Pseudo-nitzschia sp.*, microbiote.

**Résumé :** La coquille Saint-Jacques (*Pecten maximus*) fait figure d'exception parmi les organismes contaminés par l'acide domoïque du fait de sa longue rétention au sein de sa glande digestive. Bien que les interdictions de pêche en cas de contamination aient un impact économique important, le mécanisme sous-jacent à cette lente dépuración reste méconnu. Les objectifs de cette thèse ont donc été de i) examiner *in situ* la contamination de *P. maximus* en corrélation avec la présence de *Pseudo-nitzschia* spp. et d'acide domoïque dans l'eau afin d'identifier les diverses sources de contamination possibles, ii) étudier le lien entre la dépuración de l'acide domoïque et la taille de *P. maximus*, iii) explorer la piste des microorganismes pour accélérer la dépuración de l'acide domoïque dans la glande digestive de *P. maximus*. Une surveillance menée depuis 2011 dans la rade de Brest a mis en évidence l'importance des eaux de surface et de fond ainsi que la présence de *Pseudo-nitzschia* spp. et d'acide domoïque dans la contamination de *P. maximus*, identifiant ainsi trois scénarios de contamination.

Une expérience de décontamination sur deux mois a révélé que les petites coquilles semblaient dépurar l'acide domoïque plus rapidement que les grandes. Des isolements bactériens effectués sur des individus contaminés ont révélés des différences avec d'autres espèces de bivalves considérés comme dépurateurs rapides. Bien que des souches bactériennes candidates aient été identifiées, aucune réduction de l'acide domoïque n'a été observée après exposition à toxine. Un transfert de microbiote de *M. edulis* a été réalisé vers *P. maximus*, montrant un transfert de souches bactériennes dans la glande digestive de *P. maximus*.

En conclusion, ces travaux enrichissent la compréhension des diverses sources de contamination de *P. maximus* par l'acide domoïque, ainsi que des réponses de ce bivalve en fonction de sa taille face à cette toxine. Cette thèse offre des pistes qui pourraient permettre d'accélérer la dépuración de l'acide domoïque chez *P. maximus*.

**Title:** Monitoring domoic acid contamination of King scallops (*Pecten maximus*) and exploring the role of microbiota in its decontamination.

**Keywords:** *Pecten maximus*, *Pseudo-nitzschia sp.*, domoic acid, microbiota.

**Abstract:** The King scallop (*Pecten maximus*) is an exception among domoic acid contaminated organisms, due to its long retention within the digestive gland. Although fishing bans in the event of contamination have a significant economic impact, the mechanism behind this slow depuration remains poorly understood. The aims of this thesis were therefore to i) examine *in situ* the contamination of *P. maximus* in correlation with the presence of *Pseudo-nitzschia* spp. and domoic acid in the water, to identify the various sources of contamination, ii) study the link between domoic acid depuration and *P. maximus* size, iii) explore the possibility of microorganisms accelerating the depuration of domoic acid in the digestive gland of *P. maximus*. Monitoring carried out since 2011 in the Bay of Brest has shown the importance of surface and bottom waters, as well as the presence of *Pseudo-nitzschia* spp. and domoic acid in

*P. maximus* contamination, thus identifying three contamination scenarios. A two-month decontamination experiment revealed that smaller scallops seemed to depurate domoic acid faster than larger ones. Bacterial isolations from contaminated individuals revealed differences with other bivalve species considered to be rapid depurators. Although candidate bacterial strains were identified, no toxin reduction was observed after exposure to domoic acid. A transfer of microbiota from *M. edulis* to *P. maximus* was performed, showing a transfer of bacterial strains into the digestive gland of *P. maximus*. In conclusion, this work enriches our understanding of the various sources of domoic acid contamination of scallops, as well as the response of this bivalve to domoic acid according to its size. This thesis offers new insight for accelerating domoic acid depuration in *Pecten maximus*.