Paléoécologie des protistes à partir d'archives biologiques provenant d'écosystèmes marins côtiers

Khadidja Zeyneb Klouch

THESE DE DOCTORAT DE

L'UNIVERSITE PIERRE ET MARIE CURIE

Ecole Doctorale Sciences de la Nature et de l'Homme (ED227)

Spécialité : Evolution et Ecologie

Paléoécologie des protistes à partir d'archives biologiques provenant d'écosystèmes marins côtiers

Présentée par

Khadidja Zeyneb KLOUCH

UPMC-CNRS Adaptation et Diversité en Milieu Marin
(UMR 7144), équipe DIPO, Station Biologique de Roscoff

Ifremer - Centre de Brest, équipe DYNECO/PELAGOS

En vue de l'obtention du grade de

Docteur de l'Université Pierre et Marie Curie

Présentée et soutenue publiquement le 29 septembre 2016, devant le jury composé de :

Dr. Laure Guillou, CNRS- Station Biologique de Roscoff	Directrice de thèse
Dr. Raffaele Siano, Ifremer - Centre de Brest	Co-Directeur de thèse
Dr. Anke Kremp, Finnish Environment Institute, Finland	Rapporteur
Dr. Isabelle Domaizon, Centre Alpin de Recherche sur les Réseaux Trophiques des Ecosystèmes Limniques, Thonons-Les-Bains	Rapporteur
Pr. Christophe Destombe, UPMC- Station Biologique de Roscoff	Examinateur
Dr. Antonella Penna, University of Urbino, Italy	Examinateur

Je dédie ce travail à mes chers parents

Remerciements

Je tiens tout d'abord à exprimer ma profonde reconnaissance pour mes directeurs de thèse Laure Guillou et Raffaele Siano qui m'ont accordé leur confiance et m'ont soutenu et guidé tout au long de ce travail. Merci Laure pour tes conseils et ta disponibilité à chaque fois que j'en avais besoin et surtout durant cette dernière « ligne droite ». Merci Raffaele, pour la qualité de ton encadrement et de m'avoir appris tant de choses sur la science mais aussi sur la vie. Ce travail n'aurait pu être accompli sans ton excellent suivi. Tu m'as toujours encouragé pour progresser, et le perfectionniste que tu es m'a finalement beaucoup apporté. Enfin, merci de m'avoir donné l'opportunité d'effectuer cette superbe thèse.

Je remercie les membres de mon jury de thèse de me faire l'honneur d'accepter d'évaluer ce travail et pour l'intérêt qu'ils ont porté pour ce sujet. Merci à Anke Kremp et Isabelle Domaizon d'avoir accepté d'être rapporteurs de cette thèse et à Antonella Penna et Christophe Destombe d'avoir accepté le rôle d'examinateurs.

Je tiens à remercier également l'ensemble des membres de mon comité de thèse : Dominique Hervio-Heath, Pascale Claquin, Isabelle Domaizon. Vos conseils m'ont permis de mieux définir mon projet de thèse et de progresser en me concentrant sur les priorités.

Je remercie sincèrement Mickael Le Gac de m'avoir initié et formé à la biologie moléculaire. Merci pour tes nombreux conseils et d'avoir été disponible pour me guider lors des nombreuses difficultés que j'ai rencontrées.

Cette thèse doit également beaucoup à Dominique Hervio-Heath, dont la gentillesse est sans limite et sans qui ce travail n'aurait sans doute pas pu aboutir. Merci de m'avoir chaleureusement accueilli et de m'avoir permis de réaliser mes manips dans ton laboratoire. Tes nombreux conseils sur la PCR en temps réel et ta disponibilité m'ont permis de mener à bien ce travail. Je ne te remercierai jamais assez d'être restée avec moi pour la mise en page finale de ce document.

Je remercie Estelle Bigeard et Lauriane Madec de m'avoir aidé dans mes analyses de génétique et de m'avoir encouragé tout au long de cette thèse.

Ce travail doit également beaucoup aux deux stagiaires avec lesquels j'ai eu le plaisir de travailler, Zujaila Qui-Minet et Maxime Georges des Aulnois. Une partie de ce travail n'aurait pu être accomplie sans votre aide.

Je remercie toutes les personnes ayant participé à l'échantillonnage des sédiments, plus particulièrement Xavier Caisey et Martin Plus. Egalement, merci à Gwendoline Gregoire et Axel Ehrhold du laboratoire GM/LES d'Ifremer d'avoir permis l'échantillonnage de la carotte de Daoulas.

Je tiens à remercier énormément, Sabine Schmidt, Richard Christen et Dominique Soudant pour leurs contributions à ce travail.

Merci aux collègues palynologues de l'IUEM, Aurélie Penaud et à Clément Lambert, avec qui j'ai eu la chance de travailler ces deux dernières années de thèse.

Je remercie le GDR Phycotox et tout particulièrement les organisateurs Philippe Hess et Hélène Hégaret de m'avoir donné l'opportunité de participer aux conférences enrichissantes sur les algues toxiques et de m'avoir fait confiance en m'accordant une bourse pour participer au congrès international l'ICHA2016.

Merci à Alexandre Faulet, gestionnaire de l'école doctorale ED227 (UPMC), d'avoir répondu à mes nombreuses questions sur les démarches administratives et d'avoir facilité la procédure de soutenance.

Je remercie toute la team de « jeunes » de Dyneco/Pelagos : Tania, Pierre, Kim, Laurie, Gabriel, Clémence, Margaux pour la bonne ambiance que vous mettez dans cette équipe. Nos discussions autour d'un café, qui au départ démarrent par une question scientifique finissent toujours par prendre une autre tournure, fou rire garanti.

Je remercie l'ensemble de l'équipe DIPO de la Station Biologique de Roscoff de m'avoir toujours bien accueilli. Je garde un très bon souvenir de la retraite DIPO, durant laquelle j'ai eu le plaisir de vous connaitre davantage.

Je tiens à remercier sincèrement tous mes collègues de Dyneco/Pelagos pour leur accueil chaleureux et les échanges constructifs que l'on a pu avoir durant ces années. J'ai de la chance d'avoir fait partie d'une équipe si agréable. Merci à Françoise Andrieux-Loyer, Anne Daniel, Florian Caradec et Sophie Schmidt d'avoir contribué à ce travail et d'y avoir apporté tous vos conseils. Egalement, merci à Claire Labry et à Marie Latimier d'avoir contribué aux réflexions et aux analyses d'écophysiologie.

Un grand merci à Julien Quéré qui s'est toujours montré disponible pour m'aider dans mes galères. C'était un réel plaisir de travailler avec toi au cours de ces dernières années.

Je remercie énormément ma collègue de bureau, Pascale Malestroit, de m'avoir écouté et conseillé surtout lors des périodes difficiles. Ton sourire, ta bonne humeur et ta joie de vivre m'ont donné plein d'énergie positive.

Un grand merci à Agnès qui m'a soutenu jusqu'à la fin. Tes bons petits chocolats ont été très appréciés et m'ont boosté lors des derniers moments de rédaction.

Je tiens à remercier spécialement Anne-Laure Le Velly pour son extrême gentillesse et qui m'a aidé dans toutes mes démarches administratives. C'est grâce à tes interventions que ce travail a pu être accompli.

Merci à tous mes amis Lam', Meriem, Kamel, Lydia, Zujaila pour leur énorme soutien. Un grand merci à Charaf pour les agréables moments qu'on a partagé ces six dernières années de Marseille à Brest et pour ta disponibilité inconditionnelle dans les moments où ça n'allait pas.

Je remercie également toutes les personnes qui ont participé de près ou de loin à ce travail. Je vous exprime toute ma reconnaissance.

Bien évidemment, je remercie tous les membres de ma famille qui ont su m'apporter leur soutien durant ces trois années de thèse. Je remercie spécialement mes parents qui ont toujours cru en moi et sans qui, je ne serai pas devenue celle que je suis aujourd'hui. Merci pour votre amour et votre soutien inconditionnels.

Mon dernier remerciement s'adresse à mon fiancé Dragos, qui a partagé toutes les émotions ressenties durant cette aventure, de l'immense joie au plus profond désespoir. Tu as toujours su trouver les mots pour m'encourager et me remettre sur la bonne voie. Merci de m'avoir supporté ces derniers mois et d'être resté éveillé la nuit pour m'aider dans la mise en page finale de ce travail, je ne te remercierai jamais assez...

Table des matières

Liste des figures

Liste des tableaux

INTRODUCTION GÉNÉRALE

Préambule

L'environnement dans lequel nous vivons est en constante évolution. Ces changements, liés à des variations physico-chimiques ou à des interactions biotiques, se sont considérablement accélérés suite aux activités humaines et ont considérablement modifié notre planète. Nous sommes rentrés dans une nouvelle ère, nommée *Anthropocène*, pour identifier cette époque de l'histoire de la terre dominée par l'Homme (Crutzen 2002). La résultante de ces activités humaines se traduit par des déséquilibres chroniques, dont le réchauffement climatique lié aux émissions élevées de gaz à effet de serre. Le réchauffement climatique a en retour des conséquences majeures sur la température, la fonte des glaciers, les précipitations et la circulation océanique, et la biogéographie des espèces (Meehl *et al.*, 2007), altérant les conditions environnementales influençant le phytoplancton ainsi que la production primaire (Sarmiento *et al.*, 2004). D'autres facteurs ajoutent une pression supplémentaire sur l'environnement marin, tels que l'acidification de l'océan et le blanchissement du corail, l'altération de la couche d'ozone, l'eutrophisation côtière, et la pression de la pêche. Mis ensemble, tous ces facteurs modifient la structure des communautés des protistes marins et ont des conséquences en cascade sur la dynamique de la chaine trophique marine ainsi que sur les cycles biogéochimiques.

Les conséquences de ces modifications sur les communautés phytoplanctoniques sont largement méconnues. On peut les étudier à travers l'analyse des séries temporelles mises en place par les réseaux de surveillance (*e.g.* le REPHY (Réseau d'Observation et de Surveillance du Phytoplancton et des Phycotoxines)). Ces séries de données sont particulièrement utiles pour repérer les tendances saisonnières, interannuelles, voire interdécennales. Cependant, rares sont les séries de données couvrant une échelle de plus de 40 ans (celles du REPHY par exemple démarrent en 1984), ainsi les informations sur la composition des assemblages phytoplanctoniques anciens et leur dynamique antérieure à la mise en place de ces réseaux sont manquantes.

L'objectif général de ce travail est de contribuer à l'étude des variations des communautés et des populations de protistes en général, et du phytoplancton en particulier, sur une longue échelle temporelle, en adoptant une approche paléoécologique consistant en l'analyse des traces biologiques anciennes (kystes, ADN) accumulées dans les sédiments marins et en utilisant la rade de Brest comme site modèle d'étude.

1. Les protistes

Les protistes sont des organismes eucaryotes, unicellulaires durant la majeure partie de leur cycle de vie (Schlegel and Hülsmann 2007). Ils sont représentés dans tous les groupes d'eucaryotes de l'arbre du vivant (Figure 1) et possèdent une multitude de tailles, de formes, et de fonctions (Caron *et al.*, 2012). Ils présentent des modes trophiques variés tels que l'autotrophie (réalisant la photosynthèse), l'hétérotrophie (consommateurs primaires de bactéries et/ou d'organismes autotrophes, prédateurs et parasites) ou la mixotrophie (alternant l'autotrophie et l'hétérotrophie). Les protistes participent donc activement à la production primaire globale et aux cycles biogéochimiques via leur implication sur l'ensemble du réseau trophique.

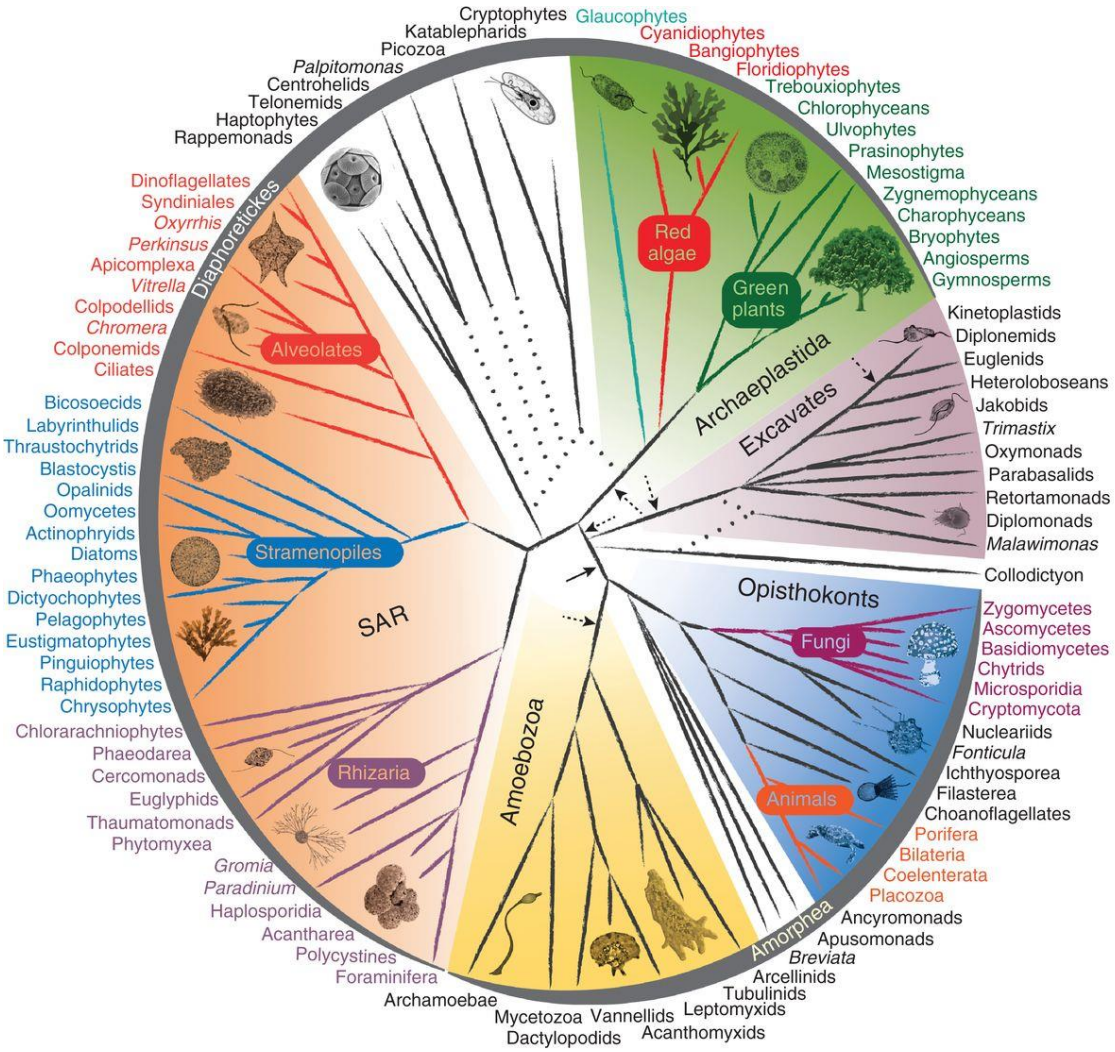


Figure 1. Arbre phylogénétique des eucaryotes selon Burki (2014).

Le groupe des protistes est paraphylétique, c'est-à-dire qu'il regroupe des espèces ayant des histoires évolutives totalement différentes. Initialement, les protistes ont été définis par Haeckel en 1860 comme des organismes autres que les animaux et les plantes et regroupaient donc un assemblage polyphylétique de procaryotes et d'eucaryotes (Caron *et al*., 2012). Jusqu'à la fin du 20eme siècle, les groupes de protistes étaient classifiés selon des critères morphologiques et par mode de nutrition. Or, certains groupes autotrophes et hétérotrophes, séparés à l'origine à cause de leur mode trophique, peuvent pourtant être phylogénétiquement très proches (Caron *et al*., 2012). Depuis les années 1980s, l'avènement des techniques de biologie moléculaire a particulièrement favorisé les études sur la systématique, l'écologie et l'évolution des protistes. En effet, le gène de la petite sous-unité ARN ribosomale 18S fournit des informations essentielles et fiables sur les liens évolutifs entre les organismes et a permis de voir que la phylogénie des protistes ne reflète pas forcément la classification fonctionnelle (Baldauf 2003). Cependant, malgré ces avancées, le terme même de protiste reste encore controversé de nos jours, certains auteurs les définissent comme organismes eucaryotes en excluant les champignons, les plantes, et les animaux, d'autres utilisent la même définition en excluant également les macroalgues. C'est cette dernière définition qui sera utilisée dans cette étude. Chez les protistes, on peut identifier des groupes unicellulaires qui effectuent la photosynthèse et qui dans leur ensemble constituent le phytoplancton.

2. Le phytoplancton

Le phytoplancton regroupe l'ensemble des micro-organismes photosynthétiques eucaryotes et procaryotes (cyanobactéries) vivant dans la zone euphotique des océans et des milieux aquatiques.

Il ne représente que 1% de la biomasse photosynthétique de la planète et pourtant il assure plus de 45% de la production primaire mondiale et plus de 90% de la production primaire du milieu aquatique (Geider *et al*., 2001) (Figure 2).

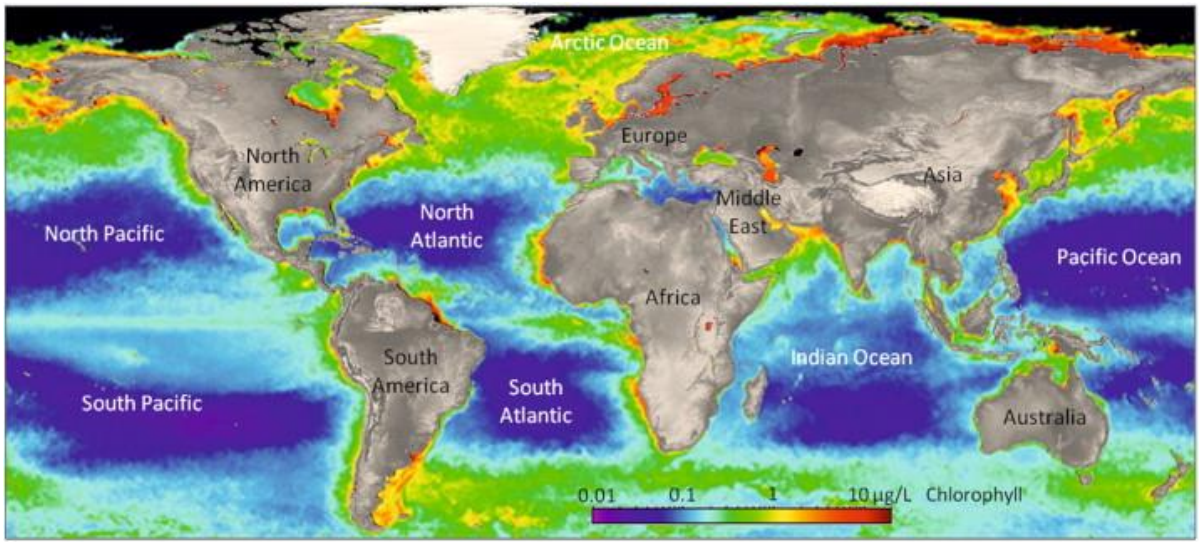


Figure 2. Concentration annuelle moyenne de la chlorophylle *a* à travers le monde (année 2009) (extrait de Villacorte *et al.*, 2015).

Les organismes phytoplanctoniques sont très variables en taille allant de 0.2 µm à 20 mm (voir au-delà) et sont classiquement divisés en 4 classes de taille; le picophytoplancton (0.2-2 µm), le nanophytoplancton (2-20 µm), le microphytoplancton (20-200 µm) et enfin le mésophytoplancton (2-20 mm) (Figure 3). Ce dernier groupe est principalement constitué de diatomées et de dinoflagellés qui sont les groupes dominants en termes de biomasse dans les écosystèmes côtiers.

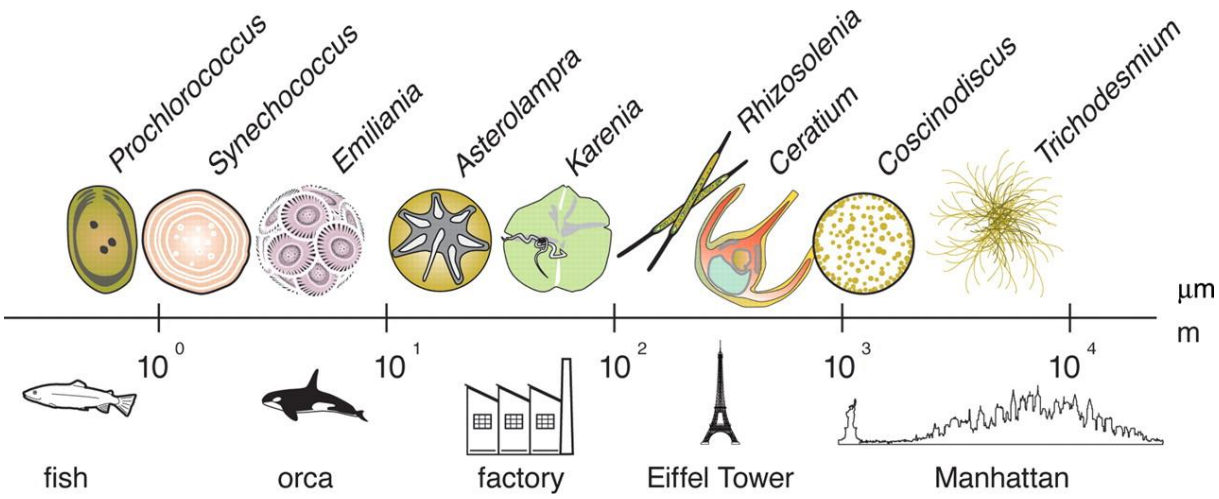


Figure 3. Comparaison de la gamme de taille relative de quelques espèces phytoplanctoniques par rapport à différents objets macroscopiques (extrait de Finkel *et al.*, 2010).

Le phytoplancton est à la base des réseaux trophiques jouant un rôle fondamental dans les écosystèmes pélagiques côtiers en tant que principal producteur primaire (environ 45% de la production primaire globale) (Falkowski 1994). En effet, les organismes photosynthétiques sont capables de fixer le carbone atmosphérique sous la forme de CO_2 et de le convertir en matière organique carbonée à travers l'utilisation de l'énergie lumineuse (Figure 4). Une partie de cette matière organique produite est transférée aux échelons supérieurs du réseau trophique à travers le broutage par le zooplancton, et de la prédation de ces derniers par des consommateurs primaires. L'autre partie est excrétée, sous forme de matière organique particulaire ou dissoute, migrera dans la zone photique vers le fond des océans. Tous ces processus de fixation du carbone inorganique par le phytoplancton et sa transformation en carbone organique, puis son transport vers les eaux profondes, sont des processus constituant la « pompe biologique » qui régule la quantité atmosphérique de CO2.

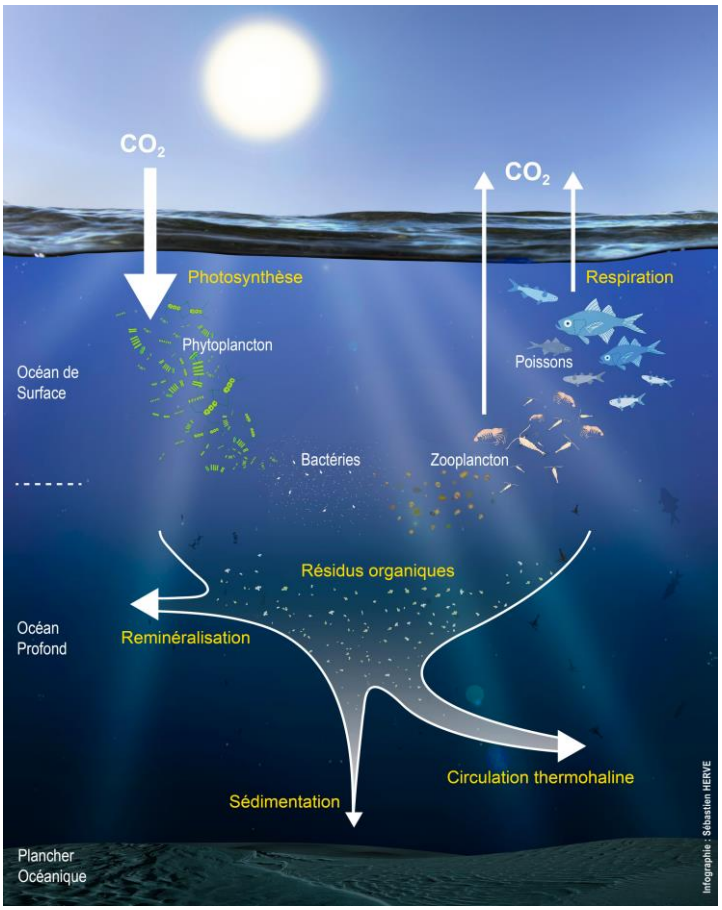


Figure 4. Schéma de la pompe biologique du carbone océanique (source: https://www.labexmer.eu/fr/recherche/axe2).

Lorsque les conditions environnementales sont favorables, certaines espèces de phytoplancton peuvent proliférer massivement, induisant une coloration intense de l'eau de mer. Ces phénomènes d'efflorescence sont communément appelés « eaux colorées » ou « marées colorées ». Dans certains cas, ces efflorescences nuisibles (HAB : Harmful Algal Bloom) peuvent s'accompagner de la production de toxines par les organismes responsables. Environ 5% seulement des espèces phytoplanctoniques sont connues pour produire des toxines (Zingone and Enevoldsen 2000), la majorité appartiennent aux dinoflagellés (Smayda 1997). Ces toxines peuvent s'accumuler dans des organismes destinés à la consommation et la commercialisation comme les pétoncles, les huîtres, les moules et les poissons et elles sont thermorésistantes (ne disparaissent pas à la cuisson). En cas de consommation importante d'organismes bioaccumulateurs de toxines, certains symptômes peuvent apparaître chez l'homme. Il existe plusieurs types de syndrome classés selon leur effet sur l'homme et la typologie de la toxine : toxines Amnésiantes (ASP, Amnesic Shellfish Poisonning), Diarrhéiques (DSP (diarrheic), AZP (Azaspiracid)), Paralysantes (PSP), Neurologiques (NSP), et CFP (Ciguatera Fish Poisonning). Au-delà des effets sur la santé publique, les efflorescences toxiques ont des conséquences sur le fonctionnement de l'écosystème, le tourisme, et l'économie.

3. Variabilité spatio-temporelle des organismes phyto-planctoniques

La diversité et la biomasse du phytoplancton varient dans le temps et dans l'espace. Les facteurs contrôlant ces variations ont été discutés depuis la découverte du phytoplancton par Van Leeuwenhoeck en 1676 (Dobell 1932) et, particulièrement après la découverte, environ 2 siècles plus tard, du fait que le phytoplancton était à la base de la chaine alimentaire dans les océans. Depuis, la recherche a fait des progrès considérables dans l'étude et la compréhension de ces facteurs. Globalement, la diversité, la biomasse, et la taille du phytoplancton sont contrôlés par des processus complexes ascendants, liés aux conditions physico-chimiques, et descendants, liés aux interactions biologiques (bottom-up et top-down en anglais). Ces processus incluent la compétition pour des ressources limitées (lumière, azote, phosphore, fer) (Tliman *et al*., 1982) et des processus top-down tels que le broutage (Armstrong 1994). Ces interactions sont à leur tour contrôlées par des processus physiques tels que la température, la circulation des masses d'eau, le dépôt de particules atmosphériques et la turbulence (Falkowski *et al*., 1998; Finkel *et al*., 2010).

Le changement de groupes ou espèces de phytoplancton dominants se répercute souvent sur la chaine trophique impactant les poissons, les mammifères et les oiseaux (Donnelly *et al*., 2007). Les organismes phytoplanctoniques ayant un cycle de vie très rapide sont connus pour être sensibles et réactifs face aux changements des conditions environnementales. Par conséquent, ils représentent

ainsi d'excellents bioindicateurs de l'impact des changements naturels et saisonniers sur les écosystèmes côtiers (Rimet and Bouchez 2012). Plusieurs études ont montré l'impact des variations de l'environnement sur le phytoplancton à différentes échelles biologiques; organismes, populations, communautés en effectuant des observations sur le terrain, des expériences en mésocosmes et/ou par de la modélisation. Ces changements se traduisent principalement par des variations de biomasse, d'abondance, et de structure des communautés. En effet, il a été estimé un déclin de la biomasse globale du phytoplancton de 1% par an au cours du siècle dernier (Boyce *et al.*, 2010), lié principalement au changement de la température des eaux de surface. Particulièrement, des études ont reporté une diminution de la biomasse des diatomées dans l'océan Atlantique, la Manche et la Mer Baltique durant 4 années consécutives (Klais *et al.*, 2011; Zhai *et al*., 2013) accompagnée occasionnellement par une augmentation de la biomasse des dinoflagellés. L'apport excessif en nutriments induit souvent l'eutrophisation des ecosystèmes qui se traduit parfois par l'apparition d'efflorescences nuisibles, notamment dans des milieux confinés (Rabalais *et al*., 2011). L'acidification des océans suite à l'augmentation du CO_2 aurait induit des variations de la diversité et de l'abondance des coccolithophoridés (Meyer and Riebesell 2015).

La structure des communautés peut également être impactée, principalement à cause des variations climatiques interannuelles, telles que la NAO (Oscillation Nord Atlantique) qui a été reliée à l'augmentation de l'abondance des diatomées et la diminution de celle des dinoflagellés (Irigoien *et al*., 2000; Zhai *et al*., 2013), et l'AMO (Oscillation Multidécennale de l'Atlantique) induisant des changements de structure à long-terme (Edwards *et al*., 2013). Les métaux lourds peuvent être toxiques pour les organismes phytoplanctoniques, introduisant des variations de la dominance des espèces et affectant la structure et la composition des communautés (Kapkov *et al*., 2011).

Les espèces phytoplanctoniques peuvent également subir des variations spatiales. C'est le cas de certaines espèces de dinoflagellés (*Gymnodinium catenatum*), et de diatomées (*Rhizosolenia indica*) dont l'occurrence en Mer du Nord a été attribuée à une hausse de température (Nehring 1998). Dans l'Atlantique Nord, l'analyse d'archives géologiques ainsi que des données du réseau d'observation du plancton via le CPR (Continuous Plankton Recorder) a montré la réapparition de la diatomée *Neodenticula seminae*, après 840 000 années d'absence (Miettinen *et al.*, 2013; Reid *et al.*, 2007). Ce changement a été attribué à la fonte de glace et/ou la circulation des masses d'eau. Quelque fois l'expansion géographique d'une espèce est attribuée à son introduction à travers divers vecteurs, dont le rejet des eaux de ballast des navires. C'est le cas pour *Coscinodiscus wailesii* dans la Manche et la Mer du Nord (Edwards *et al.*, 2001) ou *Alexandrium catenella* dans l'étang de Thau, France (Lilly *et al.*, 2002). Ces nouvelles espèces introduites peuvent devenir proliférantes et être à l'origine

d'efflorescences nuisibles, voire toxiques avec des conséquences importantes sur le fonctionnement des écosystèmes.

3.1. Les méthodes d'étude des changements au niveau du phytoplancton

Les variations du phytoplancton sont étudiées de plusieurs manières, la plus classique étant l'analyse de séries temporelles dans le cadre des réseaux de surveillance ou d'observation du plancton. La collecte des données de séries temporelles implique des mesures dans l'eau, généralement sur une base hebdomadaire ou mensuelle dans le but de renseigner sur la biomasse, l'abondance et la composition taxonomique à différentes stations de surveillance. Cela permet également de suivre les espèces phytoplanctoniques productrices de toxine, ainsi que de reporter les évènements d'efflorescences, et les compositions des communautés. La télédétection de la couleur de l'eau permet d'obtenir une mesure de la quantité de chlorophylle (indication sur la biomasse) présente dans l'eau à l'échelle globale ou régionale, avec d'importantes implications pour le changement climatique et les pêcheries. Les images satellites permettent ainsi de suivre les changements de biomasse lors d'évènements d'efflorescences saisonniers. La modélisation permet également d'étudier les réponses du phytoplancton à certains changements comme la température et les concentrations en nutriments sur la base de séries temporelles. De plus, ils permettent dans plusieurs cas de fournir une aide à la décision.

Récemment, le développement des outils moléculaires a permis d'améliorer l'étude de la biodiversité. En particulier, l'approche par metabarcoding a été employée pour étudier la diversité et les changements des communautés (de Vargas *et al.*, 2015; Yoon *et al.*, 2016) ou de groupes de phytoplancton, tels que les dinoflagellés (Le Bescot *et al.*, 2015) et les diatomées (Kemarrec *et al.*, 2013; Malviya *et al.*, 2016). Le metabarcoding peut également être utilisé pour des fins de surveillance. En effet, dans une démarche d'optimisation de l'évaluation de la qualité des eaux à l'aide de diatomées, Zimmermann *et al.*, (2015) ont révélé une plus grande diversité identifiée par metabarcoding par rapport à celle identifiée par microscopie optique et ont proposé le metabarcoding comme un bon outil complémentaire pour la surveillance de la qualité de l'eau.

Les variations peuvent également être étudiées au niveau des espèces et des populations à l'aide de ces approches génétiques. Celles-ci sont particulièrement utiles pour les études de biogéographie, notamment pour caractériser les voies d'introduction des espèces invasives, ou encore pour mieux comprendre les efflorescences toxiques et les espèces qui en sont responsables. Ainsi, à l'aide de marqueurs génétiques (microsatellites), Dia *et al.*, (2014) ont montré une différentiation génétique

significative dans le temps et dans l'espace au cours d'efflorescences toxiques d'*Alexandrium minutum* au niveau de 2 estuaires bretons et sur deux années consécutives.

Toutes ces méthodes sont indispensables pour l'étude des changements des communautés phytoplanctoniques et reposent sur l'analyse de séries temporelles. Cependant, celles-ci s'étendent rarement au-delà de 40 ans à quelques exceptions près, comme c'est le cas pour le CPR (Continuous Plankton Recorder) dont les données temporelles s'étendent sur plus de 80 ans (Batten and Burkill 2010). Par conséquent, la composition des communautés phytoplanctoniques anciennes demeure inconnue. Pour y remédier, une alternative serait de se servir de certains restes biologiques fossilisés pour reconstruire les tendances du passé et les communautés anciennes, c'est ce qu'on appelle l'approche paléoécologique.

4. La paléoécologie, historique et applications

La paléoécologie, au sens étymologique est l'étude de l'écologie du passé (Birks and Birks 1980). Il existe plusieurs autres définitions, la plus exhaustive est celle proposée par Rull (2010) : «la branche de l'écologie qui étudie les écosystèmes du passé et leur tendance au cours du temps en utilisant des fossiles et d'autres proxies».

L'idée que les objets fossiles trouvés dans des roches sédimentaires soient en réalité des traces d'organismes ayant vécu par le passé a été longtemps controversée. Plusieurs savants grecs de l'antiquité, tels le poète et philosophe Xénophane, l'historien Hérodote ou le géographe Strabon, interprétaient de façon correcte la nature des fossiles. Pourtant, c'est à la conception erronée d'Aristote (fossiles façonnés directement dans la boue à partir d'une « force formatrice » mystérieuse) que l'on s'est référé pendant deux mille ans. Ce n'est qu'à partir du début du 19ème siècle que la véritable nature des fossiles a été réellement démontrée grâce à l'établissement des premiers principes de la géologie moderne et notamment, de la parution du livre « L'origine des espèces », de Charles Darwin en 1859, qui a bouleversé les idées reçues en sciences biologiques, notamment en paléontologie, zoologie et biogéographie, allant même au-delà (au niveau philosophique et religieux) avec la théorie de la sélection naturelle. Par la suite, les études paléoécologiques ont été fortement stimulées par l'industrie du pétrole à travers l'analyse des échantillons récupérés par forage. La découverte de nombreux microfossiles à partir d'une simple roche a largement contribué au gain d'informations sur les anciens environnements et la datation. C'est à partir des années 1960s et 1970s que l'étude des communautés de fossiles et des paléocommunautés a réellement explosé et que la paléoécologie évolutionniste (les fossiles changent au cours du temps à la fois à cause de l'évolution des espèces et des conditions

environnementales) est née. Aujourd'hui, la paléoécologie est multidisciplinaire, combinant à la fois des techniques de paléontologie, de sédimentologie et de géochimie afin de reconstruire les environnements du passé et d'explorer les réponses biotiques aux changements environnementaux (Seddon 2012). Elle permet d'étudier les processus de spéciation, d'invasion, d'extinction, ainsi que les processus d'adaptation, de migration et les shifts au niveau des populations (Seppä, 2009). De plus, elle offre une précieuse source d'information pour comprendre comment les écosystèmes de la terre répondront au réchauffement climatique actuel.

Il existe 2 échelles de temps communément utilisées en paléoécologie : 1) période du quaternaire (Quaternary time) : qui correspond à l'échelle de temps allant du siècle à des millénaires (10 000 ans) et couvre des processus tels que la succession, la migration et l'extinction; 2) période pré-quaternaire (Deep-time) : qui inclut un large intervalle d'échelles de temps, généralement (> 10 000 ans) et dans laquelle on s'intéresse à l'évolution et la dynamique des organismes ainsi qu'aux réorganisations biogéographiques à large échelle (Vegas-Vilarrubia *et al*., 2011).

4.1. La paléoécologie sédimentaire

Les traces biologiques peuvent se préserver dans les sédiments marins et constituer une précieuse source d'information pour les paléoécologues en tant que proxies des changements des communautés anciennes et de l'environnement du passé. En effet, le sédiment marin représente un véritable réservoir de la biodiversité et une excellente archive à long-terme du climat de la planète. Au-delà des organismes qui y vivent, on retrouve des formes vivantes provenant de la colonne d'eau, tels que les formes de résistance du plancton (kystes et spores), qui peuvent rester en dormance de nombreuses années ainsi que des restes de coquilles et de squelettes issus de la mort des organismes. On y retrouve également de la matière organique provenant des organismes planctoniques anciens. En effet, durant son transport vers le fond de la mer, la matière organique est ingérée par différents organismes de la chaine trophique et est ainsi transformée, et seule une petite partie parvient au fond de la mer et y est accumulée (Figure 5) (Jørgensen and Boetius 2007). L'inspection du registre sédimentaire est donc la clé pour identifier les communautés anciennes et l'évolution des environnements du passé.

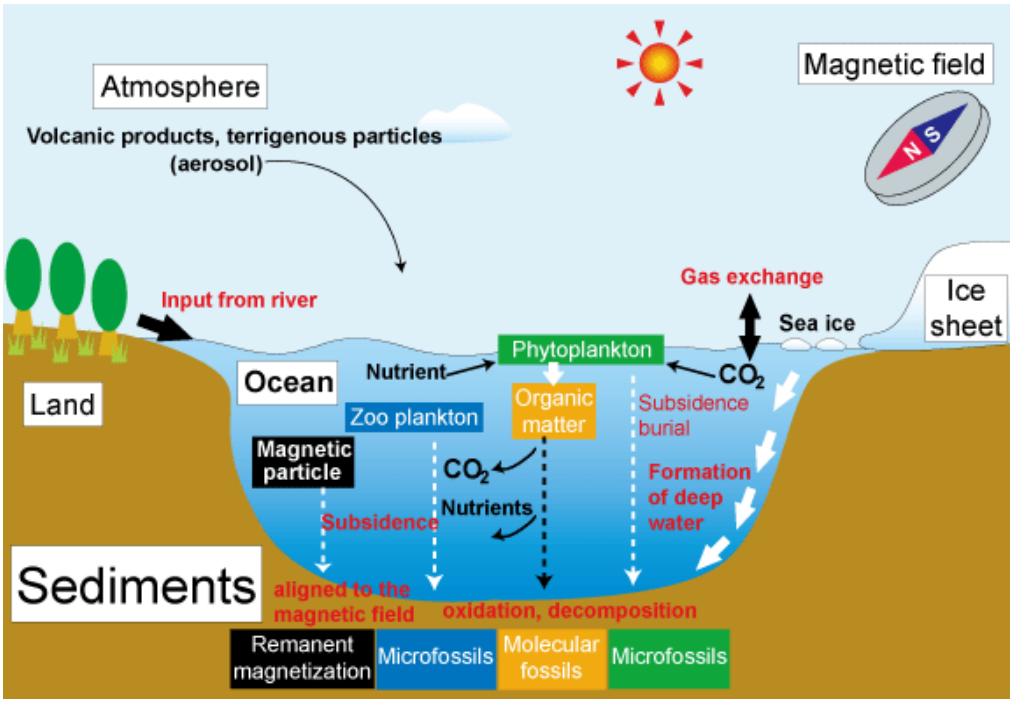


Figure 5. Illustration des différents flux dans la colonne d'eau ainsi que le dépôt des micro-macrofossiles et de la matière organique dans le sédiment marin (source : http://www.kochi-u.ac.jp/marine-core/research/index_e.html).

4.2. Les indicateurs (proxies) en paléoécologie

4.2.1. Les macro et microfossiles

Les macrofossiles représentent généralement des restes de plantes et de végétaux tels que des graines et des tissus végétaux. L'analyse de ces macrofossiles est souvent combinée à une analyse de microfossiles, car ensemble, ces deux proxies permettent de renseigner sur la couverture végétale ancienne à une échelle régionale avec des identifications pouvant aller jusqu'au genre ou à l'espèce (Birks and Birks 2000; Jørgensen *et al*., 2012).

Les microfossiles contenus dans les sédiments sont composés de l'ensemble des restes d'organismes invisibles à l'œil nu, tels que le pollen, les kystes de dinoflagellés, spores et frustules de diatomées, coccolithes d'haptophycées, et coquilles de foraminifères. L'utilisation de microfossiles dans les études de paléoécologie permet d'acquérir une grande quantité d'informations sur les environnements du passé. En effet, les microfossiles sont généralement utilisés en tant qu'indicateurs des conditions physique et chimique de leur habitat (température de l'eau, oxygène), donnant accès à des reconstructions qualitatives ou quantitatives des paramètres de l'environnement.

Les grains de pollen des plantes peuvent se conserver pendant des millions d'années dans le sédiment. Leur étude, qui relève de la discipline de la palynologie (branche étudiant les microfossiles

organiques), est principalement centrée sur la reconstruction de l'histoire de la végétation ancienne et des environnements du passé. Les grains de pollen font partie des indicateurs les plus employés dans les reconstructions environnementales et climatiques dans les études du Quaternaire (Edwards and Macdonald, 1991).

Les microfossiles tels que les foraminifères et les coccolithes peuvent incorporer la signature géochimique de l'environnement dans lequel ils se forment. Par exemple, l'étude des isotopes du carbone contenu dans les tests de foraminifères peut servir à la reconstruction des températures d'eau de surface ou la paléoproductivité et/ou la configuration de masse d'eau (McCorkle *et al*., 1997; Bickert and Mackensen 2004; Curry and Oppo 2005). Les coccolithes possèdent également cette propriété et sont souvent utilisés pour des études de biostratigraphie mais aussi pour reconstruire les paramètres environnementaux anciens (température, nutriments).

Les spores et frustules de diatomées peuvent s'accumuler dans le sédiment et sont conservés pour des périodes de temps considérables. Ils sont utilisés pour reconstruire la température des eaux de surface (Vyverman and Sabbe 1995; Bigler and Hall 2002), la salinité (Fritz *et al.*, 1991; Laird *et al.*, 1996; Verschuren *et al.*, 2000), le carbone organique dissous (DOC) (Pientiz *et al.*, 1999), la conductivité (Davies *et al.*, 2002) et le pH (Psenner and Schmidt 1992; Koinig *et al.*, 1998).

Les dinoflagellés sont connus pour être sensibles aux changements de température, d'où l'utilisation de leurs kystes comme indicateurs des températures anciennes (de Vernal *et al.*, 2001; Londeix *et al.*, 2009, Chen *et al.*, 2011). Ils sont également utilisés comme indicateurs de l'eutrophisation (Dale 2009; Zonneveld *et al.*, 2012; Donders *et al.*, 2008) ou comme indicateurs biostratigraphiques notamment dans l'industrie de l'exploration pétrolière (Gradstein *et al*., 1992).

Les reconstructions paléoécologiques basées sur l'analyse des fossiles présentent l'inconvénient d'être restreintes uniquement aux taxa qui laissent des structures fossilisables, identifiables dans le sédiment, or, la plupart des organismes planctoniques ne possèdent pas de telles caractéristiques. En l'absence de ces structures, d'autres méthodes basées sur l'utilisation de biomarqueurs (lipides, les pigments ou encore l'ADN) peuvent être employées pour identifier les autres organismes.

4.2.2. Les biomarqueurs

Les composés organiques retrouvés dans les sédiments tels que les lipides, les pigments et l'ADN et sont dérivés d'organismes spécifiques et forment une archive de l'ancienne composition des espèces dans la colonne d'eau et par conséquent, peuvent être utilisés pour reconstruire les conditions physiques et chimiques résultant de changements climatiques (Brassell 1993). Pour qu'un biomarqueur soit utilisé comme indicateur en paléoécologie, il doit soit rester non altéré, ou retenir suffisamment d'informations dans sa forme altérée.

4.2.2.1. Les lipides

Les biomarqueurs lipidiques (stéroles, alkénones), sont connus pour être stables, résistants à la diagénèse et sensibles aux paramètres environnementaux (Huang *et al.*, 2004). En effet, dans des conditions optimales de préservation, on estime que ces lipides peuvent être préservés dans le sédiment jusqu'à un milliard d'années (Brocks and Pearson 2005). Dans les reconstructions paléoécologiques, l'analyse des lipides permet de connaitre les principaux organismes ayant vécu dans des écosystèmes anciens ainsi que la manière dont ces écosystèmes ont changé. En effet, en analysant la distribution et la composition isotopique de ces composés, il a été possible de reconstruire la productivité océanique ancienne (Brassell 1993), ou même la température de la surface de la mer, par exemple en analysant les alkénones à longue chaine qui sont spécifiques aux haptophytes (coccolithophoridés) (McCaffrey *et al.*, 1990). De plus, en combinant des biomarqueurs lipidiques (alkénones à longue chaine) à une analyse de l'ADN fossile, Coolen *et al.*, (2009), ont pu reconstruire la succession des haptophytes en Mer Noire. Cependant, malgré leur utilité, ces biomarqueurs présentent la limite de ne pas être spécifique à un seul organisme donné mais à un groupe entier (plantes terrestres, algues, bactéries) et par conséquent, sont moins efficaces quand il s'agit de reconstruire la présence d'organismes cibles.

4.2.2.2. Les pigments

Les pigments produits par les organismes photosynthétiques peuvent se préserver dans le sédiment et servir pour reconstruire les communautés phototrophes ainsi que la productivité du passé. Certains pigments sont spécifiques à des groupes particuliers d'organismes (Jeffrey *et al.*, 1997). Ils reflètent assez bien les changements des conditions environnementales grâce à leur réponse à un certain nombre de forçages tels que l'apport en nutriment, l'acidification et la pression de broutage (Leavitt and Hodgson 2001). La majorité des études paléoécologies basées sur les pigments ont été réalisées dans des écosystèmes lacustres, car ceux-ci présentent des conditions idéales pour la préservation des pigments. En particulier, ces études ont renseigné sur les changements de la structure du phytoplancton en réponse à l'augmentation des apports en nutriments. De plus, il a été montré que les pigments totaux sont corrélés à l'abondance des organismes photosynthétiques et à leur biomasse (Leavitt and Findlay 1994). Certains pigments comme les caroténoides peuvent renseigner sur les changements historiques de l'irradiance solaire dans les lacs (Leavitt *et al.* 1997) ou sur des évènements d'anoxie dans des écosystèmes côtiers (Squier *et al.*, 2001).

4.2.2.3. L'ADN

L'ADN est intrinsèquement retrouvé dans tous les environnements de la planète comportant de la vie. Contrairement aux biomarqueurs pigmentaires ou lipidiques, l'ADN possède une spécificité inégalée et peut fournir une résolution taxinomique allant jusqu'au niveau de l'espèce (Edvardsen *et al*., 2000). De plus, il permet d'étudier également les organismes qui ne laissent pas de structures fossilisables. L'ADN a été utilisé pour reconstruire les environnements du passé et évaluer les changements de l'écosystème à travers le temps mais aussi pour reconstruire des changements au niveau des communautés biologiques tels que les virus datant d'environ 7000 ans (Coolen 2011), les bactéries datant de 217 000 ans en Méditerranée, (Coolen and Overmann 2007), les champignons d'au moins 16 000 ans (Lyodolph *et al*., 2005; Bellemain *et al*., 2013), les protistes datant de 32 000 ans (Lejzerowicz *et al*., 2013), les plantes d'au moins 10 000 ans (Parducci *et al*., 2013; Pedersen *et al*., 2013), et les animaux d'au moins 7 000 ans (Haile *et al*., 2009).

5. L'analyse des kystes et de l'ADN ancien comme indicateurs paléoécologiques

5.1. Les kystes de dormance et les kystes pelliculés

Certains organismes phytoplanctoniques possèdent un cycle de vie caractérisé par une alternance de deux phases; planctonique et benthique, impliquant plusieurs types sexués. Ceci est le cas de certaines espèces de dinoflagellés (Figure 6). En effet, lors de la phase planctonique (haploïde), les cellules végétatives présentes dans la colonne d'eau se divisent par mitose (formation de deux cellules filles identiques à la cellule mère). Lorsque les conditions environnementales deviennent défavorables (e.g. diminution de la concentration en nutriments et de l'intensité lumineuse, fluctuations de salinité et/ou température) (Taylor 1987), les cellules végétatives entrent dans la phase sexuée et produisent des gamètes. Dans le cas des dinoflagellés, la fusion de deux gamètes produit un planozygote diploïde qui se développe généralement en un hypnozygote (kyste de dormance) entouré d'une double membrane (Figure 7A). Chez certaines espèces de dinoflagellés, le planozygote peut directement produire des cellules végétatives sans passer par la formation d'un hypnozygote (Figueroa *et al*., 2010).

Les kystes de dormance s'accumulent dans le sédiment et peuvent rester en dormance plusieurs années (Wyatt and Jenkinson 1997). Ils passent par une période de dormance dite « obligatoire » avant qu'ils ne soient physiologiquement capables de germer. La durée de cette période est très variable selon les espèces, allant de 12h à 12 mois (Kremp and Anderson 2000) et même au sein d'une espèce. Par exemple, les kystes d'*Alexandrium catenella* semblent avoir une période de

dormance obligatoire assez courte allant de 5 à 65 jours, différente selon la région géographique (Hallegraeff *et al*., 1998; Joyce and Pitcher 2006).

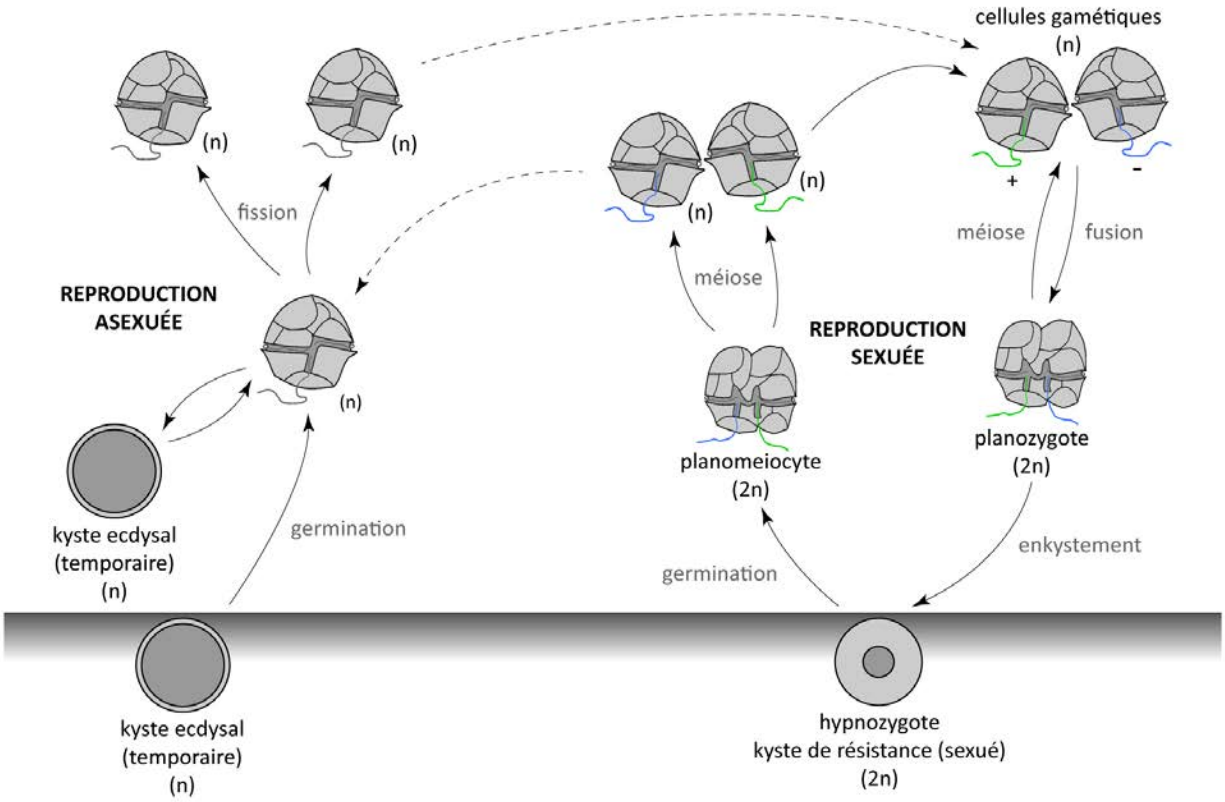


Figure 6. Cycle de vie général d'un dinoflagellé, cas de l'espèce toxique *Alexandrium minutum* (extrait de Le Bescot 2014).

Il arrive également que les cellules végétatives des dinoflagellés s'enkystent temporairement en réponse à des conditions environnementales défavorables ou lors d'un stress physique ou chimique. Les cellules végétatives perdent leurs thèques ainsi que leurs flagelles et prennent une forme arrondie. Une nouvelle paroi appelée « pellicule » se forme (Sekida *et al*., 2001) (Figure 7B). L'existence de ces formes de résistance permet aux cellules de supporter les fluctuations à court terme des conditions environnementales (température, salinité, turbulence) (Anderson 1998; Garcés *et al*., 2001). Ces kystes formés par méiose sont haploïdes, possèdent une seule fine membrane (pellicule), et n'ont pas de période de dormance obligatoire contrairement aux kystes de dormance. Plusieurs termes ont été utilisés dans la littérature pour qualifier ce type de kystes; « kystes temporaires », « ecdysaux », ou encore « pelliculés ». Bravo *et al*., (2010) explique que ces termes portent à confusion et propose l'uniformisation des ces appellations et l'utilisation du terme « kystes pelliculés».

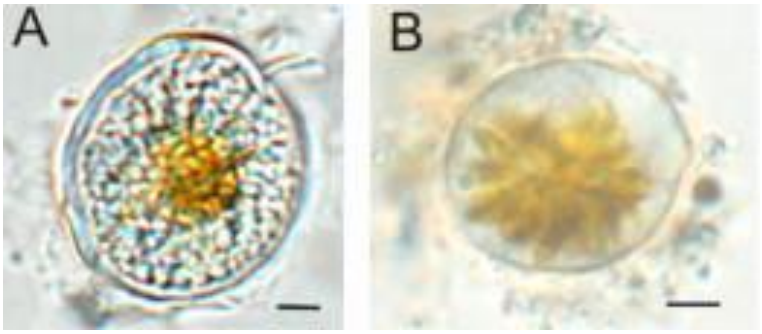


Figure 7. Morphologie des kystes de résistance d'*Alexandrium minutum.* A) kyste de dormance. B) kyste pelliculé (modifié d'après Bravo *et al*., 2010).

5.1.1. Risque de prolifération toxique à partir des kystes de dormance associés aux sédiments

Les kystes permanents peuvent rester dormants pendant de nombreuses années dans les sédiments et sont protégés par les structures extérieures qui les entourent. En effet, la double membrane résistante entourant les kystes de dinoflagellés est composée de polymères, proches de la sporopollénine, une molécule qu'on retrouve également chez les spores et les grains de pollen des plantes. Certaines espèces ont une couche supplémentaire composée de carbonate de calcium ou encore d'épines. Les kystes accumulés dans le sédiment peuvent germer s'ils sont remis en suspension dans la colonne d'eau par différents processus (hydrodynamisme, bioturbation, dragage). Cette « banque de kyste », représente une source d'ensemencement potentielle pour l'initiation des efflorescences d'une année sur l'autre. L'étude des kystes permet donc d'estimer la contamination locale par les kystes d'espèces phytoplanctoniques toxiques et d'évaluer le risque d'un développement récurrent d'efflorescences nuisibles. La caractérisation de la typologie des sédiments peut contribuer à la compréhension des phénomènes physico-chimiqueS conduisant à l'accumulation de ces kystes. En effet, en examinant les sédiments superficiels de baies, ports et lagunes, des densités élevées de kystes ont été retrouvées (ex : Erard-Le Denn 1991; Anglès *et al*., 2010; Genovesi *et al*., 2013). Plusieurs études ont aussi montré que l'accumulation des kystes dépend de la granulométrie du sédiment, elle serait plus importante dans des sédiments de nature vaseuse plutôt que sableuse (Anglès *et al*., 2010). De plus, une concentration importante de matière organique (~5-15%) et une porosité élevée favoriseraient également l'accumulation des kystes, comme c'est le cas pour les kystes *d'Alexandrium tamarense/catenella* dans la lagune de Thau (France) (Genovesi *et al*., 2013).

Plusieurs études ont pu isoler des formes de résistance anciennes d'organismes planctoniques. Ces kystes datés de quelques années à plusieurs dizaines d'années ont pu germer en laboratoire et être

remis en culture (Ribeiro *et al.*, 2011; Härnström *et al.*, 2011; Mcquoid *et al.*, 2002; Lundholm *et al.*, 2011). De plus, Miyazono *et al.*, (2012) ont réussi à faire germer des kystes d'*A. tamarense* âgés d'environ 100 ans, à partir de sédiments côtiers de la baie de Funka (Japon), ce qui représente la plus grande durée de viabilité connue pour cette espèce. Cependant, le taux de germination des kystes a tendence à diminuer avec l'âge des sédiments (Miyazono *et al.*, 2012; Lundholm *et al.*, 2011) (Figure 8). Les souches obtenues par germination ont très peu été utilisée pour analyser leurs adaptations aux conditions environnementales du passé (e.g. Ribeiro *et al.*, (2013)).

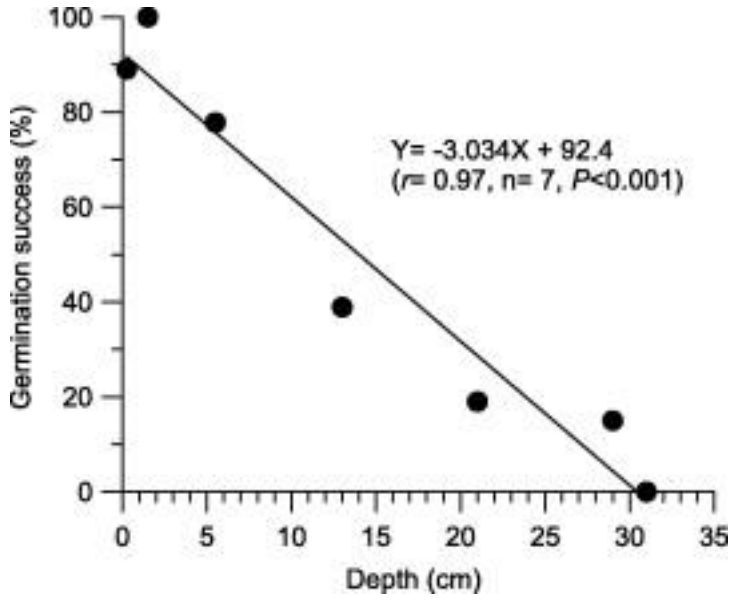


Figure 8. Corrélation entre la profondeur du sédiment et le succès de germination des kystes d'*A. tamarense* (extrait de Miyazono *et al*., 2012).

5.2. L'ADN ancien

L'ADN porte l'information génétique de l'identité des organismes, leur fonctionnement et leur adaptation aux conditions locales ainsi que leur histoire évolutive. L'ADN environnemental peut être isolé à partir d'une variété de substrats tels que les sédiments marins et lacustres (Lejzerowicz *et al.*, 2013; Parducci *et al.*, 2013), les sols (Jøergensen *et al.*, 2012), les glaciers (Willerslev *et al.*, 2007), le permafrost (Epp *et al.*, 2012), les coprolithes (Poinar *et al.*, 1998; Reinhard *et al.*, 2008), les contenus intestinaux (van Geel *et al.*, 2014), ou même encore les plaques dentaires calcifiées (Metcalf *et al*., 2014) (Figure 9).

Depuis que Poinar *et al*., (1998) ont isolé pour la première fois de l'ADN ancien (végétal et animal) datant de 20 000 ans à partir de coprolithes (excréments fossilisés), il y a eu une augmentation fulgurante d'études ayant intégré l'analyse de l'ADN ancien dans les études paléoécologiques. En

effet, l'ADN ancien a été utilisé pour répondre à une variété de questions, telles que la description des paléoenvironnements (Willerslev *et al*., 2014), la réponse des écosystèmes au changement climatique (de Bruyn *et al*., 2011), la biogéographie (Mitchell *et al*., 2014), l'impact des activités anthropiques et les processus d'extinction (Shapiro *et al*., 2004; Drummond *et al*., 2005), l'évolution de l'Homme et la paléogénomique (Green *et al.*, 2010; Meyer *et al.*, 2012) et même l'évolution des maladies humaines (Adler *et al.*, 2013; Krüttli *et al.*, 2014; Metcalf *et al.*, 2014).

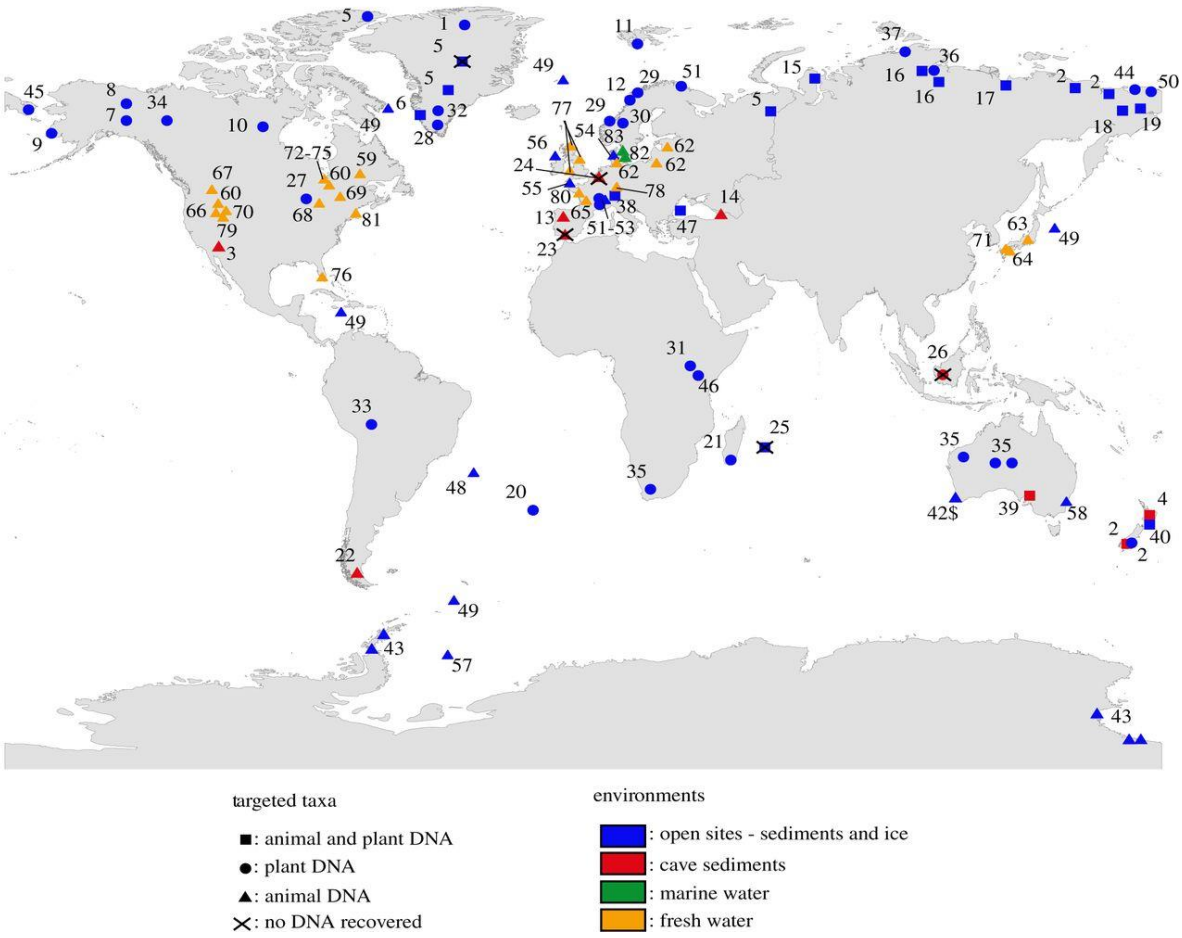


Figure 9. Carte géographique représentant les sites où l'ADN eucaryote environnemental (moderne et ancien) a été étudié. Les symboles correspondent aux taxa cibles. Les couleurs correspondent à différents types d'environnements (extrait de Pedersen *et al*., 2015).

5.2.1. Potentiel de préservation de l'ADN dans les sédiments

Après la mort des cellules ou la libération dans l'eau, les molécules d'ADN peuvent subir différentes transformations le long de la colonne d'eau et dans les sédiments, et finissent soit par être dégradées via des processus biotiques et abiotiques et/ou être finalement préservées sur du long-terme (Torti *el al*., 2015) (Figure 10). Une partie de cet ADN se dépose dans les sédiments marins et lacustres, soit

à l'état intracellulaire (à l'intérieur de cellules ou des formes de dormance) ou à l'état extracellulaire (libre à l'extérieur des cellules)(Figure 11).

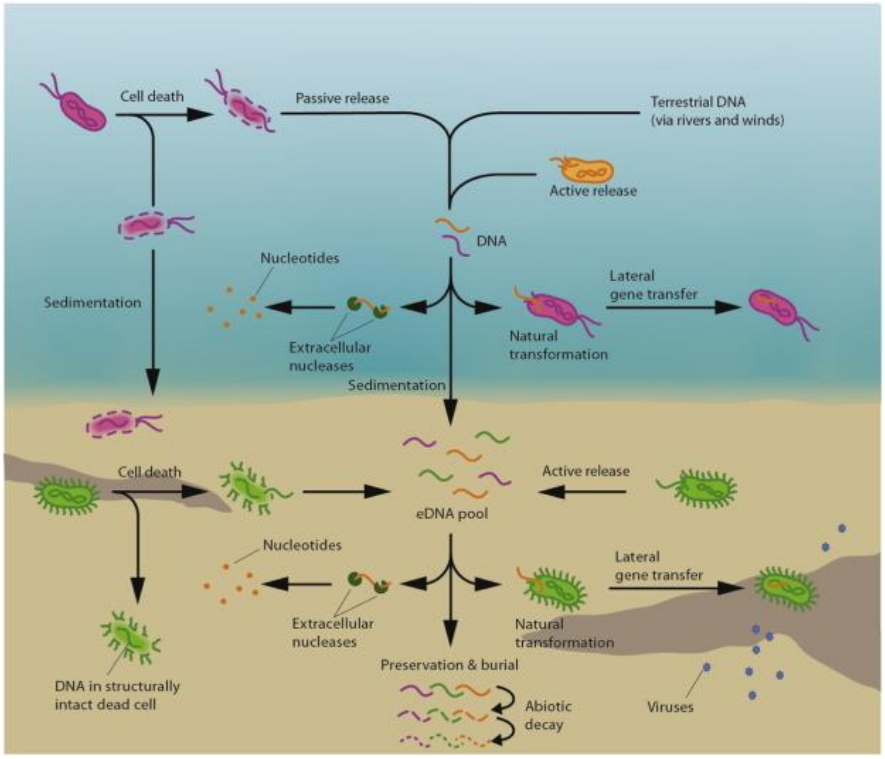


Figure 10. Schéma montrant l'origine et devenir de l'ADN extracellulaire dans l'environnement marin (extrait de Torti *et al*., 2015).

On pense que la structure de résistance des organismes en état de dormance (kystes, spores, graines de plante, pollen) dans les sédiments permet une meilleure préservation de l'ADN comparé aux organismes qui ne possèdent pas de telles structures. Cependant, il a été montré que l'ADN extracellulaire constitue la majeure partie de l'ADN total dans les sédiments, et qu'il se trouve associé généralement à des cellules mortes, agrégats organiques et inorganiques, et à des matrices minérales (Boere *et al.*, 2010). De plus, il peut se prémunir contre la dégradation en s'adsorbant à des matrices minérales et molécules organiques réfractaires (Pietramellara *et al.*, 2009). De cette façon, on estime qu'entre 50-95 % de l'ADN extracellulaire pourrait être protégé contre la dégradation (Danovaro *et al.*, 2006; Dell'Anno *et al.*, 2002). Enfin, une partie de l'ADN correspond à des organismes vivants dans les sédiments.

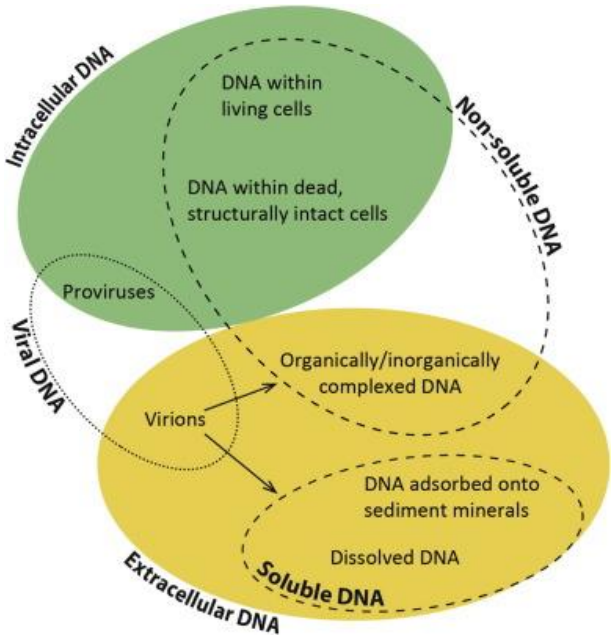


Figure 11. Les différentes formes d'ADN dans les sédiments marins. La dénomination de l'ADN intracellulaire et de l'ADN extracellulaire ne prend pas en compte le statut physiologique de la cellule (vivante, en dormance, morte) (extrait de Torti *et al*., 2015).

L'idée que l'ADN puisse être conservé après son enfouissement dans les sédiments et se préserver sur de très longues périodes de temps a été longtemps considérée comme impossible, en raison de la fragilité de la molécule d'ADN (Figure 12) et de sa dégradation rapide par les microorganismes. En effet, lorsqu'il est sous forme hydratée, l'ADN est sujet à de nombreux processus de dégradation à la fois abiotique (hydratation, oxydation, endommagement lié aux UV) et biotique (nucléases microbiennes) qui conduisent à sa fragmentation (Willerslev and Cooper 2005). A l'inverse, il a été montré que l'ADN se préserve mieux dans des environnements froids et secs tels que les permafrost et les glaciers (Willerslev *et al.*, 2004; Orlando *et al*., 2013) ainsi que sous conditions anoxiques (Boere *et al.*, 2011). D'autres facteurs physiques tels qu'une faible température, haute pression, et salinité élevée contribuent également à la préservation de l'ADN dans les sédiments (Boere *et al.*, 2010). Dans les conditions idéales de préservation (permafrost et glace), la limite théorique de conservation de l'ADN ancien est estimée à environ un million d'années (Allentoft *et al.*, 2012). Cependant, la plus longue durée observée dans ces mêmes environnements a été de 400 000 à 800 000 ans (Willerslev *et al*., 2007). Récemment, des études ont montré que l'ADN ancien peut très bien se conserver dans des conditions oxiques (Lejzerowicz *et al.*, 2013; Coolen *et al.*, 2013). Cela démontre le potentiel de l'ADN comme outil paléoécologique.

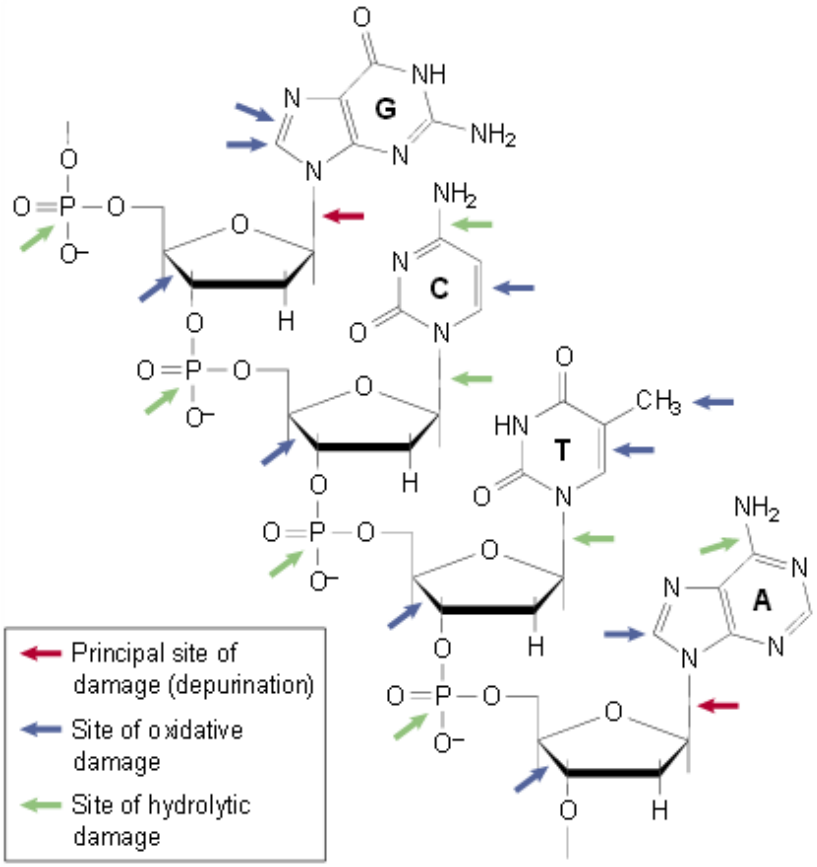


Figure 12. Les principales voies de dégradations affectant l'ADN ancien (extrait de Hofreiter *et al*., 2001).

5.2.2. Application de la paléogénétique pour l'étude du plancton

Plusieurs études montrent que l'ADN ancien peut être conservé jusqu'à l'holocène et le pléistocène et être amplifié à partir de divers environnements. En effet, l'analyse de carottes de sédiments prélevées à 5000 m de profondeur dans l'océan Atlantique au large du Brésil (Lejzerowicz *et al*., 2013) et à 900 m en Mer noire (Coolen *et al*., 2013) a permis de récupérer des séquences d'ADN ancien de dinoflagellés, coccolithes, foraminifères et copépodes allant jusqu'à 32 500 ans. L'ADN ancien a été utilisé pour étudier les successions de dinoflagellés et d'haptophytes dans l'Arctique, l'Antarctique, les sédiments de la mer Noire et de la Méditerranée (Boere *et al*., 2011; Coolen *et al*., 2006; D'Andrea *et al*., 2006). De plus, il a permis de reconstruire la dynamique des communautés planctoniques anciennes et leurs réponses aux changements environnementaux (*e.g.* Boere *et al.*, 2011; Lejzerowicz *et al.*, 2013; Coolen *et al*., 2013). Par exemple, les signatures génétiques de l'espèce d'Haptophyte, *Emiliania huxleyi*, et des virus de coccolithes associés retrouvées à partir des carottes de sédiments de la mer Noire ont révélé des changements dans la structure des populations d'hôte et de virus durant 7000 ans de l'histoire de l'Holocène, coïncidant avec des shifts dans la salinité et la disponibilité des nutriments (Coolen *et al*., 2011). Egalement, à partir de sédiments

lacustres au niveau d'un lac kenyan, un changement important des communautés de diatomées au milieu du 19ème siècle a pu être à posteriori décrit (Stoof-Leichsenring *et al.*, 2012) (Figure 13). Similairement, Domaizon *et al.*, (2011) ont pu reconstruire la diversité et la dynamique de la cyanobactérie *Synechococcus* au niveau d'un lac subalpin. De plus, l'ADN ancien de sédiments lacustres a également permis le suivi des activités humaines et les changements de paysage à proximité d'un lac alpin durant l'Anthropocène (Giguet-Covex *et al.*, 2014), et l'identification des changements dans les communautés phytoplanctoniques dans un lac tibétain au cours des dernières 3000 années (Hou *et al.*, 2014).

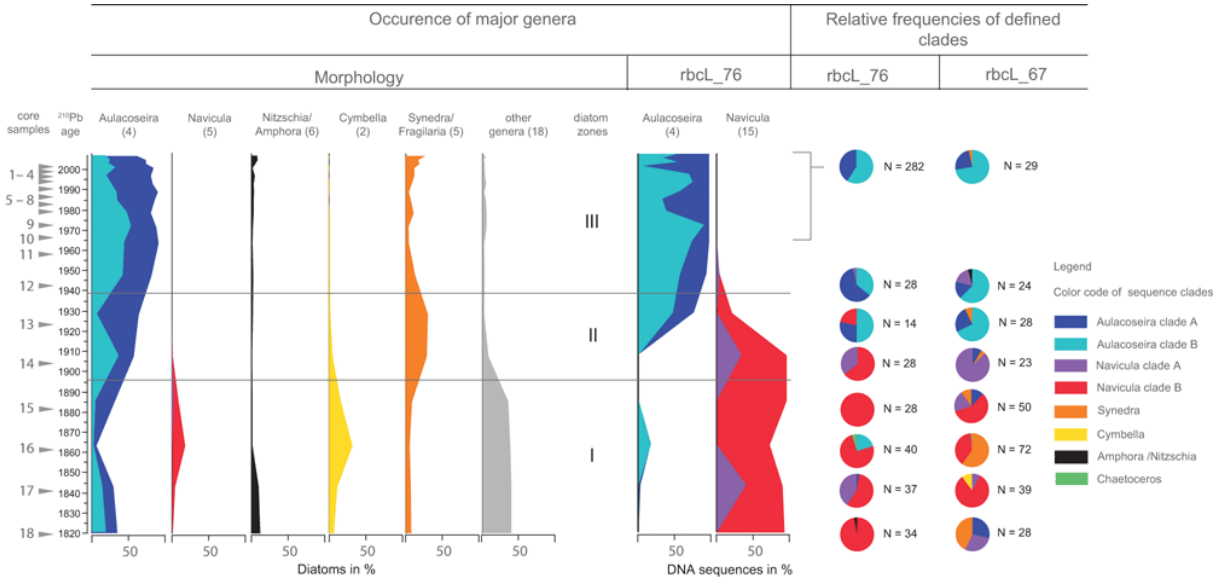


Figure 13. Abondances relatives de genres et clades de diatomées à partir de données morphologiques et de données génétiques dans le lac Naivasha (Kenya). La figure montre un shift dans la composition des diatomées au cours des deux derniers siècles se traduisant principalement par le remplacement du genre *Navicula* par le genre *Aulacoseira* (extrait de Stoof-Leichsenring *et al.*, 2012).

6. Les écosystèmes estuariens

Selon Perillo (1995), il existe près de 40 définitions différentes des estuaires. La plus communément utilisée est celle de Cameron and Pritchard (1963) puis Pritchard (1967) définissant un estuaire comme «un plan d'eau semi-fermé ayant une connexion libre avec la mer ouverte et à l'intérieur duquel l'eau de mer est diluée d'une façon mesurable avec de l'eau douce issue du drainage du bassin versant». Cependant, cette définition ne comprend pas l'effet des marées qui peut être significatif dans de nombreux estuaires. Dyer (1997), en modifiant la définition de Pritchard (1967) donne une définition, plus satisfaisante d'un point de vue hydrodynamique : «un plan d'eau semi-fermé ayant une connexion libre avec la mer ouverte, se prolongeant dans la rivière jusqu'à la limite de l'influence tidale, à l'intérieur duquel l'eau de mer est diluée d'une façon mesurable avec de l'eau

douce issue du drainage du bassin versant ». En d'autres termes, les estuaires sont constitués d'une masse d'eau confinée où se rencontrent des eaux douces fluviales et des eaux marines salées.

6.1. Classification des estuaires

Il existe différents classifications pour les estuaires, selon les critères qui sont considérés, (géomorphologie, hydrodynamisme, l'amplitude des marées, structure de salinité) (Valle-Levinson 2010). Deux types de classification sont présentés ci-dessous ; selon la salinité et selon l'amplitude des marées, c'est cette dernière que l'on utilisera pour la suite de ce travail.

6.1.1. Classification selon la structure de salinité

Pritchard (1955) propose une classification des estuaires selon la structure verticale de salinité et du mode de mélange des masses d'eau douce et d'eau salée (Figure 14) :

- **Estuaire stratifié à coin salé** : reçoit une influence relativement faible de la marée avec des apports fluviaux importants. Le volume d'eau salée apporté par la marée est faible comparé au volume d'eau douce. Dans ces conditions, l'eau douce moins dense s'écoule à la surface de l'eau salée avec pratiquement pas de mélange entre les couches, cela crée une stratification verticale importante (Dyer 1986) (Figure 14a).
- **Estuaire stratifié à marée :** caractérisé par un apport fluvial très important et une influence des marées faible à modérée. Ce cas est similaire au précédent mais se différencie par le mélange des masses d'eau qui est plus important (Figure 14b).
- **Estuaire partiellement mélangé** : dans ce cas, le volume d'eau apporté par la marée est plus important, induisant des courants plus forts et un mélange plus important des masses d'eau (Figure 14c).
- **Estuaire homogène** : estuaire peu profond avec des marées importantes et un faible apport fluvial. Cette combinaison permet de créer un mélange de l'eau de mer sur toute la colonne d'eau (Figure 14d).

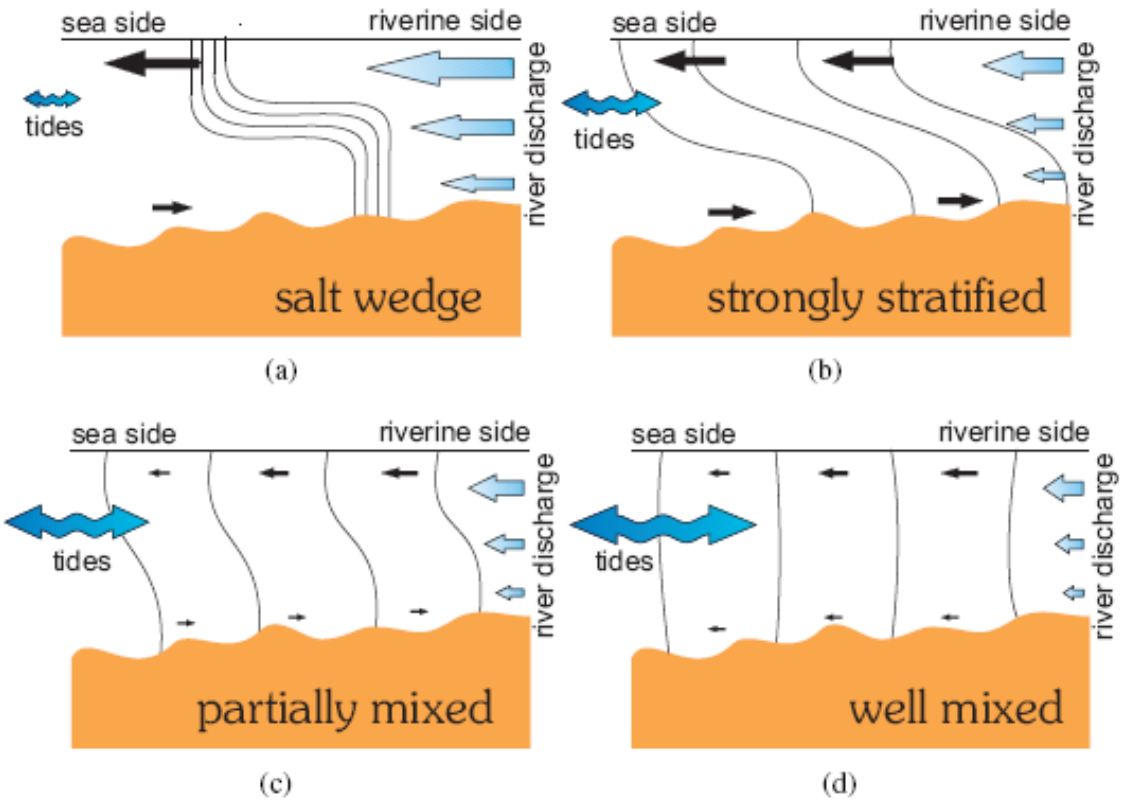


Figure 14. Représentation schématique des types d'estuaires selon les profils de salinité longitudinaux et les régimes de marées. A) Estuaire stratifié à coin salé. B) Estuaire stratifié à marée. C) Estuaire partiellement mélangé. D) Estuaire homogène (extrait de Chernetsky 2012).

6.1.2. Classification selon l'amplitude des marées

La classification quantitative de Davies (1964) puis Hayes (1975) permet de distinguer les estuaires en fonction du marnage :

- **Estuaires microtidaux**

Lorsque l'amplitude des marées est inférieure à 2 m, l'estuaire est décrit comme microtidal. Il est dominé par l'action des vents et des vagues. Généralement ces estuaires sont larges (10 Km) et peu profonds (profondeur de 1 m).

- **Estuaires mésotidaux**

Lorsque l'amplitude de la marée se situe entre 2 et 4 m, l'estuaire est décrit comme mésotidal. Ce genre d'estuaires est le plus commun et le plus étudié dans le monde.

- **Estuaires macrotidaux**

Lorsque l'amplitude de la marée est supérieure à 4 m, l'estuaire est décrit comme macrotidal. Il est dominé par des courants forts dont l'effet peut être ressenti sur des centaines de kilomètres.

6.2. Forte productivité des écosystèmes estuariens

Les estuaires sont parmi les zones les plus productives au monde, grâce aux apports constants provenant des rivières, en carbone organique, nutriments et minéraux qui favorisent des taux élevés de production primaire (Hopkinson *et al*., 2005). De ce fait, ils constituent un habitat idéal pour diverses espèces aquatiques. La respiration totale annuelle d'un écosystème estuarien est généralement supérieure à la production primaire brute (Caffrey 2004; Gattuso *et al*., 1998), ainsi, les estuaires sont des écosystèmes hétérotrophes qui transforment la matière organique en éléments nutritifs et CO_2 inorganiques, et sont à la différence des environnements océaniques, sources de CO_2 dans l'atmosphère. Une proportion variable des éléments nutritifs est consommée par la production primaire alimentant l'activité microbienne hétérotrophe (Cloern 1996), tandis que le carbone dissous et particulaire sont recyclés dans les estuaires (Raymond and Bauer 2001). En conséquence, la matière organique déposée dans les sédiments estuariens provient de sources autochtones ainsi que allochtones (terrestre, marine) (Cifuentes *et al*., 1988).

Les sédiments estuariens sont des environnements importants de régulation des flux de carbone, d'éléments nutritifs, de métaux et de soufre. Les couches supérieures de ces sédiments sont connues pour leur activité biogéochimique élevée en raison de la minéralisation de la matière organique par un consortium de bactéries utilisant différents accepteurs d'électrons (oxygène, nitrates, oxydes métalliques, et sulfate). L'oxygène joue un rôle important dans renouvellement de la matière organique dans les sédiments (Glud 2008), et la ré-oxydation de composés réduits provenant de la minéralisation anoxique, se diffusant à partir des couches profondes jusqu'à la zone oxique (Canfield *et al*., 1993; Deflandre *et al*., 2002; Pastor *et al*., 2011). La dénitrification et l'élimination du nitrate (Seitzinger 1988; Burgin and Hamilton 2007) qui se produisent dans les sédiments marins et estuariens sont également très importants dans la régulation des flux d'azote fixé en zone côtière. Enfin, la réduction des sulfates et la méthanogénèse sont les principaux processus de dégradation de la matière organique dans les sédiments des eaux peu profondes, qui contribuent à la minéralisation globale du carbone organique et peuvent contribuer à la réduction des apports de carbone vers les zones côtières (Middelburg *et al*., 1995; Meiggs and Taillefert 2011).

6.3. Enjeux socio-écologiques

Les estuaires écosystèmes sont souvent associés à de forts enjeux économiques et sociétaux, car ils représentent des zones de navigation, d'activités industrielles et de concentration de population humaine. On estime que près d'un tiers de la population mondiale vit près d'estuaires. Par ailleurs, les estuaires contribuent à 33% de la valeur économique des écosystèmes marins (Costanza *et al*.,

1997), représentant des zones d'interface entre domaines continental et marin. D'un point de vue écologique, ces écosystèmes très productifs constituent des lieux de nourriceries et de routes de migration pour de nombreux organismes, mais également des zones de croissance ou de refuge (Rochard *et al*., 1990; McLusky and Eliott 2004; Pasquaud *et al*., 2012). Les activités humaines affectent de manière assez intense l'état écologique des estuaires à travers le monde (Rabalais *et al.*, 2009) induisant des changements considérables de l'environnement physique et modifiant la composition chimique de la colonne d'eau et des sédiments, et par effet cascade, la composition des communautés associées (Davis and Koop 2006).

6.4. Variabilité des estuaires et des communautés associées

Grace à leurs configuration et aux différents gradients physiques et chimiques qui les caractérisent, les estuaires offrent aux organismes qui y vivent une succession d'habitats depuis l'aval jusqu'à l'amont. En effet, ces organismes sont naturellement soumis à une forte variabilité des conditions environnementales de leur écosystème (variabilité tidale, saisonnière et à long terme) (Castel 1993). Par conséquent, les estuaires sont généralement caractérisés par un nombre d'espèces autochtones faible associé à une forte productivité. Ceci peut s'expliquer par l'adaptation des espèces grâce à leur caractère euryhalin et/ou eurytherme (McLusky and Eliott 2004).

Il est souvent difficile de distinguer la variabilité naturelle du milieu de celle liée aux activités humaines, c'est ce qu'on appelle le paradoxe estuarien (Eliott and Quintino 2007). Dans ce contexte de changement, il semble intéressant de s'interroger sur le devenir, l'évolution, voir l'adaptabilité de ces espèces à un tel environnement changeant. En particulier de savoir si dans un écosystème naturellement variable et fortement anthropisé, il serait possible de détecter des changements des communautés anciennes en réponse aux modifications de l'écosystème estuarien. L'utilisation d'outils paléoécologiques semble être le seul moyen pour aborder ces questions. Cependant, la plupart des études paléoécologiques ont été effectuées dans des environnements froids et secs (permafrost, glaciers, fjords,…), présentant des archives sédimentaires « idéales » et permettant une stratification satisfaisante des sédiments pour la datation et une résolution temporelle élevée. Parmi les rares études paléoécologiques réalisées en milieu estuarien, certaines se sont intéressées à établir une ligne de base (ou état de référence) des conditions hydrologiques, afin de mieux prédire et gérer les changements écologiques dans ce type d'écosystèmes (e.g. Watson *et al*., 2011; Serrano *et al*., 2016). Par exemple, récemment dans un estuaire Australien (Baie d'Oyster, Albany), Serrano *et al*., (2016) ont mis en évidence des shifts au niveau de la composition chimique des sédiments et des concentrations en nutriments au niveau de sédiments de l'herbier *Posidonia autralis* durant les deux derniers siècles (Figure 15). Ellegaard *et al*., (2014) ont étudié l'impact de la déforestation d'une

mangrove sur un estuaire Vietnamien, et ont mis en évidence l'augmentation du taux de sédimentation dans l'estuaire et un changement dans les assemblages des diatomées au moment de la déforestation.

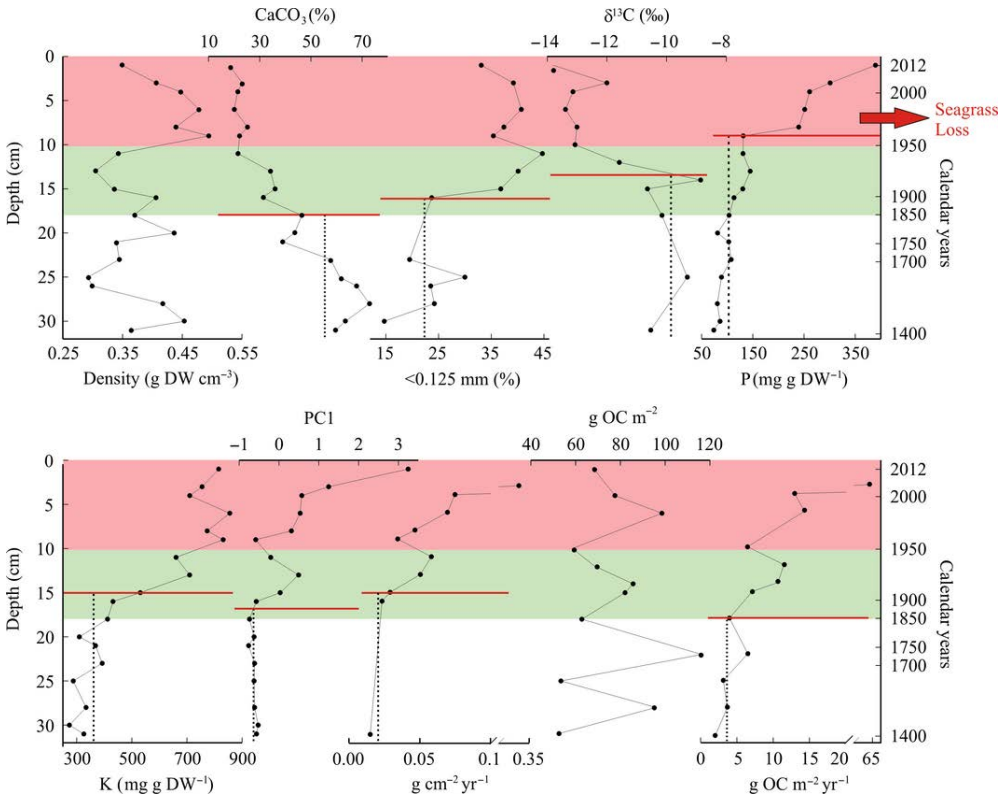


Figure 15. Dynamique temporelles des variables biogéochimiques dans des sédiments à *Posidonia autralis* dans la Baie d'Oyster (Ouest d'Australie). Les barres rouges représentent les régimes shift observées pour les différentes variables (extrait de Serrano *et al.*, 2016).

7. La rade de Brest

La rade de Brest est un écosystème marin semi-fermé, situé à la pointe de la Bretagne (France) (Figure 16). Elle est d'une superficie de 180 km^2 et est connectée à la mer d'Iroise et à l'océan Atlantique par un goulet de 6 km de long et d'environ 2 km de large. La rade de Brest est un environnement peu profond avec près de 60% de ses fonds inférieurs à 10 m et seulement 3% au-dessus de 30 m. La majeure partie (80%) des eaux continentales provient de deux principales rivières, l'Aulne (1842 km^2) et l'Elorn (402 km^2). Les 20% restants sont apportés par la Douffine (8%), la Mignonne (5%), le Camfrout (3%), le Garvan (2%), le Faou (2%) et la Penfeld (2%). L'amplitude des marées varie de 1.22 m en situation de morte-eau exceptionnelle (coef.20) et 7.32 en situation de vive-eau exceptionnelle (coef.120) (Auffret 1981), De ce fait, la rade est considérée comme un environnement macrotidal selon la classification de Davies (1964) et de Hayes (1975). La marée

génère un fort brassage interne en rade et des échanges modérés entre celle-ci et la mer d'Iroise. La faible profondeur de la rade ainsi que le mélange d'eau induit par les courants de marées empêchent la stratification verticale, l'accumulation des nutriments et par conséquence, les problèmes d'eutrophisation. Le nord de la rade abrite près de 400 000 habitants, tandis que le sud est plus influencé par les activités agricoles intensives.

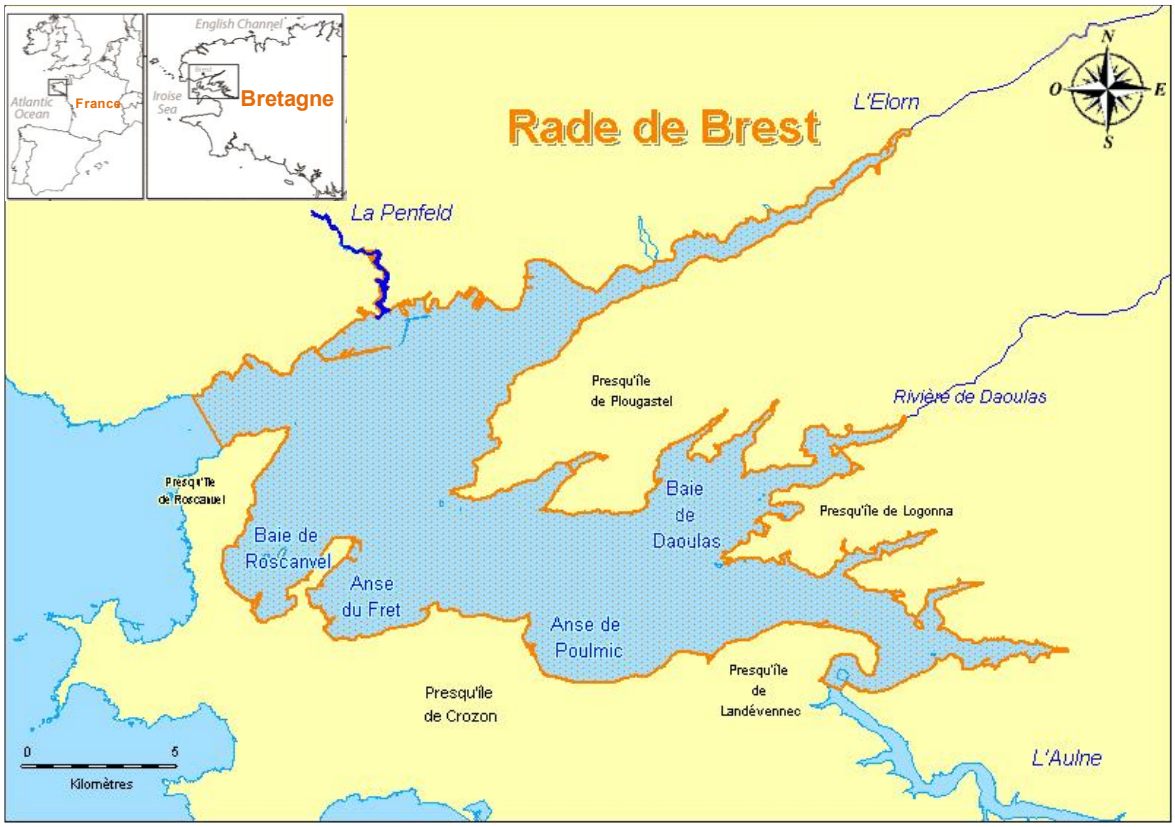


Figure 16. Carte de la rade de Brest (extrait et modifié à partir de : http://www.liteau.net).

7.1. Les communautés phytoplanctoniques en rade de Brest

A cause du développement des activités urbaines et des activités agricoles intenses, la rade de Brest subit depuis 1950, des changements hydrologiques importants, notamment en termes d'apports de nutriments par les rivières. Les fluctuations importantes des teneurs en nutriments ont conduit à un déséquilibre du rapport d'azote/phosphore (N/P), induisant des changements au niveau des communautés biologiques. Par exemple, il a été observé une modification de la dynamique des efflorescences se traduisant par une diminution de l'intensité des efflorescences printanières au profit de la période estivale (Chauvaud *et al*., 2000). Des modifications de la composition spécifique ont également été reportées ; en 1981, les efflorescences étaient dominées essentiellement par les diatomées (Quéguiner and Tréguer 1984), basculant vers des efflorescences dominées à la fois par

les diatomées et les dinoflagellés une dizaine d'années plus tard (Del Amo *et al*., (1997). Beucher *et al*., (2004) observent la même tendance et attribue la favorisation des dinoflagellés aux faibles teneurs de phosphate. Des expériences de laboratoire ont également montré une croissance importante des dinoflagellés en faible teneur en phosphate par rapport à d'autres espèces comme les diatomées (Chapelle *et al*., 2010; Lin *et al*., 2016).

7.2. Efflorescences d'*Alexandrium minutum* en rade de Brest

Alexandrium minutum est un dinoflagellé (Alveolata) unicellulaire autotrophe, bien que des cas d'hétérotrophie/mixotrophie aient déjà été mis en évidence (Jacobson and Anderson 1996). Les cellules d'*A. minutum* sont de forme sphérique à ellipsoidale et leur taille est variable entre 15-30 µm de long et 13-24 µm de large (Hwang and Lu 2000). La cellule est entourée d'une thèque constituée de plaques rigides composées de cellulose. Ces plaques sont contenues dans les alvéoles sous-corticaux propres aux alvéolés. Le sillon du flagelle équatorial, appelé cingulum, permet de séparer la cellule en une part e) (Figure 17).

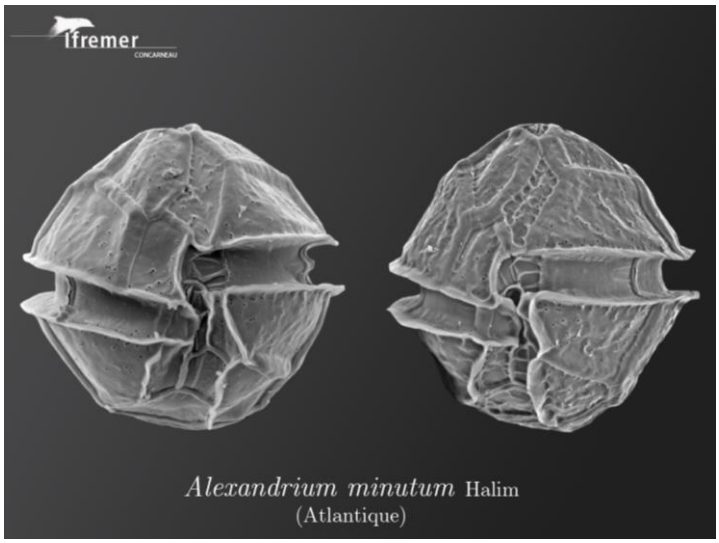


Figure 17. Image d'*Alexandrium minutum* obtenue en micorscopie électronique par N. Chomérat (Ifremer).

Alexandrium minutum est capable de produire des kystes de résistance (pelliculés et de dormance). Depuis sa détection en 1960 en Egypte (Halim 1960), *Alexandrium minutum* a été rapidement retrouvée dans différentes régions à travers le monde, provoquant de nombreux épisodes d'efflorescences avec production de toxines à PSP (Paralytical Shellfish Poisoning). Par ailleurs, elle a été listée officiellement comme espèce invasive en Europe (http://www.europe-aliens.org), c'est-à-dire, une espèce qui a été introduite du fait de l'action de l'homme dans une région où elle n'existait pas auparavant et qui représente un impact écologique et/ou économique négatif sérieux (Boudouresque 2012). Dans les eaux côtières françaises, elle a été observée dans l'Aber-Wrac'h et

l'Aber-Benoît (Bretagne nord) en août 1988 suite à une efflorescence. Depuis, elle a produit des efflorescences récurrentes (marées rouges) le long de la côte Nord-Ouest de la Bretagne et particulièrement dans les petites baies et estuaires.

En rade de Brest, *A. minutum*, a été détectée depuis 1990 par le réseau de surveillance (REPHY). Les abondances de l'espèce sont restées relativement faibles jusqu'à 2012, où il y a eu une efflorescence record à Daoulas, atteignant jusqu'à 42 000 000 cells L^{-1}. Cette efflorescence s'est accompagnée de production de toxines ayant eu un effet cascade sur l'accumulation des toxines dans les organismes filtreurs, la fermeture de la pêche et l'aquaculture engendrant des pertes économiques considérables. Depuis cet évènement, des efflorescences sont signalées chaque année au cours de la période estivale, ainsi la rade de Brest est devenue une zone à risque pour les efflorescences toxiques d'*A. minutum* (Chapelle *et al*., 2015).

OBJECTIFS DE LA THÈSE

L'objectif principal de mon projet de thèse était d'évaluer l'intérêt des approches paléoécologiques utilisant les traces biologiques (ADN, kystes) pour retracer l'évolution des communautés phytoplanctoniques et de mieux comprendre l'impact récent (sur une centaine d'années) de l'activité humaine sur des écosystèmes marins côtiers. Mon travail s'est naturellement focalisé sur des écosystèmes très productifs et fragiles à la fois (des estuaires), dans lesquels des crises majeures avaient été recensées ces 40 dernières années. C'est dans ce contexte que l'étude de la rade de Brest (Bretagne), outre sa proximité géographique, est apparue comme idéale. Ce travail fut également très prospectif, étant parmi les premiers à appliquer ce type d'approche sur des écosystèmes estuariens.

Dans ce contexte, je me suis également placée à différentes échelles de temps et d'espace, depuis l'étude des communautés à celle d'une espèce toxique, sur un site donné ou sur une zone géographique plus étendue, et enfin sur des sédiments plus ou moins récents, depuis les premières couches superficielles jusqu'à des couches datées d'une centaine d'années. J'ai aussi utilisé des approches moléculaires différentes (PCR en temps réel, metabarcoding), de culture (expériences de germination de kystes, maintien en culture), de datation, de statistiques (analyses multivariées), de modélisation (statistique et lagrangienne), ainsi que des approches d'écophysiologie.

Il a fallu également identifier des questions écologiques plus précises, liées à la configuration et à l'histoire récente de la rade de Brest, et adaptées à différentes échelles de temps et d'espace :

Sur le siècle dernier/communautés complexes : Comment évolue la diversité des protistes le long des couches sédimentaires ? Peut-on retracer certains évènements récents connus des communautés de protistes dans cette « lecture » de l'histoire basée sur l'ADN ?

Sur le siècle dernier/espèce toxique cible (*A. minutum*) : Cette espèce toxique nouvellement identifiées en rade de Brest, produisant des efflorescences toxiques est-elle invasive (introduite) ou envahissante (présente depuis longtemps, mais soudainement capable de proliférer) ?

Sur ces dernières années/ espèce toxique cible (*A. minutum*) : Est-il possible d'identifier des zones à risque pour les efflorescences toxiques actuelles en analysant des traces biologiques des sédiments de surface ?

Sur le siècle dernier/Adaptation phénotypique des microalgues : Peut-on mettre en évidence des variations phénotypiques entre des individus modernes et anciens en lien avec nos connaissances des changements des conditions hydrologiques de ces écosystèmes côtiers?

Les travaux effectués dans le cadre de ma thèse s'organisent en 4 chapitres, chacun ciblant une des questions posées.

La première partie de ce travail, présentée dans le **chapitre 1** concerne l'étude de la diversité des communautés de protistes à partir des sédiments estuariens de la rade de Brest de différentes

époques, sur une échelle de temps couvrant environ 150 ans. Il s'agissait par ailleurs d'évaluer le potentiel de préservation des différents groupes identifiés dans les sédiments marins de plus d'un siècle. Pour y parvenir, j'ai appliqué une approche de metabarcoding sur des couches sédimentaires de différents âges. J'ai analysé des données de séquençage à haut débit (de la région V4 du 18S ADNr) obtenues à partir des couches datées de deux carottes de sédiments prélevées provenant de différents estuaires de la rade de Brest. Ces travaux font l'objet d'un article en préparation pour la revue *ISME Journal.*

Dans le **chapitre 2**, compte tenu de l'importance des dinoflagellés dans les communautés de sédiments anciens et des apparitions récentes d'efflorescences en rade de Brest de l'espèce toxique et potentiellement invasive *Alexandrium minutum*, je me suis intéressée à reconstruire sa dynamique temporelle à une échelle multidécennale en rade de Brest. J'ai essayé de définir le caractère ou non invasif de cette espèce dans notre écosystème modèle, en retraçant les premières traces de cette espèce dans nos carottes sédimentaires. J'ai développé un nouveau test de PCR en temps réel (PCR quantitative) adaptée aux études de l'ADN ancien, permettant de quantifier de façon spécifique le gène de l'ITS1 de l'ADNr. Les données paléogénétiques ont été confrontées aux données d'abondance de l'espèce dans la colonne d'eau acquises par le réseau de surveillance REPHY pour la validation de notre approche de paléogénétique. Ces travaux font l'objet d'un article scientifique publié dans le journal *FEMS Microbiology Ecology.*

Dans le **chapitre 3**, dans le contexte des efflorescences toxiques récentes en rade de Brest, je me suis intéressée à caractériser la distribution spatiale actuelle d'*A. minutum* en utilisant les données quantitatives de génétique pour mapper de potentielles zones d'accumulation des kystes qui pourraient constituer des zones à risque d'efflorescences. Pour ce faire, à partir d'échantillons de sédiments superficiels, j'ai quantifié les abondances d'*A. minutum* (copies d'ITS1 rDNA) par PCR en temps réel. J'ai vérifié l'existence de kystes viables d'*A. minutum* dans les sédiments en réalisant des expériences de germination. La distribution de l'espèce en rade de Brest issue de l'analyse des sédiments a été confrontée à des données issues de la modélisation afin d'identifier d'éventuelle barrière de dispersion de l'espèce dans la colonne d'eau. Pour ce faire, le potentiel de dispersion des particules physiques en rade de Brest a été étudié à l'aide de simulations lagrangiennes mimant les trajectoires potentielles de cellules à partir des différents estuaires de la rade. Ces travaux font l'objet d'un article scientifique, soumis à la revue *Harmful Algae.*

Dans le **chapitre 4**, j'ai comparé les performances physiologiques (taux de croissance et biomasse maximale atteinte) de deux espèces différentes (*Scrippsiella donghaienis* et *Alexandrium minutum*) à partir de souches de différentes époques obtenues après germination. Pour cela, j'ai testé différentes conditions de culture (rapport N/P), en essayant de mimer des changements de la

concentration de nutriment relevés en rade de Brest pendant le siècle dernier. En particulier, les réponses phénotypiques des différentes populations ont été étudiées en conditions de limitation de P inorganique, ce dernier étant l'élément limitant principal au développement des populations phytoplanctoniques en rade de Brest. Ces travaux ont été effectués dans le cadre d'un stage de Master 2 que j'ai co-encadré.

Enfin le dernier chapitre est composé d'une discussion générale des principaux résultats de ce travail ainsi que d'une conclusion et des perspectives.

CHAPTER 1

Long-term ecological trends inferred by DNA metabarcoding of protist paleocommunities

Context of the work

Protists are important contributors to biogeochemical cycles in the marine environment. Their diversity is remarkable compared to that of Metazoa or land plants. In the past, the diversity of protist was studied almost entirely using morphological surveys, but in the recent years, the use of molecular-based has shown promising results. Indeed, studies have shown that the protist diversity is still underestimated and that only 10% of the sequences recovered from molecular studies were previously known (Slapeta *et al*., 2005; Boenigk *et al*., 2006). The metabarcoding approach, which is the combination of DNA taxonomy and high-throughput sequencing, is a promising tool for the rapid assessment and monitoring of biodiversity. Its application for the study of modern protist diversity has increased significantly in the last decade, allowing not only reveal the hidden diversity within these taxa, but also a more complete discovery of taxonomic diversity compared to traditional methods. However, this insight of diversity does not allow understanding the succession of protist communities over time in relation with the environment evolution. Using a paleoecological approach based on DNA analyses from sedimentary archives could help to achieve this goal. Indeed, few studies have used paleoecology and metbarcoding to study protist diversity in shallow/deep sea sediment or freshwater sediments (e.g. Coolen *et al.,* 2013; Lejzerowicz *et al.,* 2013; Pawlowski *et al*., 2011; Capo *et al*., 2015). However, to our knowledge, no study has been conducted to charcterize the past protist communities in an estuarine environment.

In this work, I try to investigate the modern and past protist community from dated estuarine sediment of the Bay of Brest (Brittany, France), by analyzing the V4 barcode region of the 18S rRNA gene obtained from Illumina Mi-seq sequencing. Furthermore, I try to depict trends in the protist communities over time and evaluate their preservation potential in estuarine sediments.

Author contributions

For this first part of the PhD thesis, I participated in the sampling and field analyses of the cores. I performed DNA extractions and genetic analyses of the Elorn core and I trained L. Madec (technician) to these analyses for the Daoulas core. R. Christen performed the bioinformatic analyses (on both V4 and V9 (see annex) rDNA 18S metabarcodes) and he provided me with the OTU tables. I performed all the analyses (statistical and ecological) of these data sets. D. Soudant performed statistical modeling analyses on the REPHY dinoflagellate time series and provided with the corresponding figure. R. Siano and L. Guillou contributed to the data analyses and interpretations. I have integrally written this chapter which corresponds to the first draft of a potential manuscript. R. Siano designed the research and supervised this work.

Long-term ecological trends inferred by DNA metabarcoding of protist paleocommunities

Résumé (en français)

Dans cette étude, nous avons utilisé une approche paléogénétique pour étudier la diversité des protistes dans des carottes de sédiments de deux estuaires (Estuaire de l'Elorn (EE), Estuaire de Daoulas estuaire (DE)) de la rade de Brest, sur une échelle de temps de 150 ans. Plus précisément, nous avons isolé l'ADN à partir des carottes de sédiments et analysé les séquences du gène du 18S ARNr (région V4), obtenues à partir d'un séquençage Illumina Mi-Seq. Dans les deux carottes, seule une faible fraction (16-18%) de la richesse moderne (OTUs) (Operational Taxonomic Unit) a été préservée dans les sédiments anciens. La plupart des groupes de protistes conservés dans les sédiments anciens correspondaient à des taxons connus pour produire des formes de résistance. La composition de protistes était similaire pour les échantillons provenant de la même profondeur et du même l'âge. De plus, les protistes étaient séparés en deux paléocommunautés clairement distinctes (antérieures/postérieures à 1950). Dans les deux carottes, le groupe de Dinophyceae était le plus important en termes d'abondances relatives et il présentait une tendance à la diminution au cours des dernières années. En particulier, une dynamique d'abondances inverse (des sédiments anciens vers les sédiments récents) a été identifié après 1987, pour les deux genres *Alexandrium* (augmentation de l'abondance relative) et *Gonyaulax* (diminution de l'abondance relative). Ces tendances étaient corroborées avec les données de plancton locales dans la colonne d'eau et sont probablement liés aux changements hydrologiques dans la concentration des nutriments qui se sont produit dans la baie de Brest au cours des dernières décennies. Ce travail contribue à la recherche sur la diversité des protistes dans les écosystèmes estuariens, montrant que la composition et les variations des communautés anciennes peuvent être identifiées par une approche paléogénétique dans de tels écosystèmes.

Long-term ecological trends inferred by DNA metabarcoding of protist paleocommunities

Khadidja Z Klouch[1,2], Richard Christen[3], Dominique Soudant[4], Lauriane Madec[5], Laure Guillou[2], Raffaele Siano[1]

In preparation for the ISME journal

[1]Ifremer - Centre de Brest, DYNECO PELAGOS, F-29280 Plouzané, France
[2]Sorbonne Universités, UPMC Univ. Paris 6, CNRS, Adaptation et Diversité en Milieu Marin (UMR 7144), équipe DIPO, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff cedex, France
[3]Université de Nice-Sophia Antipolis, CNRS, UMR 7138, Systematique Adaptation Evolution, Parc Valrose, BP71, Nice F06108, France
[4] Ifremer, DYNECO/VIGIES, Rue de l'Ile d'Yeu, BP 21105, 44311 Nantes Cedex 03, France
[5] Ifremer - Centre de Brest, Unité de Physiologie Fonctionnelle des Organismes Marins, LEMAR, UMR 6539, F-29280 Plouzané, France

Abstract

In this study, we used a paleogenetic approach to investigate the protist diversity in sediment cores of two estuarine ecosystems (Elorn Estuary (EE), Daoulas Estuary (DE)) of the Bay of Brest, over a time scale of 150 years. Specifically, we isolated DNA from the sediment cores and analyzed 18S rRNA gene (V4 region) sequences obtained from an Illumina Mi-Seq sequencing. In both cores, only a small fraction (16-18%) of modern richness (OTUs) (Operational Taxonomic Units) was preserved in ancient sediments. Most of the protists groups preserved in ancient sediments corresponded to protist taxa known for resting stages production. The protist composition was similar for samples retrieved from the same depth and age. In addition, the protist separated into two clearly distinct paleocommunities (prior/posterior to 1950). In both cores, the Dinophyceae was the most important group regarding to its relative abundances and it displayed a decrease trend in over the recent years. In particular, an inverse dynamic (from ancient to recent sediment) in abundances was identified after 1987, for the two genus *Alexandrium* (increasing relative abundance) and *Gonyaulax* (decreasing relative abundance). These trends corroborated the local plankton data in the water column and are likely related to the hydrological changes in nutrient concentration occurring in the Bay of Brest over the last decades. This work contributes to the research on protist diversity in estuarine ecosystems, showing that past community composition and variations could be inferred by a paleogenetic approach in such ecosystems.

1. Introduction

Protists correspond to all unicellular eukaryotic organisms, excluding animals, fungi, land plants and macroalgae (Guillou *et al.,* 2013). They have representatives in all eight eukaryotes lineages and span a wide cell-size range, forms and functions (playing significant ecological roles as primary producers, consumers, decomposers and trophic links in aquatic food webs (Caron *et al.,* 2012). Yet, their diversity in the global ocean is still under-estimated (de Vargas *et al*., 2015). The characterization and assessment of protist diversity has been extensively performed using traditional morphological-based techniques. Although highly informative, these methods have the disadvantages of being costly, time-consuming, and strongly depending on highly skilled persons in taxonomy. Additionally, small-sized and highly cryptic organisms could not be correctly identified.

In recent years, the use of DNA-based analyses have significantly improved our knowledge of the microbial diversity, unveiling novel taxa such as the marine stramenopiles (MAST) clades (Massana *et al.,* 2004), the marine alveolates (MALV) clades (Guillou *et al.,* 2008) and cryptic taxa (*e.g.* within the genus *Skeletonema* (Stramenopiles) (Kooistra *et al.,* 2008), and the genus *Spumella* (Stramenopiles) (Pfandl *et al.,* 2009)). In particular, DNA metabarcoding, which refers to the combination of High-Throughput Sequencing and DNA taxonomy, has revolutionized our understanding of diversity by detecting even a hidden diversity in a rapid and a cost-effective process. Furthermore, this approach allows the identification of taxa from environmental samples without first isolating any target organism (Tableret *et al.,* 2012). Metabarcoding analyses require the use of a good genetic marker to allow the detection of all the lineages in their entirety. It is acknowledged that the 18S RNA gene is the best universal marker to get a general picture of the eukaryotic biodiversity. It has many advantages: it is present in all eukaryotes; it occurs in many copies per genome; includes highly conserved and variable nucleotide sequences and; is extensively represented in public reference databases (Guillou *et al.,* 2013; Pawlowski *et al.,* 2014). Within the 18S RNA gene, two main hypervariable regions have been commonly used as a barcode for general protist biodiversity studies: the short (130 b) V9 region, characterized by low length heterogeneity and the long (380 bp) V4 region, characterized by particularly rapid rates of evolution and extreme variation in length (Pawlowski *et al.,* 2014). These regions have been successfully used in many pioneering metabarcoding protist studies (Logares *et al.,* 2014; Guardiola *et al.,* 2015; de Vargas *et al.,* 2015; Massana *et al.,* 2015; Yu *et al.,* 2015) from a wide type of substrates in different environments: sea-water (de Varagas *et al.,* 2015; Amaral-Zettler *et al.,* 2009; Hu *et al.,* 2016; Le Bescot *et al.,* 2015; Nanjappa *et al.,* 2014), freshwater (Capo *et al.,* 2015; Simon *et al.,* 2016), soil (Arjen de Groot *et al.,* 2016; Mahé *et al.,* 2016), and marine sediments (Chariton *et al.,* 2015; Lejzerowicz *et al.,* 2015; Forster *et al.,* 2016). Beyond the diversity assessment of marine communities, it has also been

applied in studies on: freshwater quality evaluation (*e.g.* Zimmermann *et al.,* 2015; Kermarrec *et al.,* 2014) and ecological impacts on communities (*e.g.* oil spill event (Bik *et al.,* 2012), fish farms (Pawlowski *et al.,* 2014)).

All these studies have been conducted on modern samples, therefore the past protist diversity remains relatively unknown. In fact, collecting long-term data at a multidecadal time scale could help to reconstruct the ancient protist community and to understand community changes in response to local changes in environmental conditions. The use of marine sediments biodiversity archives (ancient DNA, fossils, shells, resting stages, biomolecules) can fill the gap in short time series of plankton and allow the reconstruction of multidecadal variations of communities. As for ancient DNA, studies have shown that it can be preserved from degradation by adsorbing to mineral and organic matrices (Pietramellara *et al.,* 2009), for substantial periods of time up to the Miocene (Panieri *et al.,* 2010), providing valuable information about past biological communities (Lejzerowicz *et al.,* 2013). Yet, the use of sedimentary ancient DNA is relatively a new tool in diversity studies, probably because of limited accessibility, the difficulty of sediments sampling and logistics. Indeed, only few studies have applied a metabarcoding approach on ancient sedimentary DNA to reconstruct the protist community structure (*e.g.* Coolen *et al.,* 2013 in the Black Sea; Guardiola *et al.,* 2015 in the Mediterranean Sea; Pawlowski *et al.,* 2011 in the arctic and the southern ocean; Lejzerowicz *et al.,* 2013 in the South Atlantic; Capo *et al.,* 2015 in a French lake). Investigating these sedimentary archives is fundamental to understand the succession of communities, dynamics and the ecosystem functioning.

In this study, we analyzed the V4 barcode region of the 18S rDNA obtained from an Illumina-Mi seq sequencing from two dated sediment cores of the Bay of Brest in order to assess the diversity of paleocommunity of protists, by evaluating the preservation potential of marine protist groups in ancient sediment. Variations in community composition are analyzed in relation to the hydrological changes occurred in the sampled area.

2. Materials and methods

2.1. Study area description and sediment core information

The Bay of Brest (Brittany, France) is a semi-enclosed marine ecosystem connected to the Iroise Sea (Atlantic Ocean) by a narrow (2 km) and deep (50 m) strait. The bay is a shallow macrotidal coastal ecosystem with 50% of its surface deeper than 5 m (Chauvaud *et al.,* 2000). It is characterized by coarse sediments in deep waters and fine and muddy sediments in the upstream part of the estuaries (Hily *et al.,* 1992). The majority (80%) of freshwater inputs come from two main rivers; the

Aulne river (1842 km^2) in the south-east and the Elorn river (402 km^2) in the north-East part of the Bay. Since 1950, the area has experienced several changes in the anthropogenic loading of nutrients; with an increase of Nitrogen supply (Ragueneau *et al.,* 2002) due to the development of anthropogenic activities, and recently, a decrease of phosphorous supply resulting from the ban of washing powders containing orthophosphates. Consequently, there was a significant imbalance in the N/P ratio (Chauvaud *et al.,* 2000; Guillaud and Bouriel 2007) causing changes in the composition of planktonic and benthic communities (Delmas and Tréguer 1983; Le Pape *et al.,* 1996; Del Amo *et al.,* 1997; Trommer *et al.,* 2013).

Sediment cores were collected at two estuaries of the bay; the Elorn Estuary (EE: 48° 23' 46.79'' N 4° 23' 2.01'' W) on the 11th December, 2012 and the Daoulas Estuary (DE: 48° 20' 46.6'' N 161 4° 17' 41.20'' W) on the 21st May 2014 (Figure 18). Two cores were collected at 12 m of depth for the EE: one for genetic analyses (31 cm) and one for dating (29 cm). One single core was collected at 3 m of depth for the DE (58 cm) for both genetic and dating analyses. Immediately after sampling, the sediment cores were delicately extruded from the plexiglass tubes and then sliced into 1 cm layers. To avoid contamination by smearing between the layers during the core extraction, only the inner part of each slice was sampled, using sterile 6 cm-diameter Petri dishes. The samples dedicated for molecular analyses were stored in 50 mL-plastic cryotubes and immediately frozen in liquid nitrogen then preserved in the -80°C laboratory freezer until further analyses. The samples dedicated for dating were stored in plastic bags until radionuclide analyses (see Klouch *et al.,* 2016 for further details on sampling).

The sediment cores were dated using the $^{210}Pb_{xs}$ (natural) and ^{137}Cs (artificial) radionuclides as indicators for chronology establishment. ^{210}Pb and ^{137}Cs activities were measured using a very low background, high-efficiency, well-shaped γ detector equipped with a Cryo-Cycle (CANBERRA) (Schmidt and de Deckker 2015). The γ detector was calibrated using certified reference materials from IAEA (RGU-1; RGTh; SOIL-6). Activities were expressed in mBq g^{-1} and errors were based on 1 SD counting statistics. The chronology of the dated cores is published in Klouch *et al.,* 2016 where the same sediment cores have been studied for single-species dynamic based on ancient DNA. The Elorn core covered about 72 years (from 1939 ± 2 to 2011 ± 1), whereas the Daoulas core covered about 147 years (from 1866 ± 7 to 2013 ± 1).

Initially, sediment sampling and analyses were also conducted in two other estuarine ecosystems of Britanny, situated in the Bay of Morlaix (northwest of Britanny) and the Bay of Vilaine (south of Britanny), but the corresponding results were not used in this study (see Annexe 1).

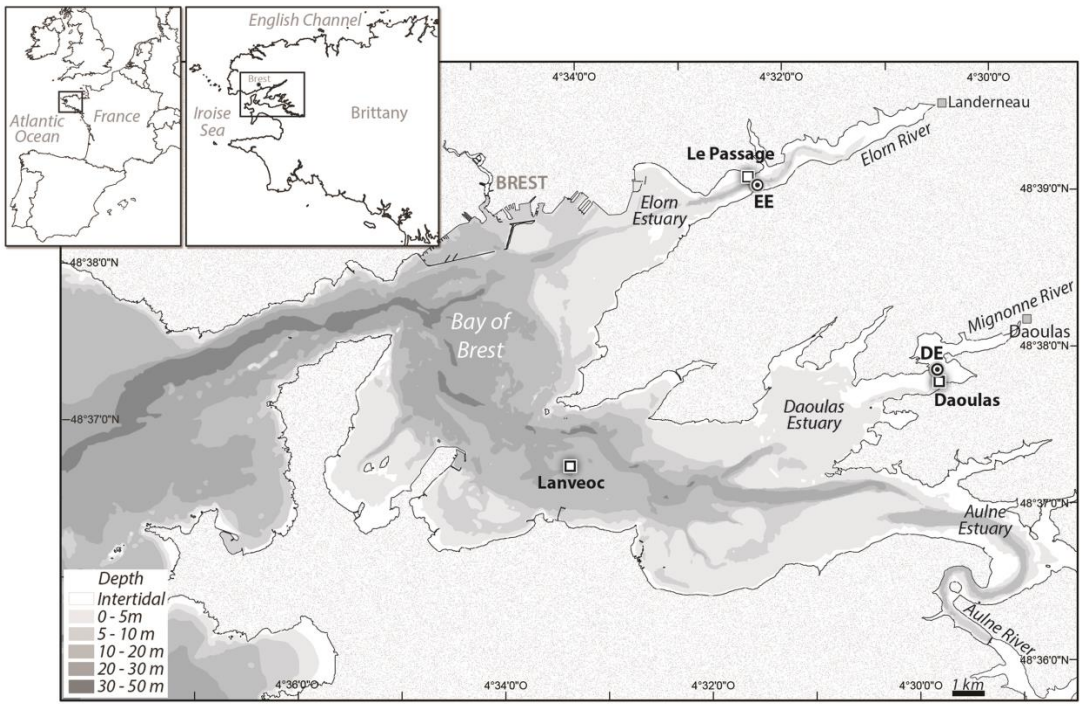


Figure 18. Map of the Bay of Brest with the core sampling sites (Elorn Estuary: EE; Daoulas Estuary: DE), with the REPHY monitoring station at the EE "Le Passage".

2.2. DNA extraction, PCR amplifications and Illumina Mi-seq sequencing

DNA manipulation pre- and post-PCR amplification were performed in a way to avoid cross-contamination between samples and contamination with modern DNA, by following specific instructions for working with ancient DNA (Gilbert *et al*., 2005). Genomic DNA extraction was carried out on 5-7 g of sediment material from each of the 31 layers of the EE and the 58 layers of the DE, using the PowerMax soil isolation kit (Mobio Laboratories Inc., Carlsbad, California, USA), following the manufacturer's instructions. DNA extracts were immediately stored at -80°C. Prior to PCR amplifications, DNA samples were diluted and standardized to 5 ng μL^{-1} to minimize the difference in DNA concentration between the samples and allow for accurate data comparison.

The eukaryotic V4 hyper-variable region of the 18S ribosomal RNA gene was amplified using the eukaryotic primers TAReuk454FWD1 and TAReukREV3 (Stoeck *et al.,* 2010). The reaction mixture was prepared in a final volume of 25 µL, containing 1X Master Mix Phusion High-Fidelity DNA polymerase (Finnzymes, Vantaa, Finland), 0.35 µM of each of the forward and the reverse primers, 3% of dimethylsulphoxide, sterile water and 1 µL of DNA template. Samples were run in triplicate in order to yield sufficient PCR products for sequencing. Positive controls (mock community composed of: *Dunaliella terticolecta, Pseudo-nitzschia pungens, Karenia mikimotii, Prorocentrum consutum, Alexandrium minutum, Heterocapsatriquetra, Scrippsiella trochoidea, Scrippsiella donghaienis)* and

negative controls (without DNA templates) were used to check the efficiency and specificity of the PCR reaction. PCR amplifications were conducted in a PeqStar (OZYME, Montigny-Le-Bretonneux, France) with the following cycling program: an initial denaturation step at 98°C for 30 s, 12 cycles of 98°C for 10 s, 53 °C for 30 s and 72 °C for 30 s, then 18 similar cycles but with an annealing temperature of 48°C and a final elongation step at 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel. Triplicates of PCR products were pooled and purified using a Nucleospin PCR clean-Up kit (MACHERY-NAGEL, Düren, Germany). DNA concentration of purified products was measured using Quant-iT PicoGreen dsDNA quantification kit (Invitrogen, Cralsbad, CA, USA) and a BioTek FLX 80 spectrofluorophotometer according to the manufacturer's instructions. PCR products of 3 and 27 samples respectively for EE and DE had insufficient DNA quantity (<15 ng) to perform sequencing. The remaining DNA amplicons (28 samples for EE and 31 samples for DE) were sent to GeT (http://get.genotoul.fr/) (Toulouse, France) for sequencing from both sides using an Illumina MiSeq (2x250) sequencer.

2.3. OTU (Operation Taxonomic Unit) construction and taxonomic assignment

The raw paired-end sequences produced from the Illumina sequencing were de-multiplexed using an *ad hoc* C++ program. The forward and reverse reads were assembled using the FLASH program (Magoč and Salzberg 2011). The sequences were filtered for quality and length. Reads were dereplicated (all strictly identical sequences were grouped together).. Reads were then clustered into OTUs (Operational Taxonomic Unit) at 4 pair base differences using an edit distance. The sequence identity (%) of the clustering varied between 70 and 100 depending on sequence length. The OTUs containing less than 3 reads over the whole data base where filtered out being considered as large sequencing errors, chimera or non-18S rDNA sequences. The most abundant sequence was used as a representative for each OTU cluster. Taxonomic assignment of OTUs was performed using the Protist Ribosomal Reference (PR2) database (Guillou *et al.,* 2013). All available V4-18S rDNA sequences were retrieved from the PR2 database by searching for the forward and reverse primers allowing for 3 mismatches between the primers and the sequences. Bacterial, Archaeal, chloroplastic and mitochondrial 18S-rDNA sequences were added to extracted sequences to identify non-18S rDNA sequences. OTUs were taxonomically assigned if their reads were ≥70% similar to a sequence from the database. For each read, only the most similar reference sequences were used to assess taxonomy as described previously (Boissière *et al.,* 2012; Gimonneau *et al.,* 2014; Tchioffo *et al.,* 2016). This was performed using the Needleman-Wunsch alignments. This process was done at 8 taxonomic levels from the Domain (Eukaryota) down to the species level.

2.4. Statistical analyses on OTUs and plankton data

An output matrix was produced containing the assigned OTUs and their abundances (number of reads) for each sample (sediment layer). In order to minimize biais from very rare sequence, all OTUs that were present in less than three reads and less than two distinct samples were discarded.

OTUs read numbers were transformed into relative abundances prior to analyses. For each sample (sediment layer), the number of reads of each OTU was divided by the sum of reads of all OTUs. Rarefaction curves for each study site were produced by rarefying to the lowest number of sequences using rrarefy command from the vegan package (http://CRAN.R-project.org/package=vegan) of R software version 3.2.3 (R Core Team 2016). We also produced a rarefaction curve by pooling the reads from all samples of the two sites to gain a view of global diversity of the paleocommunities of the Bay of Brest. Ordination of OTUs data was performed by non-metric multidimensional scaling (NMDS) using the Bray-Curtis similarity coefficient and the metaMDS command from the 'vegan' R package. The clustering of groups in NMDS plot was determined by performing a dendrogram using the Bray-Curtis distance.

Plankton data collected from 1995 to 2001 in the frame of the REPHY (*REseau de surveillance et d'observation du PHYtoplankton et des PHYcotoxine*s)) monitoring network at "*Le Passage*" station, corresponding to the EE sampling core station were analyzed to confront with trends depicted from paleogenetic data. Plankton data analysis was performed on taxonomic units (TU) which are defined as a group of species or genera as described by Hernandez Farinas *et al.,* (2015). Such grouping allowed overcoming errors related to the misidentification between resembling species or genera using optical microscopy. The time series have been modeled using Dynamic Linear Models (DLM) (West and Harrison, 1997) with the *dlm* package (Petris 2010) in R software (R core team 2016). Dynamic Linear Models are particularly adapted to environmental data characteristics such as irregular sampling frequency, missing data and, outliers (Hernandez Farinas *et al.,* 2014). The normality of standardized residuals has been checked visually using QQ-plot and tested with Kolmogorov-Smirnov test. The independence of standardized residuals has been checked using Stoffer-Toloi test.

3. Results

3.1. Protist diversity from modern to ancient sediment

The V4-18S rDNA sequencing yielded a total number of 4428 OTUs and 175 318 reads. After OTU data cleaning 1811 OTUs (170 805 reads) were considered for further analyses. A total of 1708 and 1549 different OTUs were identified in DE and EE, respectively. Diversity saturation was reached for the total data set (DE and EE) with a maximal richness estimated around 1800 OTUs (Figure S1.1a), but not for each single core separately (Figure S1.1b).

The protist and total eukaryotic OTU numbers diminished from top to bottom sediment layers of both the EE and DE cores, indicating a decrease of diversity richness from modern to older sediments. Protist OTU accounted for 12-75% of the total eukaryotic diversity. Protist OTU number was higher in the upper part, from 2013 ± 1 to 1991 ± 2 in DE and from 2011 ± 1 to 1999 ± 1 in EE (Figure 19). In ancient sediments (deepest layers), the protist OTU number seems to stabilize to 8-25% and 12-24% of the maximum protist richness (100% OTU richness) observed at 1 and 2 cm-depth layer for EE and DE, respectively. This corresponds to a total loss of 75-92% of the protist modern diversity in ancient sediments.

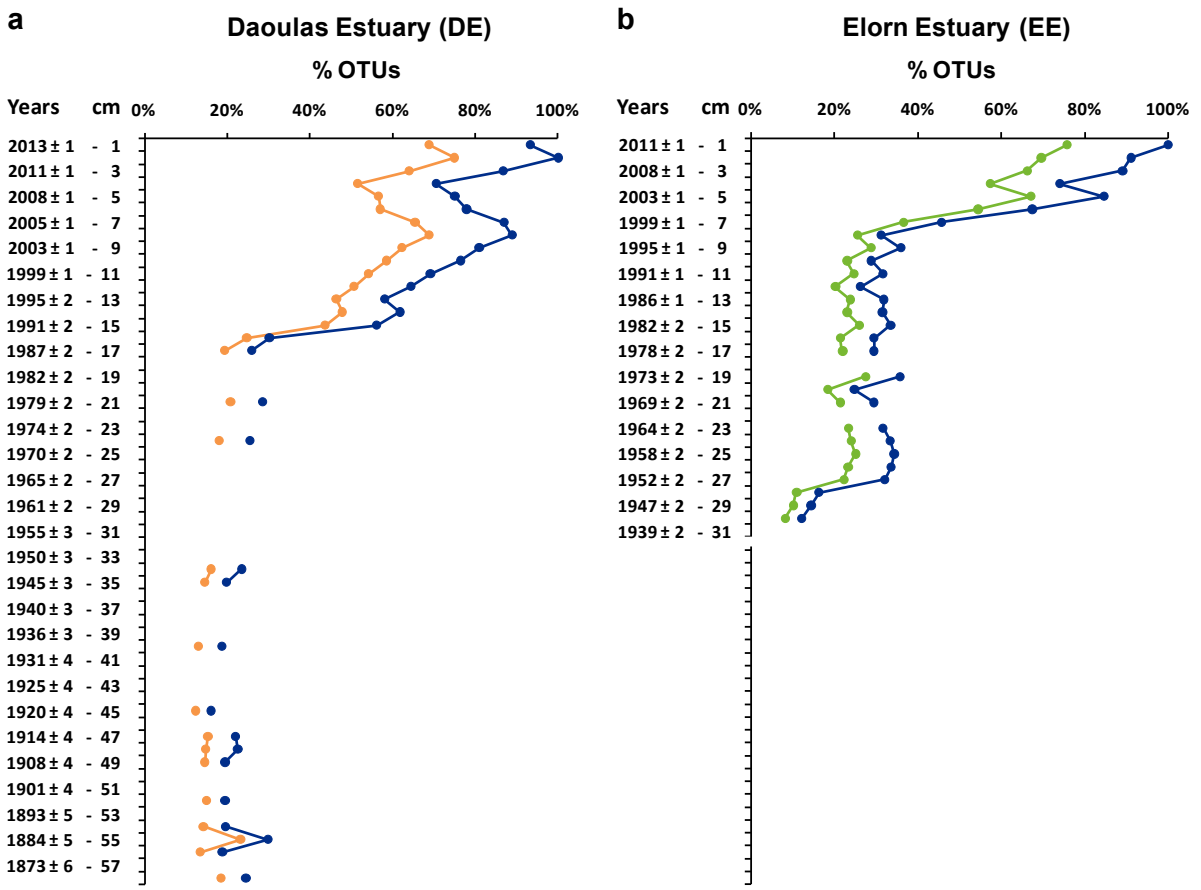


Figure 19. Pattern of OTU richness over time in DE and EE sediment cores. The total richness (all eukaryotic OTUs) is represented in dark blue. The protist richness (excluding Metazoa, Fungi, land plants and macroalgae) is represented respectively, in orange for DE (a), and green for EE (b). The protist richness was normalized to 100%, corresponding to the highest number of OTUs observed across the core layers (1 cm for EE and 2 cm for DE).

3.2. Protist assemblages across sediment cores

NMDS based on protist assemblages (Bray-Curtis distance) analyses allowed the separation from the DE core of 4 groups of samples, assembling progressive sediment layers: group 1 (1-16 cm), group 2 (17-24 cm), group 3 (34-40 cm), and group 4 (45-58 cm) (Figure 20a). The short distance between samples of the group 1 suggests that the protist community of superficial sediments is more similar than in the other groups (G). Similarly, 4 groups of samples were identified in EE: G1 (1-6 cm), G2 (7-27 cm), G3 (28, 30 cm) and G4 (29) (Figure 20b). When performing NMDS ordination of DE and EE samples together, the samples clustered in 5 groups (Figure 20c). The group 1 and 2 included superficial layers from both the EE and DE and were composed of the same superficial layers as those previously identified in each site separately. G3 clustered only samples from EE (28-30 cm) and G4

clustered only samples from DE (34-40). Finally, the G5 included deeper samples from DE (45-58 cm) and one deep sample (29 cm) from EE.

Across the NMDS 1 axis, for both single and cumulative plots, samples dated before and after 1950 are separated (Figure 20a-b-c).

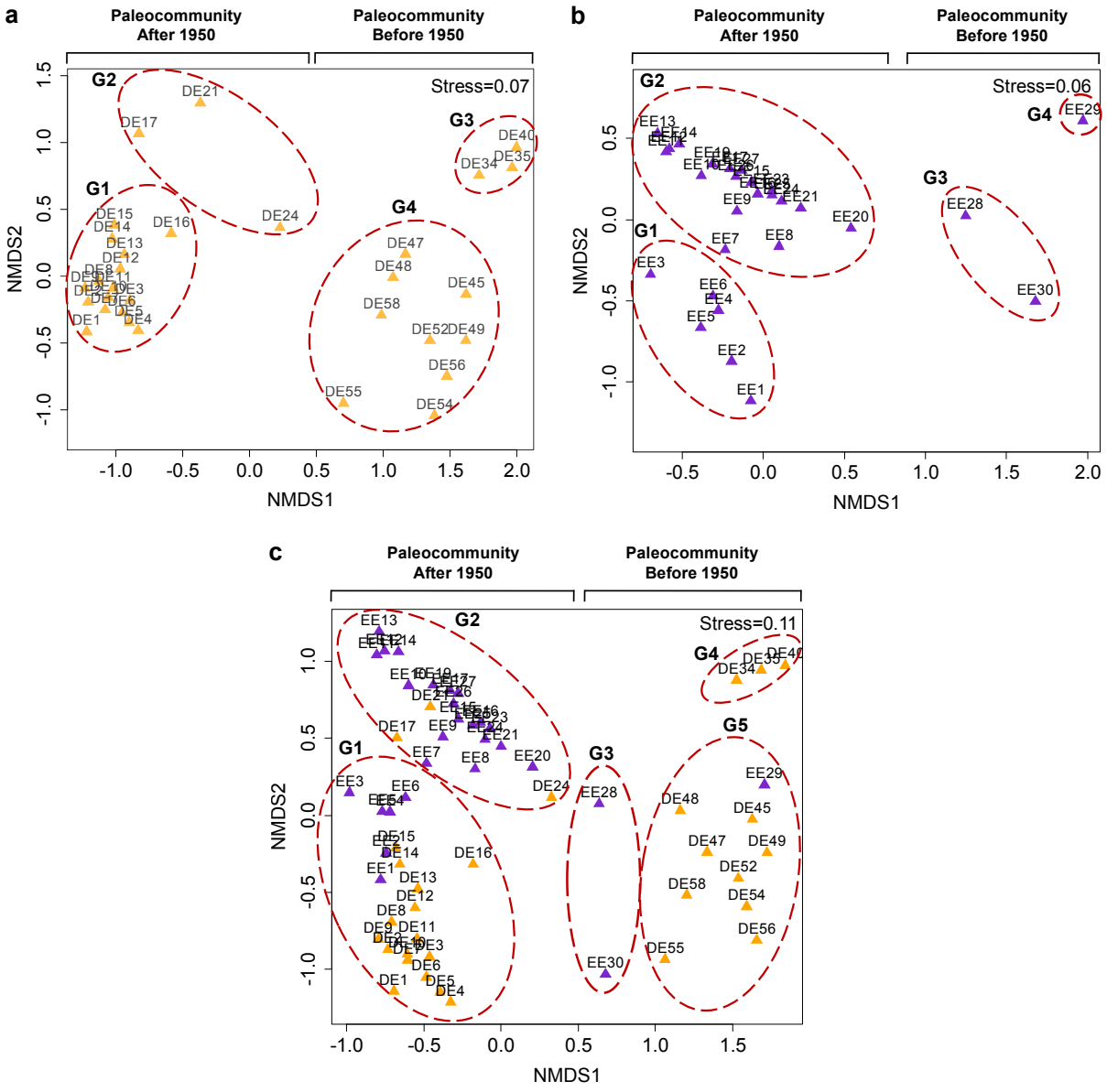


Figure 20. NMDS ordination based on the Bray-Curtis distance for: a) DE samples, b) EE samples and c) DE and EE samples together. Numbers correspond to the sediment layerdepth in centimeters.

3.3. Taxonomic composition of paleocommunities

Taxonomic assignation of OTUs showed that paleocommunities are composed of 9 eucaryotes supergroups. OTUs that could not be assigned to any supergroup (named Undetermined OTUs) represented less than 3% (Figure 21). In both sites, Alveolata accounted for the highest OTU protist diversity (30% in EE and 26% in DE) and abundance (64% in EE and 40% in DE) followed in a decreasing importance order by Opisthonkonta (including Metazoa and Fungi), Stramenopiles, and Rhizaria. These groups accounted for similar percentages of OTU richness but not for abundances in both cores (Figure 21).

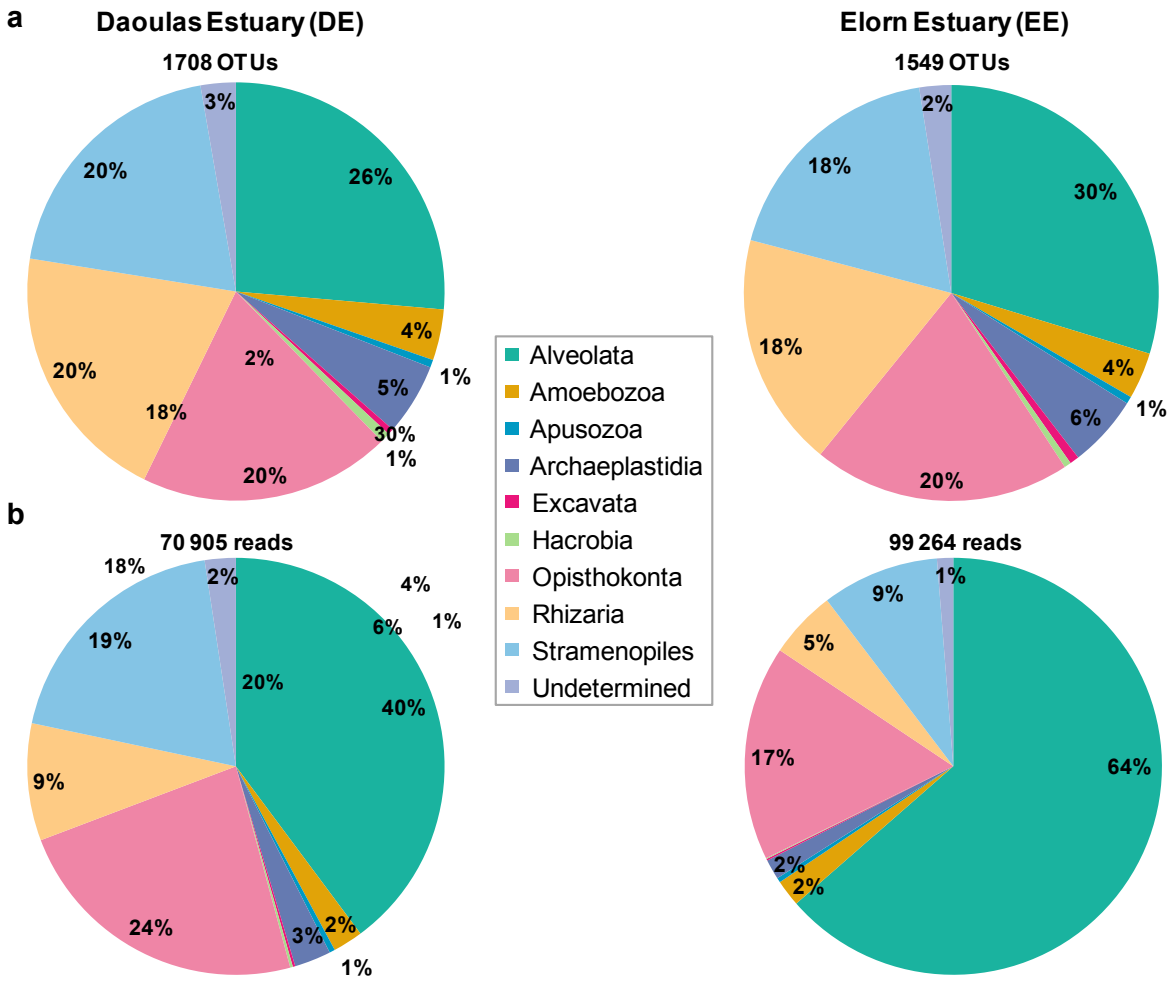


Figure 21. Taxonomic composition of paleocommunities representing: a) the richness and b) the abundance, at DE (left) and EE (right). All the groups were represented in the pie charts but only those with value≥1% are represented in numbers.

3.4. Protists diversity and dynamics in ancient sediment

A total of 79 OTU protist groups have been identified to the taxonomic level of the classes from the DE and EE core, all belonging to all 9 eukaryotic supergroups. About half of them (38 out of 79 groups in DE and 40 out of 77 at EE) were retrieved in deep ancient sediments (Figure 22a-23a).

The classes occuring in deep ancient sediments were similar in both the DE and EE cores. Within the Alveolata supergroup, we identified Apicomplexans (Apicomplexa-Und, Perkinsea), Ciliates (Ciliophora-Und, Litostomatea, Spirotrichea), Dinophyceae, Syndinea and undetermined Alveolata (Alveolata-Und). Across both sediment cores, the most important classes regarding to their relative abundance were the Dinophyceae (up to 82%), the Apicomplexa-Und (up to 21%) and Bacillariophyceae (up to 34%) (Figure 22a-23a).Within the Rhizaria, most of the classes were identified across both cores and contributed similarly to relative abundances; the mains classes were Cercozoa-Und, Endomyxa, Endomyxa-Phyomyxea, Filosa-Granfilosea, Filosa-Imbricatea, Filosa-Sarcomonodea, Filosa-Thecofilosea and Novel-Clade 10-12. As for Stramenopiles, the same classes were identified from both sites; Bacillariophyta, Bolidophyceae-and-relatives, Dictyophyceae, Chrysophyceae-Synurophyceae, Labyrinthulea, MAST (Marine STramenopiles), Oomycota with the exception of Eustigmatophyceae and Xantophyceae which were only observed in EE.

Some classes were present only in the upper part of the cores (Figure 22-Figure 23a). Fornicata (Excavata), Cariacotrichea and Plagioplylea (Alveolata), Discosea-Longamoebia (Amoebozoa), Rad-B (Rhizaria) were identified at DE. At EE, apart Rad-B (Rhizaria), we identified different classes than those of DE: Centroheliozoa-Und, Telonemia-Und, Katablepharidaceae (Hacrobia), Parabasalia (Excavata), Bicoecea (Stramenopiles)

Globally, most of the detected classes showed very low relative abundances. Only 12 and 10 classes, belonging to 4 supergroups (Alveolata, Stramenopiles, Rhizaria and Archaeplastidia) had a higher abundance than 5%, at DE and EE, respectively (Figure 22b-Figure 23b). Among these groups, the richness was higher (3-7 groups per sample/year) in DE deeper layers (G3 and G4) compared to upper layers (G1 and G2) where only 1-4 classes were identified per sample/year (Figure 22b). Similarly, at EE, the three deepest layers (29-29-30, G3 and G4) were the most diverse (up to 8 groups per layer) (Figure 23b).

Relative abundances of the more important (≥5%) classes varied over time for both the EE and DE, but variations was significantly higher in DE than ino EE. These variations are attributable to changing relative importance of Dinophyceae. In DE, the relative abundances of Dinophyceae fluctuated importantly (0.2-82%) over time. They were present with very low abundances (range: 5-52% from 1866± 7 to 1920 ± 4. From 1933 ± 3 to 1948 ± 3, the Dinophyceae were detected in sediment samples but they accounted for <5% (Figure 22a), conversely the major part of relative abundance was

attributed to the MASTs, in particular to the MAST-12 clade which increased importantly reaching up 78% of the total abundance. Six other MASTs clades (MAST-1, MAST-3, MAST-6, MAST-7, MAST-9, and MAST-20) were identified from all the sediments samples combined. The MAST-1 and MAST-6, together with MAST-12 accounted for the major part of abundance in this study. From 1987 ± 2 to the more recent layer 2013 ± 1, Dinophyceae were observed again in ≥5%, and showed a decreasing trend in their relative abundances being replaced by Bacillariophyceae which was the most important group during this period of time from 2003 ± 1 to 2013 ± 1 (Figure 22b). At EE, Dinophyceae were observed regularly across all sediment layers and contributed importantly to the total relative abundances (up to 87%). They were detected regularly from 1939 ± 3 to 2011 ± 2 then displayed a slight decrease trend in relative abundance from 1991 ± 1 to 2011 ± 2. Bacillariophyceae were underrepresented (≤11%) and MASTs were present in more samples and for a longer time period: from (1943 ± 2 to 1997 ± 1) than that of DE, with relative abundances always ≤10% (Figure 23a).

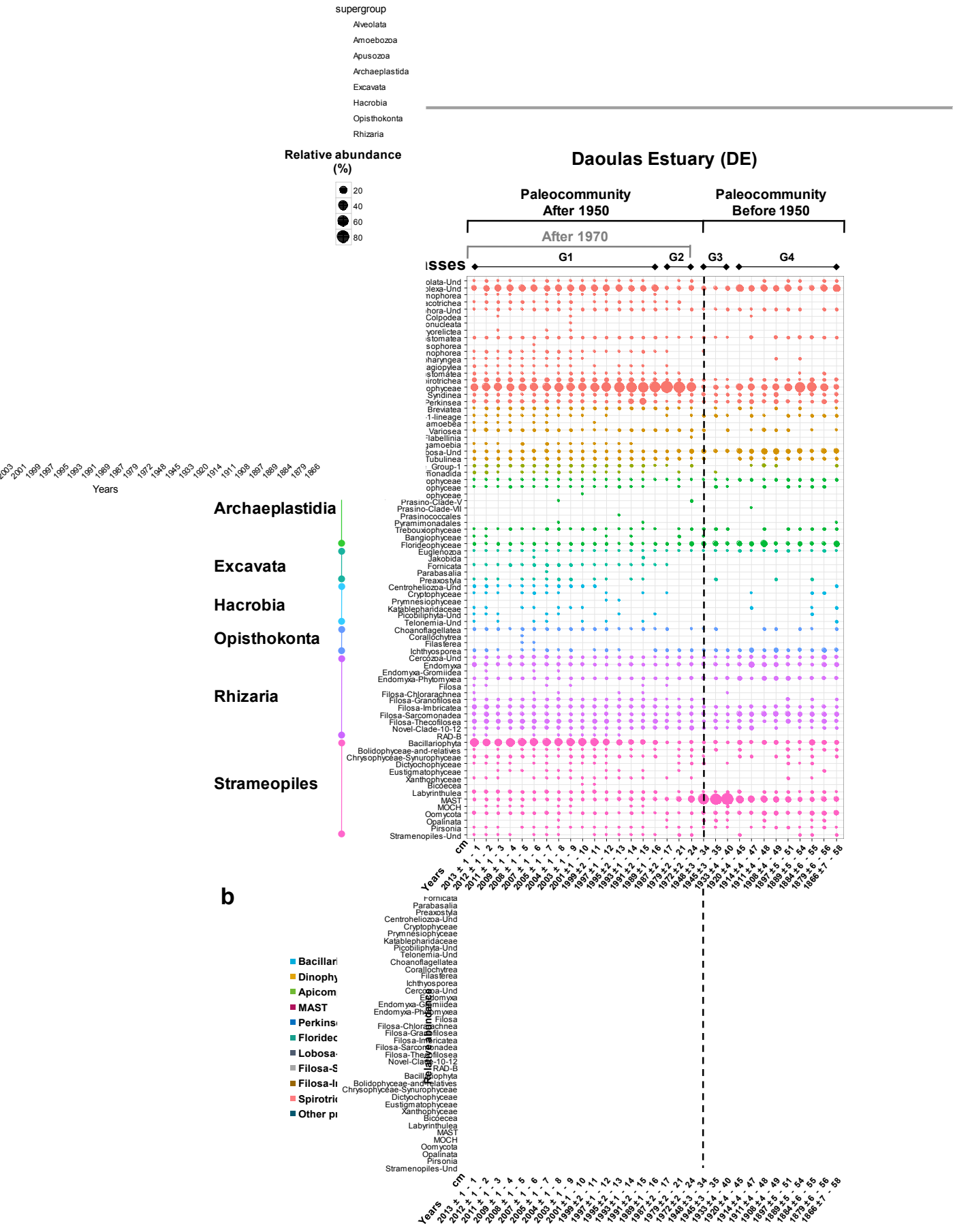


Figure 22. Relative abundance (%) of protists classes through time at the Daoulas Estuary (DE). (a) for taxa with relative abundance (≤5%) and (b) for taxa with relative abundance (>5%). The size of the symbol is proportional to the relative abundance of the group within the entire paleocommunity of the sample (cm)/year (date). The supergroups are separated by groups. The taxa named "–Und" could not be further assigned taxonomically.

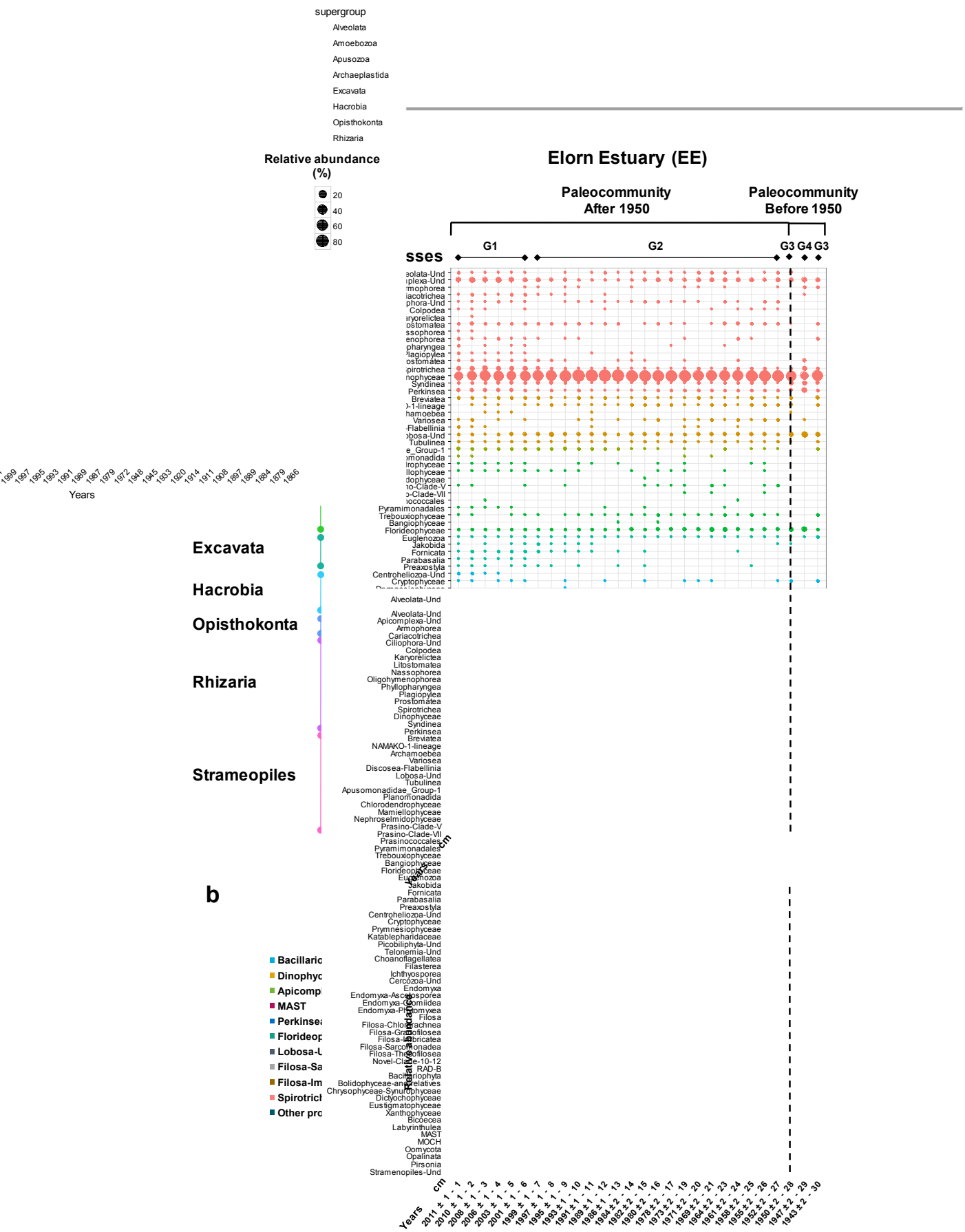


Figure 23. Relative abundance (%) of protists classes through time at the Elorn Estuary (EE). (a) for taxa with relative abundance (≤5%) and (b) for taxa with relative abundance (>5%). The size of the symbol is proportional to the relative abundance of the group within the entire paleocommunity of the sample (cm)/year (date). The supergroups are separated by groups. The taxa named "–Und" could not be further assigned taxonomically.

3.5. Multiannual dynamics of Dinophyceae

Dinophyceae OTUs from both the DE and EE cores were assigned to seven genera. The most abundant genera were *Alexandrium*, *Gonyaulax* and *Protoperidinium*. Over 4-37% of the Dinophyceae OTUs (orange bars in Figure 24) could not be assigned to the genus level. About 1-5% (dark blue bars in Figure 24) represent OTUs with conflictual taxonomical assignation (two or more dinoflagellate genus).

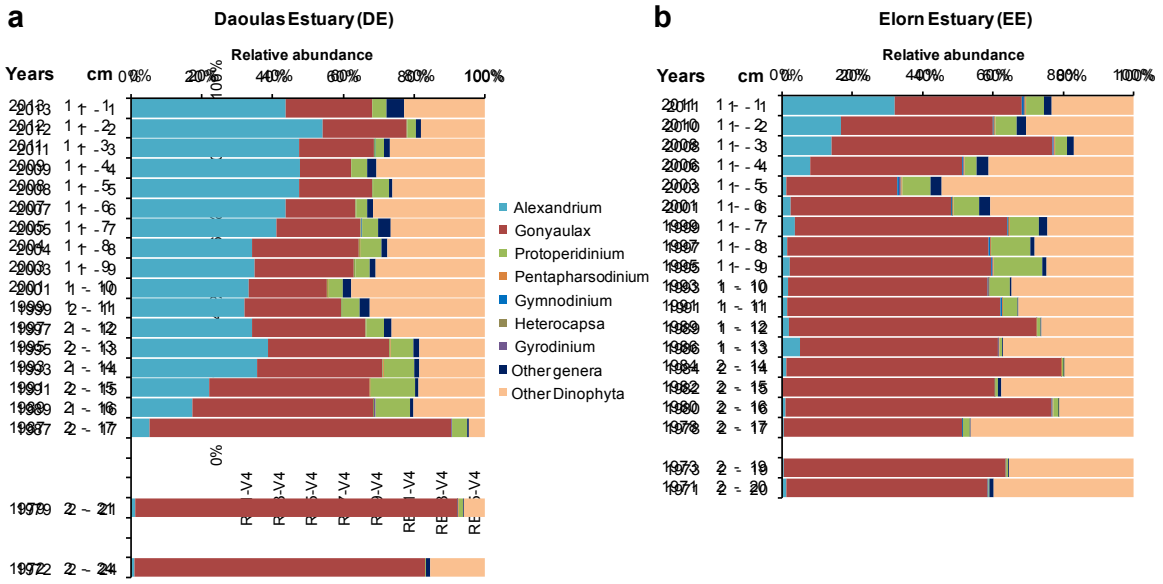


Figure 24. Relative abundance (%) of dinoflagellate OTU assigned to genera through time for: a) Daoulas estuary and b) Elorn estuary.

Alexandrium and *Gonyaulax* OTUs had an inverse dynamics along the cores. In both the DE and EE cores, the relative abundance of *Alexandrium* increased from ancient to modern times after 1987 (from 5 to 54%) and 1989 (from 2 to 32%) at DE and EE, respectively. Whereas for Gonyaulax, the relative abundance decreased at the same years (from 84 to 24%) at EE and (from 70 to 36%) at DE.

In parallel, the modeled dynamics of dinoflagellates abundances (cells L^{-1}) analyzed from water column samples at the EE showed a decreasing trend from 1995 to 2001 (Figure 25).

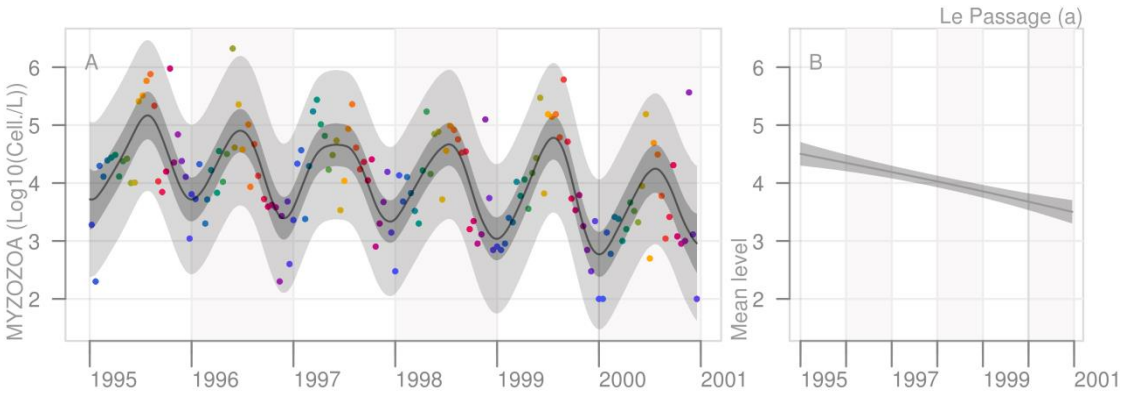


Figure 25. Abundances (Log transformed) of dinoflagellate taxa (Taxonomic Units; TU) in the water column at the Elorn Estuary inferred from the REPHY monitoring network data. A) Observed abundances with the following color code: (blue gradient: winter; green gradient: spring; orange gradient: summer, and red gradient: autumn). The Black line represents the mean concentration, light grey area its 95% confidence interval and dark grey area the 95% concentrations confidence interval as modeled by the Dynamic Linear Model (DLM). B) Mean level or deseasonalized mean concentration and its 95% confidence interval.

4. Discussion

4.1. Diversity of protist paleocommunities

We analyzed protist V4-18S rDNA sequences obtained from dated sediment cores to assses to what extent modern protist diversity can be archived in marine estuarine sediments. This study has been conducted considering previously developed standards and precautions to follow when dealing with ancient DNA and paleogenetic procedures (Anderson-Carpenter *et al.,* 2011; Boere *et al.,* 2011; Gilbert *et al.,* 2005, See Klouch *et al.,* 2016 for further details). Only few studies have investigated the diversity and composition of protist community preserved in ancient sediments, therefore the degree of variation between modern protist assemblages and paleocommunities remains poorly explored. We did not reach diversity saturation when performing rarefaction curves on each sampling site data, suggesting that our sequencing effort was not totally exhaustive for separate sampled site.However, when samples were cumulated, our sequencing effort was theoretically sufficient enough to cover the global paleocommunity diversity. We can therefore assume that the ~1800 V4-rDNA-based OTUs would encompass the paleocommmunity diversity of the Bay of Brest over the recovered time scale. Over the ~150 years analyzed in this study, the protist and the total (including Metazoa, Fungi, land plants and macroalgae) richness decreased over time at both the DE and EE (Figure 19). Only 16-18% (on average) of the protist diversity observed in superficial, modern sediments was recovered in deeper ancient sediments, with a corresponding loss of 82-84% of diversity. The part of protist diversity found in old sediment layers represent probably the protist taxa having a satisfactory preservation potential in ancient sediments. By comparing the protist diversity found in the water column and in ancient sediments, Capo *et al.,* (2015), observed that up

to 71% of protists found in the water column were also found in sediments. We instead compared the diversity across the different dated sediment layers; hence we have taken into account only the part of the protist community accumulating at the sediment surface and preserving in stratified layer.

DNA degradation may have contributed to these decreasing profiles of paleocommunity protist richness. Indeed, DNA is known to be more preserved in cold and dry environments such as permafrost, fjords and lakes because of the anoxic conditions of the sediments and the low temperatures which slow the DNA microbial degradation and promote good preservation (Boere *et al.,* 2011). In estuarine ecosystems, such as those analyzed here, DNA degradation could have played a major role. Indeed, many factors influence the DNA conservation in the sediments including microbial activity, adsorption of DNA to mineral and organic matrices, oxidative damage, as well as temperature, salinity and pressure (Boere *et al.,* 2011). Notwithstanding, on the same sediment core, we have been able to amplify specific ancient DNA (up to 150 year-old) of two dinoflagellate species (*Alexandrium minutum* and *Scrippsiella donghaeinis*), proving that even specific DNA fragment were relatively well preserved in our samples (Klouch *et al.,* 2016). DNA degradation occurs under the form of DNA fragmentation (Boere *et al.,* 2011) and the use of short DNA amplicon (100-500 bp) is usually recommended to target potentially fragmented ancient DNA (Coolen *et al.,* 2013). For this reason, initially we attempted to use the short DNA barcode V9 (130 bp) to recover even fragmented ancient DNA and complete the diversity recovered with the V4 barcode. However, the results showed a high number of prokaryotes (15%) (Bacteria and Archaea), and most of the reads could not be determined taxonomically (see Annexe 2), which constrained us to not exploit the data of V9 18S rDNA in this study. On another hand, Coolen *et al.,* (2009) and Coolen *et al.,* (2013) showed an excellent high-throughput sequencing yield of ~560 bp and 340 bp long fragments respectively, proving that our massive sequencing of <500 bp fragments was relevant. Moreover, the massive sequencing of the V4 region was already tested in Capo *et al.,* (2015). We choose the amplification of the V4 region for its higher resolution and the better database coverage for taxonomic assignment (Stoeck *et al.,* 2010; Guillou *et al.,* 2013). Yet, it is noteworthy that the universal V4-18S rDNA primers used in this study recognize most of the eukaryotic phyla but do not encompass Foraminifera and several lineages of Excavates (Pawlowski *et al.,* 2011). We privileged having a low number of well-assigned taxa with a low proportion of undetermined taxa, rather than having a higher number of taxa with a high number of undetermined taxa. Indeed, the number of undetermined taxa was rather low (3%) in our study. Despite metabarcoding is considered a promising and highly informative approach, there are debates about the accuracy of abundances estimations in metabarcoding studies considering that the number of reads will depend on the size of the organism, the highly variable

copy numbers in the 18S between taxa and even within the same species, and the potential primer bias (the different amplification success among taxa) (Bik *et al.,* 2012). Analyzing relative abundances is thought to be more accurate and reliable. For this reason, we analyzed relative abundances instead of read abundance of each taxa. Although we recovered a high diversity of protist from sediment, some taxa may have not been identified because of the limited coverage of the database concerning some taxa such as benthic dinoflagellates. Indeed, because of the lack of taxonomic investigations, these groups are not well assigned in the PR2 database.

In this study, the global eukaryotes' paleocommunities of our two analyzed cores were comparable, suggesting that the relative importance of protist supergroups were stable in our studied area. Alveaolata dominated both in terms of diversity (30% in EE and 26% in DE) and abundance (64% in EE and 40% in DE) . Opisthonkonta (24% in DE and 17% in EE), Stramenopiles (19% in DE and 9% in EE) and Rhizaria (9% in DE and 5% in EE) followed in terms of relative abundance. The importance of this supergroups emerged also from the analyses of superficial sediment in European coastal waters (Forster *et al.,* 2016), with the difference that all Alveolata, Stramenopiles, Rhizaria had similar richness proportions and the Opisthokonta had lower richness. Protist composition in marine sediments seems different if we compare superficial communities (Forster *et al.,* 2016) to stratified, ancient sediment (this study), proving again that only part of modern protist community are preserved in ancient sediments of marine ecosystems. The protist diversity could be different in freshwater ecosystems, where the Opisthokonta seems to be the dominant group, as suggested by paleogenetic analyses carried on sediments of the Le Bourget Lake (France) Capo *et al.,* (2015).

Most of protist groups recovered in our ancient sediments are known to produce resting stages (cysts, spores) which are acknowledged to be preserved in the sediments for a long period of time (Härnström *et al*., 2010; Lundholm *et al*., 2011; Myazono *et al.,* 2012). We cannot estimate to what extent our DNA originates entirely or mostly from intact resting stages or from extracellular DNA. Indeed, intracellular DNA is known to be better preserved than environmental, extracellular since a resistant structure protects it against degradation. RNA instead of DNA diversity survey might have solved this issue, however in our study we aimed at studying the whole protist diversity, not only that of active organisms. DNA diversity survey seemed to be adapted to this scope, since no significant difference were observed when RNA and DNA diversity survey in sediments have been compared (Massana *et al.,* 2015).

4.2. Paleocommunity changes over time

The sediment samples of both analyzed cores clustered together according to depth and ages suggesting that the protist communities recovered from a same period of time are similar. Two major paleocommunities were distinguished from our analyses from both cores (before/after '50)

suggesting a coherent change in the protist community in the Bay of Brest occurred in the last century. Indeed, the bay has experienced many changes in hydrological conditions since 1950. In particular, the development of agricultural and urban activities around the Bay has contributed to a significant increase of nutrient (nitrogen and phosphorus) supply to the Bay. Data of nutrient concentration from the rivers flowing into the Bay are available since the ’60. Between the ’70 and ’90, the nitrate concentration has been multiplied by 2. After the ‘90, political measures have been taken to reduce the pollution supply to the rivers resulting in a decrease of nitrate and phosphate concentrations. All these changes have resulted in an imbalance of the N/P ratio in the Bay of Brest. The phytoplankton communities have been particularly affected by these modifications. The phytoplankton blooms were dominated by diatoms between 1981 and 1982 (Quéguiner and Tréguer 1984), whereas, after a decade, phytoplankton blooms were dominated by dinoflagellates (Del Amo *et al.,* 1997). After 1995, dinoflagellate abundances diminished in the Bay of Brest as demonstrated from the DLM analyses of plankton data series. The changes that we observed in paleocommunities likely reflect variations in plankton occurred in the water column. However the picture we have of community changes is only a partial vision of the whole plankton community, since, as discussed before, most of the protist richness is lost in ancient sediments.

Unexpectedly, we observed higher diversity of the abundant groups paleocommunities anterio to ‘50 at both sites. In contrast, community posterior to ’50 were characterized by equivalent relative abundance of few groups (Dinophyceae and Bacillariophyceae). This change is mainly due to the variation of the relative importance of Dinophyceae, especially at DE where an inverse pattern between Dinophyceae and MAST have been observed over the time

The MASTs are heterotrophic marine stramenopiles described by Massana *et al.,* (2004) including initially 12 clades (ribogroups). Six new clades were evidenced by Orsi *et al.,* (2011). In a recent reevaluation of their phylogenetic position, Massana *et al.,* (2014) have proposed the elimination of 7 MASTs clades which belonged to other Stramenopiles classes or correspond to chimeric sequences from sequencing errors. Moreover, 3 previously described lineages were renamed and included into the MAST group and 3 more new MASTs clades have been revealed, yielding to a total of 18 clades currently recognized to belong to MAST. Interestingly, in our study, the MASTs relative abundances increased abruptly only during the period (1933-1948) in DE due to a peack (98%) of MAST-12 clade. The MAST-12 clade was also the most recurrent and abundant along the whole DE and EE sediment cores. The MAST-12 clade seemed to be associated to sediments and especially oxygen-depleted and deep-sea sediments (Massana *et al.,* 2004; Massana *et al.,* 2014; Takishita *et al.,* 2007; Logares *et al.,* 2012)) this comfort the finding of this group of protist in our study The MAST-3 and MAST-7 also found in our samples are thought to be planktonic since all of the sequences available in GenBank

were retrieved from plankton samples and none from sediment samples (Massana *et al.,* 2014). The identification of MAST-12 in our samples could indicate that our sediments were characterized by low amounts of oxygen, but , no information on the physical-chemical characteristic of the DE sediment of the Bay of Brest are available to associate the presence of MAST-12 to anoxic conditions of sediments. MAST-12 are known to be bacterial grazers (Massana *et al.,* 2014) and not parasite of dinoflagellate. The opposite patterns between MAST-12 and dinoflagellate observed in our samples cannot be explained by a parasitic relationship and remains still unsolved.

The decreasing trend in the abundances of Dinophyceae both at the DE and EE are corroborated by the decreasing trends of dinoflagellate abundances observed in plankton samples at the Elorn Estuary (Le Passage station). Within the dinoflagellate paleocommunity, the OTUs assigned to the genus *Alexandrium* displayed an increase trend in relative abundance from the past to recent years inversely to *Gonyaulax* OTUs whose importance diminished over the time. In addition, these results corroborates those revealed in our previous study (Klouch *et al.,* 2016) conducted on the same sediment cores DE and EE, in which an increase trend of the abundances of *Alexandrium minutum* has been evidenced using real-time PCR quantifications. Shift in genus occurrence and abundance have been observed in other paleogenetic studies. For example, in a paleoecological study carried out in a Kenyan lake, a shift in the diatom assemblages has been revealed, as the genus "*Navicula"* occurring in ancient sediment was replaced by genus "*Aulacoseira" in* recent sediment (Stoof-Leichsenring *et al.,* 2012) proving the potential of paleogentic approach to reconstruct ancient patterns and communities, revealing even hidden biodiversity.

5. Conclusions

This study innovatively investigated the protist diversity in modern and ancient estuarine sediments by using a paleogenetic approach. The diversity in ancient sediment was mostly represented by taxa which are known capable of producing resting stages, likely due to a better preservation in these sediments. The decreasing relative abundances of dinoflagellates seemed to be correlated with the local plankton data in the water column and are likely related to the hydrological changes in nutrient concentration occurring in the Bay of Brest over the last 60 years. This study contributes to the research on protist diversity, showing that ancient community composition and variations could be inferred by a paleogenetic approach. Furthermore, this approach could be applied in other ecosystems allowing to gain new insights on the poorly explored past protist diversity.

6. Acknowledgment

The authors wish to thank Xavier Caisey and the other scuba divers from Ifremer for sampling the EE core, and Gwendoline Gregoire and Axel Ehrhold from the laboratory GM/LES of Ifremer for allowing the sampling of the DE core in the framework of the project SERABEQ ("Laboratoire d'Excellence" LabexMER (ANR-10-LABX-19)). Sarah Romarc is acknowledged for helping with the optimization of the pre-sequencing analysis. Tania Hernández-Fariñas and Pierre Ramond are acknowledged for helping with statistical tests. Finallly, we wish to thank Alexis L. Pasulka for providing her help for the elaboration of the figure 22 and 23.

7. Supplementary data

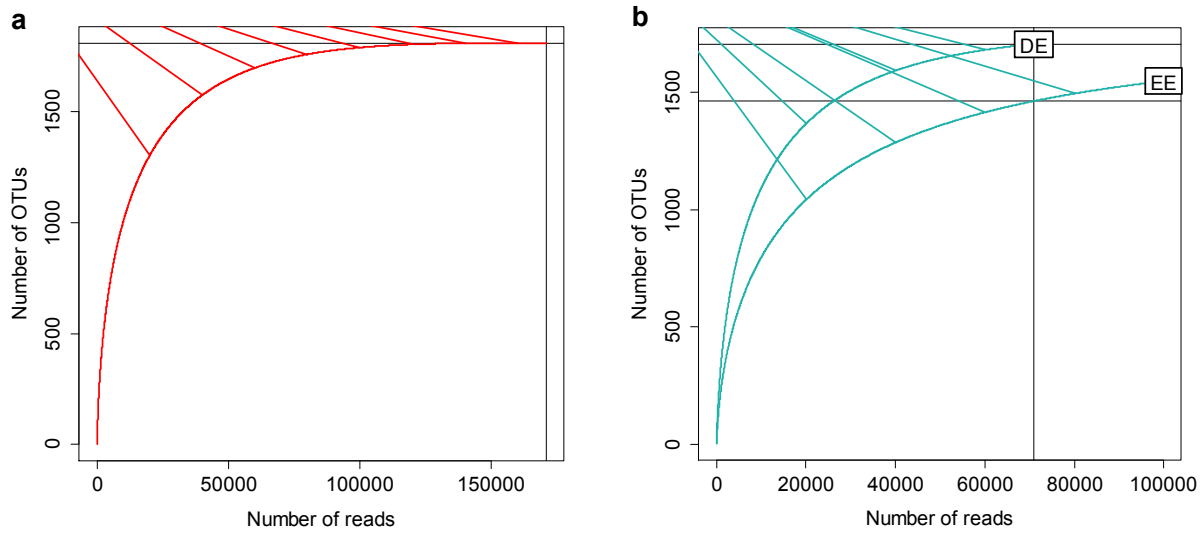


Figure S1.1. Rarefaction curves relating the number of OTUs to the sequencing effort. (a) Global rarefaction curve relating for all DE and EE samples and, (b) for EE and DE independently.

CHAPTER 2

Historical records from dated sediment cores reveal the multidecadal dynamic of the toxic dinoflagellate *Alexandrium minutum* in the Bay of Brest (France)

Context of the work

Harmful Algal Blooms (HABs) occur in many coastal ecosystems around the world, causing major effects on marine ecosystems, human health, and many economic activities related to the exploitation of marine resources such as fisheries and aquaculture. The intensity and frequency of HAB events is continuously increasing. Recently, in the Bay of Brest (Brittany France), particularly intense blooms of the toxic dinoflagellate *Alexandrium minutum* have been reported by local monitoring network (REPHY), reaching up 42 million cells L^{-1} in 2012 at Daoulas estuary. This species first described in 1960 in the Mediterranean is widespread and is considered officially an invasive species (a species which is introduced into a new area by human-mediated pathways, causing ecological and/or economical damage) in European seas. In the Bay of Brest, *Alexandrium minutum* has only been observed since 1990, therefore it is considered to be recently introduced. Despite the existence of valuable time-series data on the species dynamic, these do not extend beyond 30 years. Therefore, there is no data available reporting if the species was present in the area before 1990 and eventually, what were the species dynamic back then.

In this chapter, I used a paleoecological approach based on DNA and cyst examination to try to reconstruct the multidecadal dynamic of *Alexandrium minutum* from dated sediments of the Bay of Brest. More specifically, I designed specific primers targeting the ITS1 rDNA region and developed a real-time PCR assay to quantify the abundances of *Alexandrium minutum* (ITS1 rDNA copies g^{-1} sediment) from the sediment samples. Quantifications were also performed for another non toxic dinoflagellate species, *Scrippsiella donghaienis*. The paleogenetic data (copies g^{-1} sediment) were confronted to monitoring plankton data (cells L^{-1}) from the water column.

Author contributions

In this study, I performed the real time PCR genetic analyses, cyst germination experiments, isolation of cultivated strains, statistical and ecological analyses on both REPHY and paleogenetic data. Parts of these analyses were carried out during my master internship in 2013, before the Ph.D started. In collaboration with E. Bigeard (technician) I set up and optimized the *A. minutum* and *S. donghaienis* real time PCR assays. I trained Z. Qui-Minet (Master 2 student) to real time PCR technique and data analyses for S. trochoidea (see annex). M. Le Gac., D. Hervio-Heath contributed to the genetic protocol optimization. S. Schmidt and F. Andrieux-Loyer collaborated for the radionuclides' analyses and the interpretation of sediment dating data. J. Quéré contributed to cyst germination, isolation and culture maintenance. I have been supervised by R. Siano and L. Guillou for data interpretations. I provided the first version of the paper which has been finalized with R. Siano.

Historical records from dated sediment cores reveal the multidecadal dynamic of the toxic dinoflagellate *Alexandrium minutum* in the Bay of Brest (France)

Résumé (en français)

La dynamique multi-annuelle du dinoflagellé toxique et producteur de kystes *Alexandrium minutum* a été étudiée à travers une échelle de temps de 150 ans par une approche paléoécologique basée sur les données de quantification de l'ADN ancien (aDNA) et de revivification des kystes, obtenues à partir de deux carottes de sédiment de la rade de Brest (Bretagne, France). Les premières traces génétiques de la présence de l'espèce dans la zone remontent à l'année 1873 ± 6. En développant un test de PCR en temps réel, la quantification de l'ADN spécifique a pu être obtenue dans les couches superficielles des carottes, et pour lesquelles la germination de l'espèce (dans des couches âgées de 17-19 ans) a également été acquise. Dans les deux carottes étudiées, nos données paleogénétiques quantitatives ont montré une tendance significative à l'augmentation de l'abondance des copies d'ITS1 ADNr d'*A. minutum* à travers le temps, corroborant les données de surveillance collectées depuis trois décennies, documentant une tendance à l'augmentation dans l'abondance des cellules dans l'eau. En comparaison, les données paléogénétiques du dinoflagellé *Scrippsiella donghaienis* n'ont pas montré de tendance cohérente entre les deux carottes de sédiment, soutenant l'hypothèse de l'existence d'une dynamique espèce-spécifique d'*A. minutum* dans le site d'étude. Ce travail contribue au développement de la recherche en paléoécologie, illustrant son potentiel pour des études de biogéographie, d'écologie et d'évolution sur les microbes marins.

Article published in *FEMS Micorbiology Ecology* journal (see Annexe 3).

FEMS JOURNALS investing in science

Historical records from dated sediment cores reveal the multidecadal dynamic of the toxic dinoflagellate *Alexandrium minutum* in the Bay of Brest (France)

Khadidja Z. Klouch[1,2], Sabine Schmidt[3], Françoise Andrieux-Loyer[1], Mickaël Le Gac[1], Dominique Hervio-Heath[4], Zujaila N. Qui-Minet[1], Julien Quéré[1], Estelle Bigeard[2], Laure Guillou[2], and Raffaele Siano[1,*]

[1]Ifremer - Centre de Brest, DYNECO PELAGOS, F-29280 Plouzané, France,

[2]Sorbonne Universités, UPMC Univ. Paris 6, CNRS, Adaptation et Diversité en Milieu Marin (UMR 7144), équipe DIPO, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff cedex, France,

[3] UMR5805 EPOC, Univ. Bordeaux, 33605 Pessac, France,

[4]Ifremer, RBE, SG2M, Laboratoire Santé, Environnement et Microbiologie, Plouzané, France

Corresponding author: Ifremer - Centre de Brest, DYNECO PELAGOS, F-29280 Plouzané, France.
Tel: +33 02 98 22 42 04; Fax: +33 02 98 22 45 48; E-mail: raffaele.siano@ifremer.fr

One sentence summary: By means of a paleoecological approach (ancient DNA quantification and cyst revivification), a species-specific increasing trend in the concentration of the toxic marine dinoflagellate *Alexandrium minutum* was revealed over a time scale of about 150 years in the Bay of Brest.

Abstract

The multiannual dynamic of the cyst-forming and toxic marine dinoflagellate *Alexandrium minutum* was studied over a time scale of about 150 years by a paleoecological approach based on ancient DNA (aDNA) quantification and cyst revivification data obtained from two dated sediment cores of the Bay of Brest (Brittany, France). The first genetic traces of the species presence in the study area dated back to 1873 ± 6. Specific aDNA could be quantified by a newly-developed real-time PCR assay in the upper core layers, in which the germination of the species (in up to 17-19 year-old sediments) was also obtained. In both cores studied, our quantitative paleogenetic data showed a statistically significant increasing trend in the abundance of *A. minutum* ITS1 rDNA copies over time, corroborating three decades of local plankton data that have documented an increasing trend in the species cell abundance. By comparison, paleogenetic data of the dinoflagellate *Scrippsiella donghaienis* did not show a coherent trend between the cores studied, supporting the hypothesis of the existence of a species-specific dynamic of *A. minutum* in the study area. This work contributes to the development of paleoecological research, further showing its potential for biogeographical, ecological and evolutionary studies on marine microbes.

Keywords: paleoecology, ancient DNA, real-time PCR, Harmful Algal Blooms (HAB), dinoflagellates, coastal ecology.

1. Introduction

Marine sediments are important resources for paleobiologists as they represent a valuable archive of past environmental communities. The resting stages of plankton, diatom frustules and spores, dinoflagellate cysts, foraminifera shells, and micro- and meso-zooplankton (rotifers, copepods, cladocerans, ciliates), as well as molecules such as sterols and pigments, can be accumulated and preserved in sediments. Their examination combined with a chronological analysis can provide ecological and environmental information about past communities (Liu *et al*., 2013; Ellegaard *et al*., 2013b). Benthic resting stages are derived from the rapid response of planktonic stages to unfavorable environmental conditions and are part of the life cycle of species. They are known to be powerful tracers of habitat changes because of their good preservation in sediment (Ellegaard *et al*., 2013a). In particular, phytoplankton resting stages have been widely used to reconstruct past environmental changes in temperature (de Vernal *et al*., 2005; Durantou *et al*., 2012; Weckström *et al*., 2006), salinity (Laird *et al*., 1996; Verleye *et al*., 2009; Mertens *et al*., 2012), productivity (Mertens *et al*., 2009; Pospelova *et al*., 2015), and eutrophication (Ellegaard *et al*., 2006; Zonneveld *et al*., 2012). Cultures derived from revived phytoplankton resting stages have been used to investigate the impact of environmental changes on the physiological performance of species (*e.g.* the dinoflagellate *Pentapharsodinium dalei*) in Koljö Fjord, Sweden (Ribeiro *et al*., 2013), or the genetic structure and diversity of populations of diatoms (*Skeletonema marinoi)* revived from sediments of Mariager Fjord, Denmark (Härnström *et al*., 2011).

The analysis of ancient DNA (aDNA) recovered from marine sediments is a complementary approach to the study of other biological remains in sediments that shows great potential. Several recent studies have demonstrated that plankton aDNA can be recovered from lacustrine and marine sediments, from the Holocene to the Pleistocene (Coolen and Overmann 2007; Boere *et al*., 2011) and up to the Miocene (Panieri *et al*., 2010). Most of the DNA stored in marine sediments is believed to be extracellular, as it is protected from nuclease degradation by adsorbing to mineral and organic matrices (Pietramellara *et al*., 2009), but DNA may also originate from resting stages (Godhe *et al*., 2002; Erdner *et al*., 2010). Whatever its origin, this aDNA could be exploitable to study plankton ecological patterns over relatively long-term periods.

The long-term dynamics of marine protist species are usually deduced from multiannual time series, generally obtained from regular spatial-temporal coastal surveys carried out within the framework of monitoring network activities (*e.g.* Shuler *et al*., 2012; Hernández-Fariñas *et al*., 2014). Long-term series generally focus on potentially toxic species, such as *Alexandrium* spp. (*e.g.* Touzet *et al*., 2011; Abdenadher *et al*., 2012; Martin *et al*., 2014; Anderson *et al*., 2014; Bazzoni *et al*., 2015). However, most of these historical datasets are relatively short, covering only a few decades (<50 years), and

are compromised by the limitations of identifying plankton species in routine optical microscopic analyses. Early information on past communities and species before the implementation of monitoring networks is therefore lacking. Paleoecological data could be an alternative to fill the gaps in the historical information about long-term protist community trends. Previous paleoecological analyses have shown multidecadal shifts and trends for diatom (Lundholm *et al*., 2010) and dinoflagellate species (Ribeiro *et al*., 2011), as well as for plankton communities (Mousing *et al*., 2013) using fossilized remains. aDNA has also been used to describe plankton succession dynamics (*e.g.* Bissett *et al*., 2005; Epp *et al*., 2010; Theroux *et al*., 2010; Stoof-Leichsenring *et al*., 2012, and Lejzerowicz *et al*., 2013 in old sediment (up to 32 000 years)) and to investigate the response of paleo-planktonic communities to past environmental changes (Coolen *et al*., 2013; Hou *et al*., 2014).

Alexandrium minutum is a cyst-forming dinoflagellate (Alveolata), responsible for several Harmful Algal Blooms (HAB) associated with toxin production causing Paralytic Shellfish Poisoning (PSP). Since its first detection in Alexandria harbor, Egypt (Halim 1960), this species has been detected in several areas: southern Australia (Hallegraeff *et al*., 1988), Ireland (Gross 1989), northern France (Belin 1993), the Mediterranean (Honsell 1993), Spain (Franco *et al*., 1994), the North Sea (Nehring 1998; Elbrachter 1999; Hansen *et al*., 2003), Taiwan (Hwang *et al*., 2000), New Zealand (Chang *et al*., 1999), Sweden (Persson *et al*., 2000), India (Godhe *et al*., 2001) and Malaysia (Usup *et al*., 2002). *Alexandrium minutum* is officially listed as an invasive species in Europe (http://www.europe-aliens.org) and is considered cryptogenic as its geographical origin is unknown (Gómez 2008; Katsanevakis *et al*., 2014). Phylogeographic analyses by Lilly *et al*., (2005) and McCauley *et al*., (2009) have shown that *A. minutum* populations can be clearly differentiated into two major clades: the global clade (containing samples from Europe and Australia) and the Pacific clade (containing samples from New Zealand and Taiwan). Yet, it remains quite challenging to determine whether recent *A. minutum* populations were introduced through natural or human-assisted pathways. In the Bay of Brest (Brittany, France), *A. minutum* has been detected since 1990 by the monitoring network REPHY (*REseau de surveillance et d'observation du PHYtoplankton et des PHYcotoxine*s) (http://envlit.ifremer.fr/surveillance/phytoplancton_phycotoxines/presentation) but its abundance was rather low (<2000 cells L^{-1}) until recent years when it increased significantly in the area. In July 2012, it reached a record concentration of around 42 million cells L^{-1}. This exceptional bloom induced a cascading effect: toxin accumulation in shellfish, closure of aquaculture activities and economic losses, leading to the Bay of Brest being classified as a new high-risk zone for toxic blooms of *A. minutum* (Chapelle *et al*., 2015).

The objective of this work was to contribute to studies on marine microbial paleoecology and long-term dynamics of HAB dinoflagellate species by collecting historical information on *A. minutum* in the

Bay of Brest. More specifically, cyst germination experiments were used to detect the viability of the species in ancient sediments and a newly-developed real-time PCR (Polymerase Chain Reaction) assay on aDNA was employed to try to reconstruct the multidecadal dynamic of the species in the area. In order to evaluate the specificity of the *A. minutum* ecological pattern, paleogenetic data obtained for *Scrippsiella donghaienis* were analyzed, as an example of a different, non-toxic, cyst-forming dinoflagellate species of this area. In parallel, plankton-monitoring data collected since 1990 in the Bay of Brest were analyzed in order to verify whether the dynamic of *A. minutum* in the water column were consistent with the results obtained by paleogenetic data during the overlapping period of the two data series.

2. Materials and methods

2.1. Study sites and phytoplankton monitoring

The Elorn and Daoulas estuaries are located in the Bay of Brest (Brittany, North Atlantic, north-west coast of France). They are both situated near areas of intensive agriculture, domestic waste and industrial discharges, which all contribute to the substantial nutrient supply of these estuaries. Since 1950, coastal ecosystems of the bay have experienced a large increase in their nutrient supply caused by the development of anthropogenic activities. More recently, the ban on washing powders containing orthophosphates has resulted in a decrease in the phosphorous supply leading to a significant imbalance in the N/P ratio (Guillaud and Bouriel 2007). This environmental change has been associated with variations in phytoplankton communities over time, such as an increase in phytoplankton biomass and in the intensity and frequency of HABs (Guillaud and Bouriel 2007). The REPHY monitoring network has been recording occurrences of phytoplankton species in French coastal ecosystems since 1984. In the Bay of Brest, monitoring is carried out at three sampling stations: *Le Passage* in the Elorn River estuary, from 1990 to 2004 and then from 2012 to date, *Lanveoc*, from 1993 to date, and *Daoulas*, which became the third spot point in the area after the impressive toxic bloom event of *A. minutum* in July 2012 (42 x 10^6 cells L^{-1}) (Figure 1). This last station is monitored during the summer period only (May-August), which corresponds to the highest productivity period when toxic blooms are most likely to occur. Fortnightly analyses of lugol-fixed samples are carried out using light microscopy. The estimations of *A. minutum* abundance are performed at a specific level, sometimes only during the occurrence of species blooms or high-risk periods. The abundance of non-toxic species is not monitored regularly at all stations and sometimes not at the species level. This is the case for the taxon *Scrippsiella* spp., which, in the case of REPHY monitoring, can include species of other dinoflagellate genera (*Ensiculifera*, *Pentapharsodinium*, *Bysmatrum*) that cannot be distinguished in routine light microscopy analyses by phytoplankton

observers. In this study, only samples with non-zero values corresponding to abundances ≥ 100 cells L^{-1}, which is the threshold abundance limit of species detection in the REPHY phytoplankton analysis procedures, were considered and analyzed.

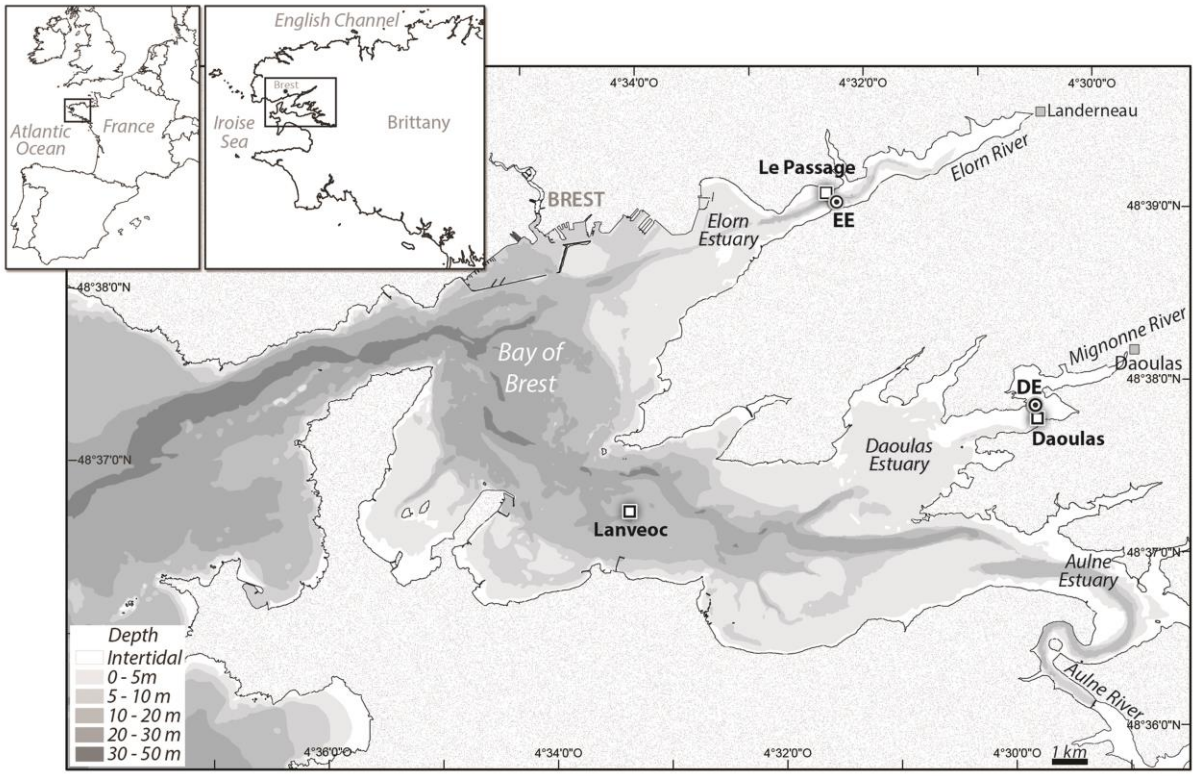


Figure 26. Map of the Bay of Brest with the core sampling sites (Elorn Estuary: EE; Daoulas Estuary: DE), and the monitored plankton stations (*Le Passage*, *Daoulas*, *Lanveoc*).

2.2. Core sampling and processing

Sediment cores were collected on 11th December 2012 at station EE of the Elorn River Estuary (48°23'46.79''N 4°23'2.01''W), and on 21st May 2014 at station DE (48°20'46.6''N 4°17'41.20''W) of the Daoulas Estuary (Figure 26). The sampling sites were selected on the basis of their suitable environmental characteristics for paleoecological analysis (calm hydrodynamics favorable for regular sedimentation (Rawlence *et al*., 2014)) and their proximity to the REPHY stations. Three cores were collected at station EE for genetic (31 cm) germination (25 cm) and dating (29 cm) analyses, respectively. Two cores were collected at DE, one (58 cm) for genetic and dating analyses and the other (59 cm) for cyst germination experiments. Visual inspection revealed no differences in the structure of the cores from the same sampling site. The EE cores were collected by divers at 12 m depth using hand-driven transparent Plexiglas tubes, 9.5 cm in diameter and 60 cm in length. The DE cores were collected at 3 m depth with a corer released from the boat. Immediately after sampling,

the sediment cores were delicately extruded from the Plexiglas tubes and then sliced into 1-cm layers. Sterile new equipment was used to slice each layer sample. For DNA and cyst germination analyses, to avoid contamination by smearing between the layers during the core extraction, only the inner part of each slice was sampled, using sterile 6-cm-diameter Petri dishes. The sediment samples for genetic analyses (about 10 g) were preserved in plastic 50-mL cryotubes. The tubes were frozen in liquid nitrogen and then stored in a -80°C freezer until further analyses. The sediment samples for cyst germination were collected in 50-mL tubes and stored in the dark at 4°C. Finally, sediment aliquots for dating were stored in plastic bags until radionuclide analyses.

2.3. ^{210}Pb and ^{137}Cs dating

The chronological framework of the sediment cores was determined based on $^{210}Pb_{xs}$ and ^{137}Cs as previously performed in Schmidt *et al*., (2007) and Andresen *et al*., (2014). ^{210}Pb ($T_{1/2}$ = 22.3 years) is a naturally occurring radionuclide delivered continuously to the landscape by atmospheric fallouts (Saari *et al*., 2010). This atmospherically-derived ^{210}Pb, readily scavenged by sediment, is referred to as ^{210}Pb in excess ($^{210}Pb_{xs}$) of that found within particles due to the decay of its parent isotope, ^{226}Ra. On the contrary, ^{137}Cs ($T_{1/2}$ = 30 years) is an artificial radionuclide: its occurrence in the environment is primarily the result of the nuclear weapon test fallout in the early sixties and the Chernobyl accident in Europe in 1986 of well-known pulse inputs (Avşar *et al*., 2015). In the laboratory, water content was determined by weighing the sediment samples before and after freeze drying, and Dry Bulk Density (DBD) was subsequently calculated, assuming a mineral density of 2.65 g cm^{-3}. Following this procedure, ^{210}Pb, ^{226}Ra and ^{137}Cs activities were measured using a very low background, high-efficiency, well-shaped γdetector equipped with a Cryo-Cycle (CANBERRA, Meriden, USA) (Schmidt and de Deckker 2015). The γ detector was calibrated using certified reference materials from IAEA (RGU-1; RGTh; SOIL-6). Activities were expressed in mBq g^{-1} and errors were based on 1 SD counting statistics. Excess ^{210}Pb ($^{210}Pb_{xs}$) activities were calculated by subtracting the activity supported by its parent isotope, ^{226}Ra, from the total ^{210}Pb activity in the sediment.

2.4. Cyst germination experiments and strain identification

Cyst germination experiments were performed similarly to previous paleoecological studies on diatoms (Härnström *et al*., 2011) and dinoflagellates (Miyazono *et al*., 2012). Sediment samples (~5 cm^3) were added to filtered seawater and placed in an ultrasonic bath for 6 min to separate dinoflagellate cysts from inorganic particles. The sediments were sieved with filtered seawater through a 100-µm onto a 20-µm sieve to separate the sediment size fraction containing the majority

of dinoflagellate cysts. Different media (F/2, site-specific seawater, K, K/2) and temperature (16 and 18°C) conditions were tested to optimize cyst germination. Irradiance (60 µmol photons m^{-2} s^{-1}) and photoperiod (12 h:12 h) were kept constant during this optimization. Finally, the combination of 16°C and K medium (Keller *et al*., 1987) was retained. A few drops of the 20-100 µm fraction were distributed onto a 12-well plastic plate containing K medium (2 mL per well). The plates were placed in a culture room at 16°C, under an irradiance of 60 µmol photons m^{-2} s^{-1} and a light:dark cycle of 12 h:12 h. The plates were examined for vegetative cell germination once to three times per week using an inverted microscope (Zeiss Axiovert 135). *Alexandrium minutum* cells were identified by light microscopy, but we failed to establish monoclonal strains in culture. For *Scrippsiella* spp., clonal cultures were established from different sediment layers of both the EE and DE cores. A total of 65 strains of *Scrippsiella* spp. were finally obtained from 12 and 6 sediment layers of the EE and DE cores, respectively. The cultured strains were identified genetically. DNA was extracted from the strain cultures using the DNeasy plant mini kit (QIAGEN, Düsseldorf, Germany) following the manufacturer's instructions. The LSU rDNA region was amplified by PCR using dinoflagellate-specific primers (Ldino6, Ldino1) from Probert *et al*., (2014) and the following PCR program: 35 cycles composed of an initial denaturation at 95°C for 2 min, a denaturation at 95°C for 30 sec, a hybridization at 56°C for 30 sec, an elongation at 72°C for 1 min and a final elongation at 72°C for 5 min. After purification by the QIAquick PCR purification kit (QIAGEN, Düsseldorf, Germany), the PCR products were sent to GATC Biotech (http://www.gatcbiotech.com/en/products/sanger-services) for Sanger sequencing. All sequences were deposited in GenBank and 32 strain cultures (the remaining 33 strains were lost) are available at the Roscoff Culture Collection (RCC: http://roscoff-culture-collection.org/).

2.5. Genetic analyses of sediment samples

2.5.1. DNA extraction and quantification

Genetic analyses were carried out considering the specific precautions that should be taken when working with aDNA in order to avoid cross-contamination between samples and contamination with modern DNA (Gilbert *et al*., 2005). Total DNA was extracted from 5-7 g of sediment material from each layer of the EE (31 samples) and the DE (58 samples) cores, using the PowerMax soil isolation kit (Mobio Laboratories Inc., Carlsbad, California, USA), following the manufacturer's instructions. DNA extracts were immediately stored at -80°C. DNA samples were quantified by absorbance measurements using a Take3 trio microplate reader (BioTek, Winooski, Vermont, USA) on 3 µL of DNA extract, and sterile water was used as a blank. DNA quality was checked by the 260/280 nm ratio to ensure that no contamination by proteins or other components had occurred during DNA

extraction. For DNA concentration analyses, in order to determine whether a significant change had occurred in DNA concentrations and, if so, to estimate the change-point in DNA concentration across the sediment core layers, the Change-Point-Method (CPM) was applied on the total DNA concentration data along the DE and EE cores. This test is generally used to detect changes in the mean value in a data series (Taylor 2000).

2.5.2. Primer design and specificity

Specific primers for the amplification of *Alexandrium minutum* (synonym of *Alexandrium lusitanicum*) from genomic rDNA samples are available in the literature (Penna *et al*., 2010) but they generate longer amplicons (> 150 bp) than required (50-150 bp) for optimal real-time PCR efficiency. To optimize amplification success in aDNA, the use of short amplicons (ca. 100 bp) is recommended (Coolen *et al*., 2013). Therefore, new primer sets for specific real-time PCR targeting the ITS1 (Internal Transcribed Spacer 1) rDNA region were designed for the two target species in this study, *A. minutum* and *S. donghaienis. Scrippsiella donghaienis* was chosen as the second target species in the light of the successful germination analyses in old sediments and given its monophyletic separation in ITS topologies within the genus *Scrippsiella* (Gu *et al*., 2008), which enables a better specific primer design. Its presence in northern European waters (i.e. Sweden) (Gu *et al*., 2008) makes *S. donghaienis* an interesting model to compare with potential invasive species in the North Atlantic. A total of 176 ITS rDNA sequences of *A. minutum* and other *Alexandrium* species and a total of 59 ITS rDNA sequences of *S. donghaienis* and other *Scrippsiella* species were downloaded from GenBank and aligned using Bioedit software (version 7.0.9.0). The primer sets were designed to target a fragment of 100-110 bp (using Primer3 software (http://primer3.wi.mit.edu/, Rozen and Skaletsk 2000)) (Table 1). The primers were analyzed for self/hetero-complementarities and secondary structure using OligoAnalyzer 3.1 software (http://eu.idtdna.com/calc/analyzer). Primer specificity was checked 1) *in silico*, by visual inspection of an alignment of ITS sequences, 2) *in silico*, using the Primer Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and 3) in the laboratory, by conventional PCR using DNA from positive (*A. minutum* (3 strains) and *S. donghaienis* (14 strains)) and negative (9 dinoflagellate species for a total of 27 strains) controls (see Supplementary Tables 2.1-4). The PCR mixture was identical for the two species except for the concentrations of the reverse and forward primers: 0.3 µM for *A. minutum* and 0.5 µM for *S. donghaienis*, 1.25 u of GoTaq Polymerase, 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 1X of GoTaq Flexi buffer, 2 µL of DNA template and sterile water. The PCR conditions for *A. minutum* were as follows: an initial denaturation step at 95°C for 2 min, 35 cycles at 95°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec and a final elongation step at 72°C for 2 min. For *S. donghaienis*, the cycling program consisted of an initial denaturation step at 95°C for 5 min, 35 cycles

at 95°C for 1 sec, 61°C for 45 sec, 72°C for 1 min and a final elongation step at 72°C for 7 min. The PCR products were loaded onto 1.5% agarose gel to confirm the presence of fragments of the expected length (100 bp for *A. minutum* and 110 bp for *S. donghaienis*) and the absence of non-specific bands.

Table 1. Primer sets designed to target the ITS1 rDNA region of Alexandrium minutum and Scrippsiella donghaienis by the different PCR amplification methods.

Target species	Genetic region	PCR method	Primer sets	Primer sequences Forward (5'-3') Reverse (5'-3')	Amplicon size (bp)
Alexandrium minutum	ITS1	Real-time	Am_48F	TGAGCTGTGGTGGGGTTCC	100
			Am_148 R	GGTCATCAACACAGCAGCA	
Alexandrium minutum	ITS1	Semi-nested	Am_55F	TGGTGGGGTTCCTAGGCT	93
			Am_148 R	GGTCATCAACACAGCAGCA	
Scrippsiella donghaienis	ITS1	Real-time	SD_357F	TATTCTGGCAACACCTTCCAC	110
			SD_468R	AGATGCTTAGCAAGTTGAGCG	

2.5.3. ITS1 rDNA cloning and standard curve construction

ITS1 rDNA PCR products of *Alexandrium minutum* (strain RCC 1490) and *Scrippsiella donghaienis* (RCC 3047) were cloned separately into plasmids (pCR 4), then chemically transformed into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen, Cralsbad, CA, USA) following the manufacturer's instructions. Several clones were obtained but only one clone of each species was retained for analysis. Plasmid DNA was isolated using the NucleoSpin Plasmid/Plasmid (NoLid) (MACHERY-NAGEL, Düren, Germany) then linearized with a restriction enzyme "Not I". The plasmid was checked on a 1% agarose gel. Standard curves for real-time PCR (serially diluted plasmids containing the ITS1 rDNA fragment of *A. minutum* or *S. donghaienis*) were constructed from the linearized plasmid DNA previously isolated. Plasmid DNA concentration was measured using a Quant-iT PicoGreen dsDNA quantification kit (Invitrogen, Cralsbad, CA, USA) and an FLX 80 fluorescence reader (BioTek, Winooski, VT, USA) according to the manufacturer's instructions. Quantification was performed using lambda DNA standards provided in the kit ranging from 2.5 to 1000 pg μL^{-1}. Plasmid concentration (ng μL^{-1}) was converted into (copy number μL^{-1}) according to the following equation: Number of copies per microlitre = (DNA concentration (ng μL^{-1}) / [660 x (plasmid size + insert size) x 10^9 (ng mol^{-1})]) x 6.022 x 10^{23} (molecules). For each target species, a standard curve was constructed with 10-fold serial dilutions of the linearized plasmid containing the ITS1 rDNA sequence of the species. Standard curves ranged from 10^6 to 10 copies μL^{-1} for *A. minutum* and from 10^5 to 10 copies μL^{-1} for *S. donghaienis*.

2.5.4. Real-time PCR

Real-time PCR reactions were performed using the iTaq Universal SYBR Green supermix kit (Bio-Rad, Hercules, California, USA) in a final volume of 20 µL. For *A. minutum* detection, the reaction mixture was composed of 10 µL of SYBR Green supermix (1X) containing (dNTPs, iTaq DNA polymerase, $MgCl_2$, SYBR Green I), 0.3 µM of the forward primer (Am_48F), 0.2 µM of the reverse primer (Am_148R), sterile water and 2 µL of sample DNA template (variable concentration between 2.42 and 34.30 ng μL^{-1}). The experiments were conducted in 96-well plates containing the standard curve dilutions in duplicate, the target samples and the negative controls composed of water instead of DNA that were both analyzed in triplicate. The plates were loaded onto a Stratagene Mxpro3000P (Agilent Technologies, Santa Clara, CA, USA) thermal cycler with the following cycling conditions: 1 cycle at 95°C for 5 min followed by 40 cycles at 95°C for 5 sec and 62°C for 30 sec. A melting curve analysis was added at the end of each run to ensure specific *A. minutum* amplification. The optimal annealing temperature of 62°C was initially determined in conventional PCR. To determine the optimal primer concentration, 9 different combinations (forward/reverse), ranging from 0.1 µM to 0.3 µM, were tested on 3 dilutions of the standard curve (10^6, 10^3, 10 copies μL^{-1}). The primer combination that yielded the lowest threshold cycle value (Ct) and maximum real-time efficiency (Am_48F; 0.3 µM, Am_148R; 0.2 µM) was retained for further analysis. The real-time PCR mixture for *S. donghaienis* detection was identical, except for the primer concentrations; 0.2 µM for the forward and reverse primer (SD_357F /SD_468R). The annealing temperature was determined at 61°C. The cycling conditions were: 1 cycle at 95°C for 5 min followed by 40 cycles at 95°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec, and a final cycle at 95°C for 1 min followed by a melting curve analysis. The reaction efficiency was estimated by the equation $E= 10^{(1/b)}-1$, where b is the slope of the standard curve. DNA amplifications of target species were considered positive if more than one out of the three replicates tested per sample was positive. The Limit Of Quantification (LOQ) was set at 10 copies per real-time PCR well, which was the smallest quantity of linear plasmid DNA of the standard curves for each real-time PCR assay. Concentrations of *A. minutum* or *S. donghaienis* in each sediment layer were expressed (assuming a 100% DNA extraction efficiency) in terms of copy number per gram of wet sediment, using the following formula: Copy number g^{-1}= copy number μL^{-1} x DNA extraction volume (µL)/sediment wet weight (g).

2.5.5. Sanger sequencing of *Alexandrium minutum* amplicons from sediment layers

In order to confirm the presence of *A. minutum* in sediment layers for which the real-time PCR amplifications were below the LOQ, amplicons resulting from a semi-nested PCR amplification of the DNA extract of the corresponding sediment layers were sequenced. The semi-nested PCR assays

were carried out only for *A. minutum* as this was the major target species of our study. After the first amplification using designed primers for real-time PCR (Am_48F/Am_148R) (100 bp), 2 µL of the amplified material was used for the second PCR round performed with a new forward primer (Am_55F) and the same reverse primer (Am_148R) of the first PCR step (Table 1). Both PCR reaction mixtures were carried out in a final volume of 20 µL, using 2 µL of DNA template, 1X of GoTaq Flexi buffer, 2 mM of $MgCl_2$, 0.2 mM of dNTP, 0.5 mM of each primer, 1.25 u of GoTaq polymerase (Promega) and sterile water. The PCR were performed in a PeqStar (OZYME, Montigny-Le-Bretonneux, France) thermocycler with the following conditions: 1 cycle at 95°C for 2 min followed by 30 cycles at 95°C for 30 sec, 61°C for 30 sec, 72°C for 30 sec and a final cycle at 72°C for 2 min. PCR products were purified using the Nucleospin Gel PCR clean-up kit (MACHERY-NAGEL, Düren, Germany) following the manufacturer's instructions, checked on an agarose gel. This semi-nested PCR procedure yielded an amplicon of 93 bp and a sufficient *A. minutum* DNA concentration for Sanger sequencing. Amplification products were sent to GATC Biotech (http://www.gatcbiotech.com/en/products/sanger-services/, Germany) for sequencing. The resulting sequences were identified by BLAST (Basic Local Alignment Search Tool) and aligned in Bioedit with other *A. minutum* ITS1 rDNA sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2).

Table 2. *Alexandrium minutum* semi-nested PCR sequences obtained from DE and EE cores with their respective ITS1 rDNA semi-nested sequences.

Species	Core ID	Sediment layers (cm)	Sediment layer dating	Semi-nested sequences (5'-3')
A. minutum	DE	24	1972 ± 2	ATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	25	1970 ± 2	GGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	31	1955 ± 3	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	33	1950 ± 3	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	41	1931 ± 4	ATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	42	1928 ± 4	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	53	1893 ± 5	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	54	1889 ± 5	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	57	1873 ± 6	GGCATGGCTTGCTTCTGCAAGCGCTTTCATG
A. minutum	EE	11	1991 ± 1	CATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	13	1986 ± 1	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	17	1978 ± 2	GCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	25	1958 ± 2	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	31	1939 ± 2	GGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT

2.5.6. Statistical analyses on data series

In order to depict trends in the abundance of target species, the moving averages of real-time PCR data (copy number g^{-1} sediment) were calculated. This method is usually used to calculate long-term trends or cycles from time series data, smoothing out short-term fluctuations in a dataset, and has been used in paleoecological studies (*e.g.* Yu and Berglund 2007). The moving average of the *A. minutum* and *S. donghaienis* genetic data series (copy number g^{-1} sediment) was calculated with a three-sample interval, meaning that an averaged value of the copy number g^{-1} was calculated for every three consecutive data (*e.g.* average of layers (1,2,3), average of layers: (2,3,4), etc.). These averaged values were used to produce the curves in Figure 3 representing the trend of the quantitative genetic data obtained for our studied species in the sediment samples. In addition, the Mann-Kendall trend test was performed on the real-time PCR data (copy number g^{-1} sediment) and the plankton data (cells L^{-1}) across the overlapping periods of the series (from 1989-2014) to detect statistically significant trends. The test was applied using the *Kendall* package in R software version 3.2.3 (R Core Team, 2015). The Mann-Kendall trend test is a non-parametric test for monotonic trends, which is typically used to detect whether there is a significant increasing or decreasing trend in a time series (Yue *et al*., 2002). In this test, the initial assumption is the null hypothesis (H0), which assumes that there is no trend in the data series over time. The alternative hypothesis (H_1) is that there is a significant trend (increasing or decreasing) over time. The significance level of the test was set at $\alpha = 0.05$. If the p value < 0.05, the H0 is rejected, meaning that there is a significant trend in the time series. Kendall's tau (τ) is a value between -1 and 1 that indicates the direction of the trend over time. An increasing trend is indicated by $\tau > 0$ and a decreasing one by $\tau < 0$.

3. Results

3.1. Sediment chronology

In the DE core, ^{210}Pb in excess activity ranged from 45 mBq g^{-1} in the uppermost sediment layers to <1 mBq g^{-1} at 58 cm (Figure 27). There was a general trend of an exponential decrease in $^{210}Pb_{xs}$ as expected due to the decay of the unsupported ^{210}Pb. The $^{210}Pb_{xs}$ profile in the EE core showed the same trend: due to the shorter length of the sediment core, levels in which $^{210}Pb_{xs}$ became negligible were not reached. In both cores, the $^{210}Pb_{xs}$ decrease presented some irregularities. This could be related to temporary changes in sedimentation intensity, which justified the use of the CSR (constant rate of supply) model for dating (Figure 27). The 31 cm-long EE core encompassed the last 72 years (from 1939 ± 2 to 2011 ± 1), whereas the DE core covered > 100 years (from 1866 ± 7 to 2013 ± 1). The sedimentary profile of ^{137}Cs in the two cores presented the expected shape with two peaks (nuclear weapon test fallout and Chernobyl) in the deepest layers. In the DE core, ^{137}Cs disappeared

rapidly to negligible levels below the deepest peak. The EE core was too short to evaluate ^{137}Cs disappearance; however, the expected peak of the second atmospheric fallout was observed. In general, the sedimentary ^{137}Cs peaks in the two cores mimicked the atmospheric fallout rather well, validating the ^{210}Pb chronology (Figure 27).

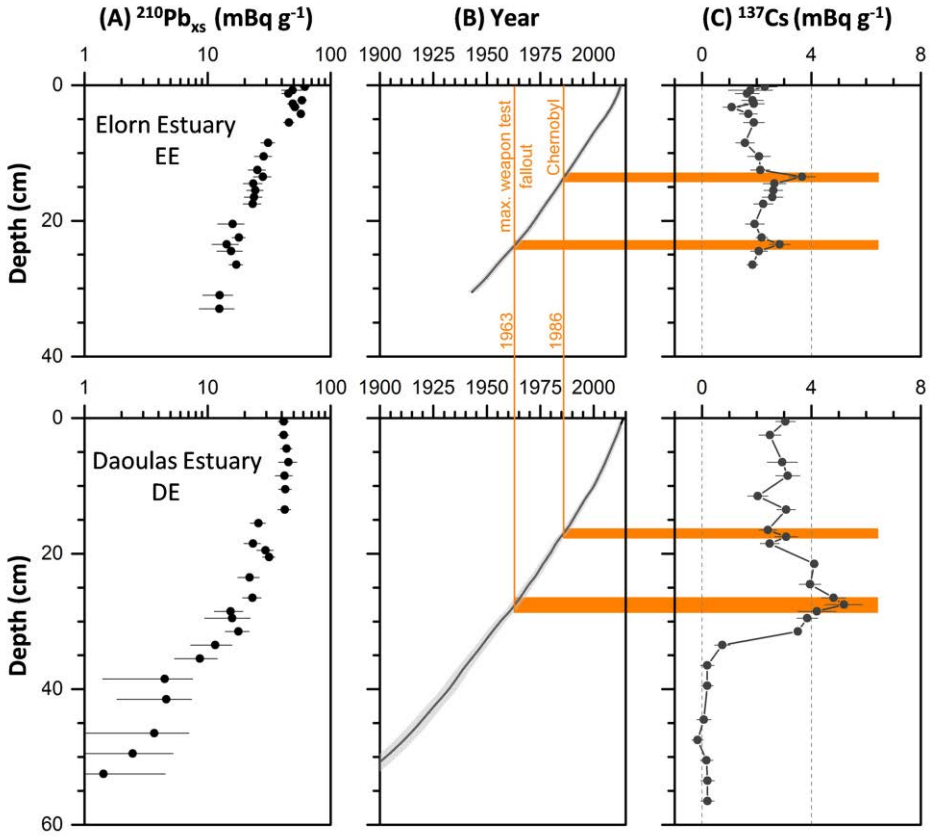


Figure 27. $^{210}Pb_{xs}$ profile (A), ages of sedimentary layers, based on CSR model (B) and ^{137}Cs profile (C) with depth along the cores in the Elorn (EE, upper panel) and Daoulas (DE, lower panel) estuaries.

3.2. Germination experiments

Successful germinations were observed in both EE and DE cores (Table 3). Germinated cells belonged primarily to dinoflagellates (up to 17 cm (1978 ± 2) for EE and 20 cm (1981 ± 2) for DE) and diatoms (up to 11 cm (1991 ± 1) for EE and 13 cm (1995 ± 2) for DE). For the EE core, *S. donghaienis* germinated predominantly along the core up to 17 cm (1978 ± 2) while cells of *A. minutum* were observed up to 7 cm (1999 ± 1). For DE, *S. donghaienis* cells were observed up to 20 cm (1981 ± 2) and *A. minutum* was detected up to 13 cm (1995 ± 2) (see dotted line in Figure 28). Germinated diatom species belonged to *Skeletonema* spp., *Chaetoceros* spp., *Thalassiosira* spp., *Nitzschia* spp., *Lithodesmium* spp., *Paralia* spp., and *Asteromphalus* spp. Other autotrophic (*Peridinium*

quinquecorne, *Gymnodinium* spp.) and heterotrophic (*Protoperidinium* spp.) dinoflagellates were recorded along the two cores.

Table 3. *Scrippsiella* spp. strains obtained from the Elorn Estuary (EE) and Daoulas Estuary (DE) dated sediment layers with their respective Genbank accession number and Roscoff culture collection (RCC) number.

Species	Strain ID	Core sediment layer (cm)	Sediment layer dating	GenBank accession number	RCC ID
S. donghaienis	IFR-SDO-Sc34	EE2	2010 ± 1	KX009607	4711
S. donghaienis	IFR-SDO-Sc35	EE2	2010 ± 1	KX009606	4712
S. donghaienis	IFR-SDO-Sc38	EE4	2006 ± 1	KX009603	4713
S. donghaienis	IFR-SDO-Sc39	EE4	2006 ± 1	KX009602	4714
S. donghaienis	IFR-SDO-Sc40	EE4	2006 ± 1	KX009601	-
S. donghaienis	IFR-SDO-Sc22	EE9	1995 ± 1	KX009619	-
S. donghaienis	IFR-SDO-Sc23	EE9	1995 ± 1	KX009618	-
S. donghaienis	IFR-SDO-Sc24	EE9	1995 ± 1	KX009617	4715
S. donghaienis	IFR-SDO-Sc25	EE9	1995 ± 1	KX009616	4716
S. donghaienis	IFR-SDO-Sc26	EE9	1995 ± 1	KX009615	-
S. donghaienis	IFR-SDO-Sc51	EE10	1993 ± 1	KX009592	4717
S. donghaienis	IFR-SDO-Sc53	EE10	1993 ± 1	KX009591	4718
S. donghaienis	IFR-SDO-Sc54	EE10	1993 ± 1	KX009590	-
S. donghaienis	IFR-SDO-Sc1	EE11	1991 ± 1	KX009637	-
S. donghaienis	IFR-SDO-Sc2	EE11	1991 ± 1	KX009636	4733
S. donghaienis	IFR-SDO-Sc3	EE11	1991 ± 1	KX009635	-
S. donghaienis	IFR-SDO-Sc4	EE11	1991 ± 1	KX009634	-
S. donghaienis	IFR-SDO-Sc5	EE11	1991 ± 1	KX009638	-
S. donghaienis	IFR-SDO-Sc6	EE11	1991 ± 1	KX009639	-
S. donghaienis	IFR-SDO-Sc7	EE11	1991 ± 1	KX009640	-
S. donghaienis	IFR-SDO-Sc55	EE12	1989 ± 2	KX009589	-
S. donghaienis	IFR-SDO-Sc57	EE12	1989 ± 2	KX009588	-
S. donghaienis	IFR-SDO-Sc8	EE13	1986 ± 2	KX009633	-
S. donghaienis	IFR-SDO-Sc9	EE13	1986 ± 2	KX009632	-
S. donghaienis	IFR-SDO-Sc10	EE13	1986 ± 2	KX009631	4719
S. donghaienis	IFR-SDO-Sc11	EE13	1986 ± 2	KX009630	-
S. donghaienis	IFR-SDO-Sc12	EE13	1986 ± 2	KX009629	-
S. donghaienis	IFR-SDO-Sc13	EE13	1986 ± 2	KX009628	4720
S. donghaienis	IFR-SDO-Sc14	EE13	1986 ± 2	KX009627	4721
S. donghaienis	IFR-SDO-Sc15	EE13	1986 ± 2	KX009626	4734
S. donghaienis	IFR-SDO-Sc16	EE13	1986 ± 2	KX009625	-
S. donghaienis	IFR-SDO-Sc17	EE13	1986 ± 2	KX009624	-
S. donghaienis	IFR-SDO-Sc18	EE13	1986 ± 2	KX009623	-
S. donghaienis	IFR-SDO-Sc19	EE13	1986 ± 2	KX009622	-
S. donghaienis	IFR-SDO-Sc20	EE13	1986 ± 2	KX009621	4722
S. donghaienis	IFR-SDO-Sc21	EE13	1986 ± 2	KX009620	-
S. donghaienis	IFR-SDO-Sc58	EE14	1984 ± 2	KX009587	4730
S. donghaienis	IFR-SDO-Sc59	EE14	1984 ± 2	KX009586	-
S. donghaienis	IFR-SDO-Sc60	EE14	1984 ± 2	KX009585	4731
S. donghaienis	IFR-SDO-Sc61	EE14	1984 ± 2	KX009584	-
S. donghaienis	IFR-SDO-Sc27	EE17	1978 ± 2	KX009614	4723
S. donghaienis	IFR-SDO-Sc28	EE17	1978 ± 2	KX009613	4724
S. donghaienis	IFR-SDO-Sc29	EE17	1978 ± 2	KX009612	4725
S. donghaienis	IFR-SDO-Sc30	EE17	1978 ± 2	KX009611	-
S. donghaienis	IFR-SDO-Sc31	EE17	1978 ± 2	KX009610	4726
S. trochoidea	IFR-STR-Sc36	EE4	2006 ± 1	KX009605	-

S. trochoidea	IFR-STR-Sc37	EE4	2006 ± 1	KX009604	4732
S. trochoidea	IFR-STR-Sc41	EE4	2006 ± 1	KX009600	-
S. trochoidea	IFR-STR-Sc42	EE4	2006 ± 1	KX009599	4727
S. trochoidea	IFR-STR-Sc43	EE4	2006 ± 1	KX009598	-
S. trochoidea	IFR-STR-Sc45	EE4	2006 ± 1	KX009597	4728
S. trochoidea	IFR-STR-Sc32	EE5	2003 ± 1	KX009609	-
S. trochoidea	IFR-STR-Sc33	EE5	2003 ± 1	KX009608	-
S. trochoidea	IFR-STR-Sc46	EE6	2001 ± 1	KX009596	-
S. trochoidea	IFR-STR-Sc47	EE6	2001 ± 1	KX009595	-
S. trochoidea	IFR-STR-Sc48	EE8	1997 ± 1	KX009594	-
S. trochoidea	IFR-STR-Sc49	EE8	1997 ± 1	KX009593	4729
S. donghaienis	IFR-SDO-S5	DE17	1987 ± 2	KX009641	4735
S. donghaienis	IFR-SDO-S8	DE19	1982 ± 2	KX009642	4736
S. donghaienis	IFR-SDO-S7	DE20	1981 ± 2	KX009643	4737
S. trochoidea	IFR-STR-S2	DE10	2001 ± 1	KX009644	4738
S. trochoidea	IFR-STR-S3	DE13	1995 ± 2	KX009645	4739
S. trochoidea	IFR-STR-S9	DE15	1991 ± 2	KX009646	4740
S. trochoidea	IFR-STR-S4	DE17	1987 ± 2	KX009647	4741
S. trochoidea	IFR-STR-S6	DE17	1987 ± 2	KX009648	4742

3.3. Molecular analyses

Total DNA concentrations extracted from the sediments ranged from 2.42 to 34.30 ng μL^{-1} for EE and from 3.46 to 26.70 ng μL^{-1} for DE. According to the 260/280 nm ratios, the DNA extracts were of sufficient yield and purity to conduct amplification analyses. A decreasing trend in total DNA concentration was observed from the top to the bottom of both EE and DE cores (see Supplementary Figure 2.1), with higher concentrations found in the first sediment layers of both cores. At 9 cm (10.84 ng μL^{-1}) for EE and 11 cm (16.73 ng μL^{-1}) for DE, the CPM method identified the change in the DNA concentration slope, from a decreasing to a steady trend. For real-time PCR tests, good linear relationships were found between the threshold cycle (Ct) and the initial number of copies ($0.998 \geq R^2 \geq 0.999$). The reaction efficiencies ranged from 95 to 99% for *A. minutum* and from 91.4 to 95.3% for *S. donghaienis*. To ensure specific amplifications, the melting temperature values (Tm) were systematically checked by analyzing the melting curves. For the *A. minutum* assay, the standard curve samples as well as the positive samples showed a specific peak at Tm = 83.05 ± 0.5°C (see Supplementary Figure 2.2). In the case of *S. donghaienis*, the melting curve peak was observed at Tm = 82.9 ± 0.5°C. Copy numbers <LOQ (= 10 copies/well) for both *A. minutum* and *S. donghaienis* were observed in some deep sediment layers. In these cases, the target species were considered present in the core layers, but it was not possible to quantify their ITS rDNA copy numbers. The presence of *A. minutum* in deep layers (indicated with star symbols in Figure 28) was confirmed by the sequencing of semi-nested PCR products. Nine and five sequences of 31-45 bp were obtained for DE

and EE core layers, respectively. The sequences were aligned with other available sequences (Supplementary Figure 2.3) and identified as *A. minutum* at 100% identity (Table 2).

3.4. Multidecadal dynamics of *Alexandrium minutum* and *Scrippsiella donghaienis* detected in sediments

Accurate DNA quantification of *A. minutum* ITS1 rDNA copies g^{-1} sediment was obtained from the 10-cm layer (1993 ± 1) to the 1-cm layer (2011 ± 1) of the EE core and from the 22-cm layer (1976 ± 2) to the 1-cm layer (2013 ± 1) of the DE core (Figure 28), covering a total period of about 40 years. For both EE and DE, in the layers from which quantitative data were obtained, an increasing trend in the concentration of *A. minutum* ITS1 rDNA copies g^{-1} sediment was observed from the bottom to the top layers. This trend was visualized by the moving average calculation (Figure 28) and statistically confirmed over the period 1989-2014 by the Mann-Kendall test analyses (DE: $\tau = 0.65$, p value = 0.0005; EE: $\tau = 0.911$, p value = 0.0003). In particular, at EE, *A. minutum* ITS1 rDNA copies g^{-1} sediment increased from the 10-cm layer (1993 ± 1; 2.72 x 10^4 copies g^{-1} sediment) to reach a peak at the 1-cm layer (2011 ± 1; 4.96 x 10^7 copies g^{-1} sediment). At DE, ITS1 rDNA copy numbers were higher than at EE. They increased from the 22-cm layer (1976 ± 2; 2.49 x 10^4 copies g^{-1} sediment), peaking at the 1-cm layer (2013 ± 1; 6.73 x 10^7 copies g^{-1}). ITS1 rDNA copies g^{-1} were slightly correlated with total DNA concentrations in both cores (EE: $R^2 = 0.51$; DE: $R^2 = 0.55$), which were higher in the top than in the bottom layers (see Supplementary Figure 2.1). In layers from which non-quantitative data were obtained, the presence of *A. minutum* was detected discontinuously, being present along the cores in 5 out of 21 layers and in 9 out of 35 layers for EE and DE, respectively (star symbols in Figure 28). These rDNA traces of *A. minutum* were detected even in the deep sediment layers of both analyzed cores, corresponding to 1939 ± 2 (31 cm) for EE and 1873 ± 6 for DE (57 cm) (Figure 28).

For *S. donghaienis,* quantification of ITS1 rDNA copies g^{-1} of sediment was obtained from the 27-cm layer (1952 ± 2) to the 1-cm layer (2011 ± 1) for the EE core, and from the 21-cm layer (1979 ± 2) to the 1-cm layer (2013 ± 1) for the DE core (Figure 28). The pattern of abundance of ITS rDNA copies observed for *S. donghaienis* differed from that of *A. minutum* and varied across the sites studied (Figure 28). At EE, a bimodal pattern of copy number abundance was evident. From the deepest quantifiable 27-cm layer (1952 ± 2; 5.1 x 10^4 copies g^{-1} sediment), the first peak of abundance was evident at the 11-cm layer (1991 ± 1; 4.4 x 10^5 copies g^{-1} sediment) and a second peak corresponding to the highest copy number observed, was found in the top 3 cm (peak of 1.2 x 10^6 copies g^{-1} sediment at 1 cm (2011 ± 1)). At DE, spikes of abundance were observed at 21 cm (1979 ± 2; 1.1 x 10^5 copies g^{-1} sediment), 13 cm (1995 ± 2; 9.13 x 10^4 copies g^{-1} sediment) and 9 cm (2003 ± 1; 1.24 x 10^5 copies g^{-1} sediment). From the 9-cm layer to the top layer of the DE core, a decrease in copy number

abundance was observed (Figure 28). Over the period 1989-2014, the Mann-Kendall test showed a significant increasing trend in EE (τ = 0.601, p value = 0.0001), but no significant trend was detected in DE (τ = -0.295, p value = 0.0650). The pattern of copies g^{-1} sediment abundance was slightly correlated with total DNA abundance at EE (R^2 = 0.53), but not at DE (R^2 = 0.01). *Scrippsiella donghaienis* was detected up to the bottom layers of both the EE and DE cores (1939 ± 2 and 1866 ± 7, respectively). In the layers for which non-quantitative data were obtained, the presence of *S. donghaienis* was detected in 4 out of 4 layers for the EE core and in 16 out of 37 layers for the DE core (Figure 28).

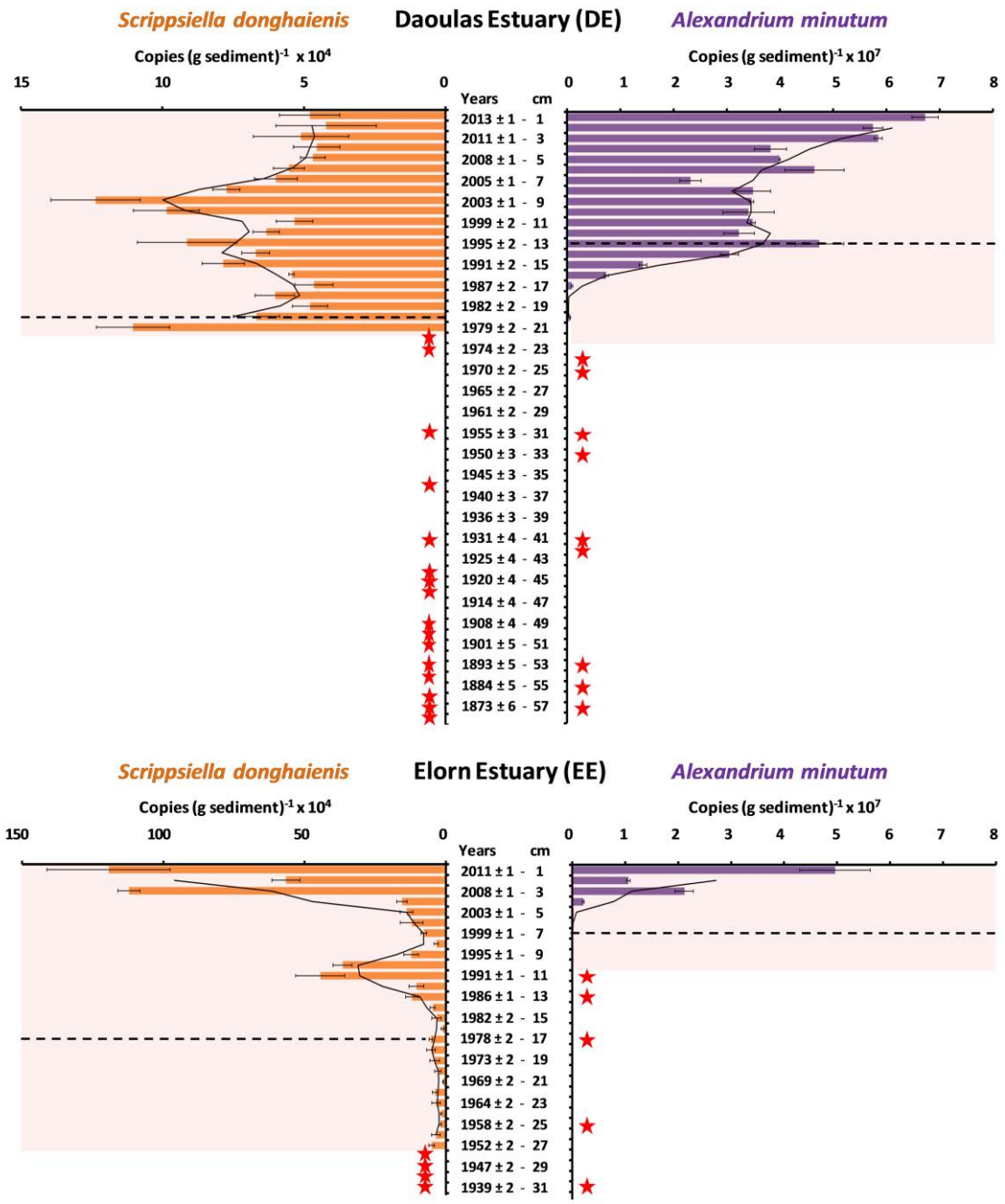


Figure 28. ITS1 rDNA copies g^{-1} sediment for *Scrippsiella donghaienis* (orange) and *Alexandrium minutum* (purple) estimated by real-time PCR in Daoulas (DE) and Elorn (EE) estuaries. Colored shadows indicate the layers for which reliable ITS1 rDNA quantifications were obtained. Stars indicate the layers where the presence of the species was detected but quantitative data were below the limit of quantification of our real-time PCR assays. The dashed lines indicate the limit layer of respective species germination. The black curves represent calculated moving average on the quantitative data.

3.5. Multidecadal dynamics of *Alexandrium minutum* and *Scrippsiella donghaienis* in the water column (plankton data)

At the *Lanveoc* station, *A. minutum* concentrations were < 7000 cells L^{-1} until 2012, reaching a peak in July 2014 (32 800 cells L^{-1}). At the *Le Passage* station, regular monitoring activities started when *A. minutum* was first observed in the Bay of Brest in 1990 (<100 cells L^{-1}). From 1992 to 1996, no *A. minutum* cells were detected at the station. From 1997, this species was regularly observed until 2003, but always at low concentrations (<2000 cells L^{-1}). Monitoring in the area stopped in 2003 and was re-established in 2012 when a bloom of 560 000 cells L^{-1} occurred on 11th July 2012. No bloom was observed in the area in 2013 but in 2014, *A. minutum* concentrations reached 199 000 cells L^{-1} on 7th July. The 2012 bloom at *Le Passage* coincided with a bloom at the *Daoulas* station (42 x 10^6 cells L^{-1} on 11th July 2012), which represents the highest concentration ever recorded in the bay. Blooms of lower intensity occurred in the following years (293 333 cells L^{-1} in 2013 and 1 052 000 cells L^{-1} in 2014) (see Supplementary Figure 2.4). A statistically significant increasing trend in *A. minutum* concentrations was depicted by our longer plankton time series at *Le Passage* and *Lanveoc* in the Bay of Brest by the Mann-Kendall test (*Lanveoc*: $\tau = 0.247$, p value = 0.0001; *Le Passage*: $\tau = 0.281$, p value = 0.0050). The *Daoulas* time series was too short (3 years) to analyze a multiannual trend, thus the Mann-Kendall test was not applied. When qualitatively compared over the overlapping period of analyses, the long-term data series of *A. minutum* obtained from our paleoecological approach (ITS1 rDNA copies g^{-1} sediment) and from the monitoring activity (plankton data Log(cells L^{-1}) of *Lanveoc* and *Le Passage* stations nicely showed a coherent increasing trend in species abundance from 1990 until 2014 (Figure 29). For *Scrippsiella* spp., monitoring was carried out uninterruptedly from 1987 until 2000 at *Le Passage,* and from 1995 to 2014 at *Lanveoc.* No monitoring of this taxon has ever been performed at the *Daoulas* station. Lower concentrations of *Scrippsiella* spp. were recorded at *Lanveoc* compared to *Le Passage*. At *Lanveoc*, *Scrippsiella* spp. abundance remained below 8300 cells L^{-1} until 1998, when it reached 42 000 cells L^{-1}. At *Le Passage*, *Scrippsiella* spp. abundance reached a maximum of 760 000 cells L^{-1} in 1999. Lower abundances were registered during the following years (see Supplementary Figure 2.4). At both stations, no trend was detected by the Mann-Kendall coefficient for this taxon (*Lanveoc*: $\tau = -0.02$, p value = 0.5297; *Le Passage*: $\tau = -0.0125$, p value = 0.6773).

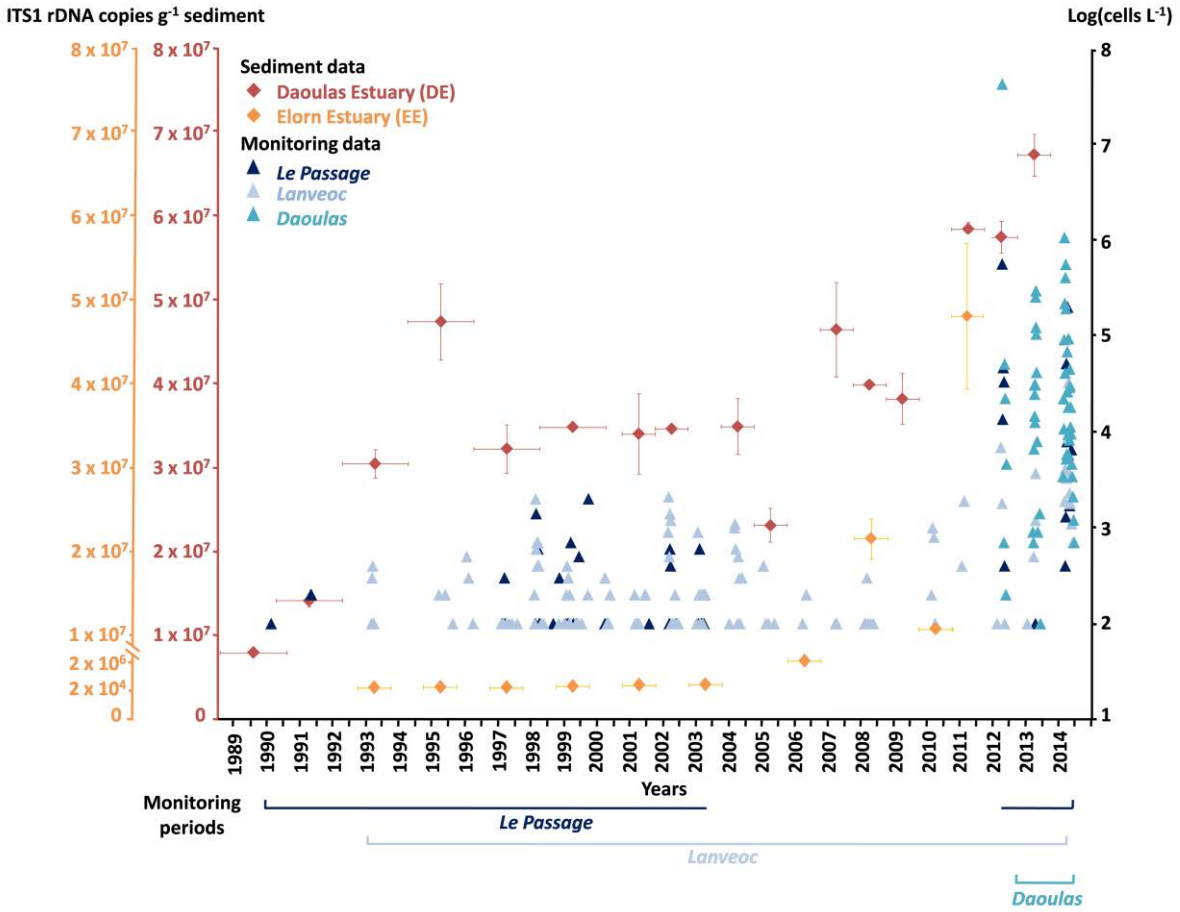


Figure 29. Comparison of paleogenetic and plankton data (REPHY) for *Alexandrium minutum* in the Bay of Brest for the overlapping period of the time series (1989-2014). Copy numbers of ITS1 rDNA g^{-1} sediment of the DE (red diamonds; red scale) and EE (orange diamonds; orange scale) data series are plotted with their standard deviation error bars (vertical bars). Horizontal bars represent error on sediment dating. All cell concentration data ≥ 100 cells L^{-1} gathered at the three monitoring stations (*Le Passage*, *Lanveoc*, *Daoulas*) are expressed in Log(cells L^{-1}) (triangles with different blue tones) during the monitored periods (horizontal colored lines).

4. Discussion

The paleoecological approach used in this study, based on paleogenetic analyses and ancient cyst revivification, provided historical data on two target dinoflagellate species over a time scale of about 150 years. The focus was mainly on the bloom-forming, toxic species *A. minutum* and then paleogenetic data obtained for the non-toxic species *S. donghaienis* were used to compare specific ecological patterns. Both species were detected along dated sediment cores, even in deep sediment layers (dating back to 1873 ± 6 for *A. minutum* and 1866 ± 7 for *S. donghaienis*). Their aDNA concentrations were quantified specifically from 1952 ± 2 or 1976 ± 2 (depending on the species and the cores) using a robust, newly-developed real-time PCR assay. Moreover, up to 31-34 year-old resting cysts were successfully revived from core sediments.

4.1. Real-time PCR and cyst germination

Real-time PCR is a quantitative technique known to be highly sensitive, enabling species abundances to be estimated accurately in different environments even when the DNA target gene copy number is low. The advantage of real-time PCR is the fast processing of a large number of samples compared to cell or cyst counting microscopic procedures, which are time-consuming and often biased by taxonomic identification problems. Several studies have used real-time PCR to quantify the abundance of a large number of toxic phytoplankton species in seawater samples: (*Karenia brevis* (Gray *et al*., 2003); *A. minutum* (Touzet *et al*., 2009; Galluzzi *et al*., 2004); *Alexandrium catenella* (Garneau *et al*., 2011); *Ostreopsis* cf. *ovata* (Perini *et al*., 2011); *Pseudo-nitzschia* spp. (Fitzpatrick *et al*., 2010); and different diatoms and dinoflagellates (Godhe *et al*., 2008; Murray *et al*., 2011), as well as in superficial sediment samples (Kamikawa *et al*., 2007; Erdner *et al*., 2010).

Real-time PCR was the most appropriate method for our paleogenetic approach, which aimed to detect ancient genetic traces of the presence and abundance of the target species in the Bay of Brest. Yet, when quantifying genetic material in old sediments, DNA degradation is a major issue to be taken into account. Taphonomic processes that may occur in old sediments and the variable preservation conditions of organic matter occurring from one ecosystem to another may influence DNA conservation and thus its quantitative estimation (Boere *et al*., 2011). DNA degradation mostly occurs in very old sediments, such as those that are of thousand years old, and can cause DNA to be fragmented, which can significantly reduce the efficiency of PCR amplification (d'Abbadie *et al*., 2007). The real-time PCR protocol applied in this study was based on a compromise between i) the best discriminating genetic marker (ITS1 rDNA) for *Scrippsiella* spp. (Gu *et al*., 2008) and *Alexandrium* spp. (John *et al*., 2014), ii) the appropriate length of DNA fragment for real-time PCR assays (100-150 bp) (Arya *et al*., 2005), and iii) the recommended analysis of short DNA fragments (~100-500 bp) in paleogenetic studies (Coolen *et al*., 2013). As the DNA fragments in our samples were relatively recent (about 150 years old) and short (about 100 bp), the degradation and fragmentation issues should theoretically have been minimized. However, given the decreasing DNA concentrations revealed along both sampled cores, a potential degradation of the aDNA analyzed cannot be excluded. The real-time PCR amplifications in sediment did not target DNA exclusively from cysts or cells, therefore the abundance estimations (copies g^{-1} sediment) cannot be extrapolated in terms of copies per cyst or copies per cell. Furthermore, there is a high inter-and intra-species variability in the rDNA copy number of dinoflagellates. Few studies have estimated the rDNA copy number for protists such as diatoms (61-36 896 copies $cell^{-1}$ of 18S rDNA) and dinoflagellates (1057-12 812 copies $cell^{-1}$ of 18S rDNA) (*e.g.* Godhe *et al*., (2008)), and the only copy number estimation for *A. minutum* (1084 ± 120.3 copies $cell^{-1}$) targeted the 5.8S rDNA region (Galluzi *et al*., 2004) and not the ITS1 rDNA region.

In fact, it is not certain that the 5.8S and the ITS1 rDNA copy number remained locally relatively constant over time.

Germination of *A. minutum* and *S. donghaienis* varied between sediment cores. Although real-time PCR analyses revealed the presence of the target species in the oldest sediments, no cyst germination was observed in sediment older than 1981 ± 2 (DE core, 20-cm layer) and 1978 ± 2 (EE core, 17-cm layer). The longest cyst survival time observed in our study was 31-34 years for *S. donghaienis* (EE core) and 17-19 years for *A. minutum* (DE core). Earlier studies have reported the viability of different dinoflagellate and diatom resting stages after variable periods of burial in sediments ranging from months to several decades (Lewis *et al*., 1999; McQuoid *et al*., 2002; Mizushima and Matsuoka 2004; Ellegaard *et al*., 2013b), and up to 100 years (Härnström *et al*., 2011; Lundholm *et al*., 2011; Ribeiro *et al*., 2011; Miyazono *et al*., 2012). Assuming that the cyst mandatory dormancy period had passed in our samples, we consider that the unsuccessful germination in old sediment was more likely to be related to cyst viability. Over time, in old sediments, resting stages can undergo diagenetic processes and their survival depends on many physical and chemical conditions in the sediment including oxygen levels, temperature, and pH (Kremp and Anderson 2000). The reconstruction of preservation conditions in old sediments over time is very difficult and it is possible that some physical-chemical processes might have prevented cyst survival in these cores. In addition, although different germination conditions (different media and temperature conditions) were tested and a final optimal combination (temperature, nutrient concentration) was found and systematically applied to all samples, it cannot be excluded that optimal germination conditions vary across sediments of different ages. In fact, *S. donghaienis* and *A. minutum* are able to germinate over a wide range of environmental conditions (Blanco *et al*., 2009; Gu *et al*., 2008).

Interestingly, germination of *A. minutum* and *S. donghaienis* was successful only for layers in which aDNA quantification was possible. It can be questioned whether DNA was amplified and quantified mainly from living stocks, represented by resting cysts, from extracellular genetic material or from both. Most DNA in marine sediments has been shown to be extracellular (Dell'Anno and Danovaro 2005) and well preserved from microbial nuclease degradation (Corinaldesi *et al*., 2011), but DNA inside the resting stages of some plankton species is generally thought to be better preserved than extracellular DNA (Boere *et al*., 2011). Real-time PCR data do not distinguish between extracellular and cyst-preserved genetic material, thus a definitive conclusion regarding the type of DNA quantified in this study is not possible. RNA analysis might have clarified whether active or inactive communities were identified, as explored in Capo *et al*., (2015) where RNA transcripts were found only in the top 2 cm of lacustrine sediments and RNA/DNA analyses showed that genetic material in sediments mainly represented inactive communities. However, RNA analyses and quantitative

analyses on cysts were beyond the scope of this study. Our main goal was to obtain historical information about the presence, abundance and viability of *A. minutum* and *S. donghaienis* in order to depict multidecadal ecological patterns.

4.2. Historical records of *Alexandrium minutum* and *Scrippsiella donghaienis* and multiannual dynamics

By means of this paleogenetic approach, the first traces of genetic material of *A. minutum* were detected in the Bay of Brest. Starting from two different cores, it could be inferred that the species was present in the bay considerably earlier than its first description in the Mediterranean Sea in 1960 (Halim 1960), as the first detection of the species in the DE sediment core of the Bay of Brest dated back to 1873 ± 6. Similarly, using cyst records in dated sediments, Ribeiro *et al*., (2011) established that the dinoflagellate *Gymnodinium catenatum* had been present in the Western Iberian Peninsula since 1889 ± 10, before its first description documented in 1939 on planktonic viable material. Our data also suggest that the presence of *S. donghaienis* in the Bay of Brest dated back to 1866 ± 7, but its formal description is relatively recent (Gu *et al*., 2008) and only a few historical and biogeographical data are available, as the species has only been reported in Australia, Sweden and East China (Gu *et al*., 2008). In fact, *A. minutum* and *S. donghaienis* could have been present in the bay even before 1873 ± 6 and 1866 ± 7, respectively. The DE core was not sufficiently long to highlight the absence of the genetic signal along a consistent number of old sediment layers. Regarding the analyzed cores, the presence of both species was detected irregularly across layers from which data below the limit of quantification were obtained. This could indicate some contamination during the sampling process across the different layers. Yet, our sampling protocol was designed to avoid direct contamination during sampling, both across different layers and with modern DNA (using a clean device for each layer) and along the plastic tube core (collecting only the inner part of each sediment layer). In addition, molecular manipulations in the laboratories were performed following previously developed precautions for paleogenetic analyses to avoid cross-contamination between samples (Anderson-Carpenter *et al*., 2011; Boere *et al*., 2011; Domaizon *et al*., 2013; Gilbert *et al*., 2005). Moreover, similar results were obtained from two different cores, collected and analyzed at two different periods of time. Therefore, we believe that the irregular presence of both species across the two cores is most probably due to the presence of a very small amount of specific genetic material rather than to sample contamination.

In order to validate the multiannual study of species dynamics, the relative species variations in quantitative real-time PCR estimations were compared to those of the plankton time series obtained from monitoring analyses during the overlapping periods of the data series. Previously, real-time PCR

has rarely been applied to ancient DNA to compare long-term plankton series and only restrictively to quantify cyanobacteria dynamics in freshwater sediments (Domaizon *et al*., 2013; Martínez de la Escalera *et al*., 2014; Pal *et al*., 2015). To the best of our knowledge, this is the first paleogenetic study carried out in monitored estuarine ecosystems addressing such a comparison between real-time PCR and plankton monitoring data. Interestingly, the statistically significant increase in concentrations of ITS1 rDNA copy number of *A. minutum* depicted from 25 year-old sediment samples of two different cores corroborates the 24 years of plankton observations carried out at different monitoring stations in the Bay of Brest. When compared qualitatively over the overlapping periods (1989-2014), both the paleogenetic and plankton time data series nicely showed an increasing trend in the concentration of the species *A. minutum* over time (Figure 29). This trend is particularly evident, and statistically confirmed, from the plankton data of the *Lanveoc* and *Le Passage* stations, which have been followed over the long term, and is also proved by the fact that huge blooms of the species have only recently been observed and monitored in the third station of the bay (*Daoulas*) (Chapelle *et al*., 2015). Conversely, *S. donghaienis* ITS1 rDNA abundances did not show a coherent pattern and trend between the analyzed cores, suggesting a non-homogenous temporal pattern in the Bay of Brest for this species. The increasing concentration trend depicted for *S. donghaienis* from paleogenetic data of the EE core was not confirmed by the DE core data, unlike for *A. minutum* for which both the EE and DE paleogenetic data series showed an increase in species concentration. The comparison with plankton data was not possible since *S. donghaienis* can only be identified genetically (Gu *et al*., 2008) and monitoring data are acquired by optical microscopy analyses, which can only identify genera. Yet, *Scrippsiella* spp. data do not show an increase in the abundance of the taxon and no major bloom of any species of the genus has been registered in the Bay of Brest to date.

Along the Brittany coasts, *A. minutum* has been sporadically observed since 1985, at the very beginning of the REPHY monitoring activities. Today, its ecological success is evident in some areas. For instance, in the Penzé estuary (Bay of Morlaix, north Brittany) this species has produced blooms at more than 10^4 cells L^{-1} every year for the last 27 years (Dia *et al*., 2014). On the north-western Brittany coast (Aber-Wrac'h), blooms are regularly observed, and in the Bay of Brest, high concentrations of the species have been reported since 2012. Our results show that *A. minutum* was present in the Bay of Brest much earlier than its first detection by REPHY monitoring. On the basis of these results, we can put forward the hypothesis that *A. minutum* had a long residence period before blooming in the area. The introduction of the species, if any, may have occurred earlier than our first detection of it (1873 ± 6). Future paleoecological reconstruction is required to answer this question

over a longer time scale. Furthermore, it would be interesting to compare the dynamic pattern of *A. minutum* with other dinoflagellate species (see Annexe 4).

Our results demonstrate that the species *A. minutum* has increased greatly in the Bay of Brest in recent years. This could be due to the introduction of a new population, potentially genetically different from original populations, or the natural evolution of the local populations, or the adaptation of the local populations to environmental changes (natural or anthropogenic). In fact, local ecosystem-specific constraints have contributed to shaping the population dynamic of the species *A. minutum* in a shorter period of time in relatively close ecosystems (Dia *et al*., 2014). Since our study was performed only at the species level and not at the population level, it is not possible to evaluate to what extent population introduction, adaptation and evolution contributed to the process of *A. minutum* development in the Bay of Brest. Future paleoecological population-based studies could address the issue of species development over a multidecadal time scale at the intraspecific level, contributing to a better understanding of long-term microbial species phenologies in the marine environment.

5. Conclusions

This study innovatively associated old-cyst revivification, paleogenetic and plankton-monitoring data to reconstruct the long-term dynamics of two target estuarine dinoflagellate species, *A. minutum* and *S. donghaienis*. Cyst survival time varied between the target species (31-34 years for *S. donghaienis* and 17-19 years for *A. minutum*) and, interestingly, was correlated with their quantifiable genetic material. This finding contributes to the debate about the proportions of intracellular, cyst-protected and extracellular DNA amplifiable from sediments. The paleogenetic data enabled the multidecadal dynamics of the two target species to be studied over a time scale of about 150 years, with quantitative data obtained for about the last 40 years. The most ancient genetic traces found in these two dated core samples suggest that *S. donghaienis* and *A. minutum* have been present in the Bay of Brest since at least 1866 ± 7 and 1873 ± 6, respectively. Paleogenetic data clearly showed that *A. minutum* has increased in concentration in recent years, corroborating plankton data over the overlapping period of the two different data series (1989-2014). The dynamic of *A. minutum* will be compared to those of other biological communities, such as terrestrial flora (using palynological proxies) in a multiproxy approach in order to explore the potential link between biological variables changes and climate change (see Annexe 5). This study contributes to the development of paleoecological research, showing that this discipline, mostly developed in lacustrine ecosystems to

date, can be applied to marine, estuarine ecosystems, providing new perspectives for future research on the biogeography, ecology and evolution of marine microbes.

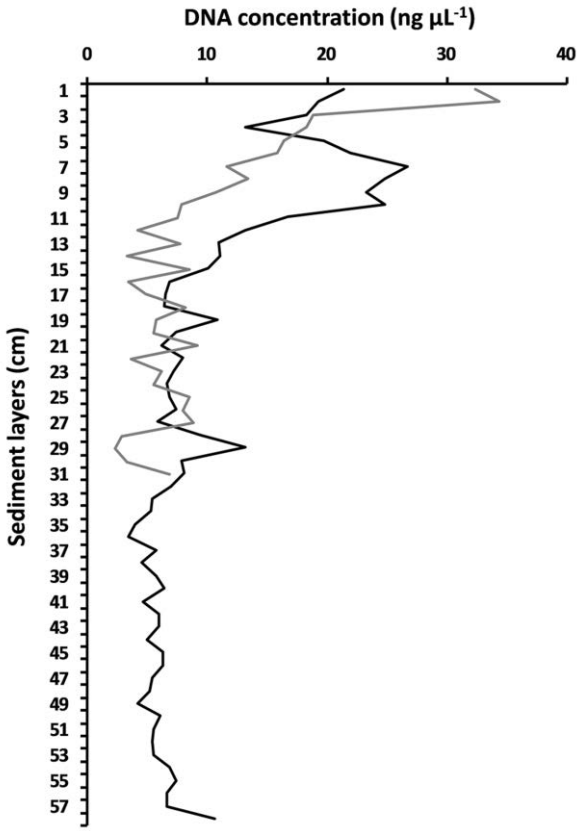
6. Funding

This work was financed by the exploratory projects VEHSPA (2012-2013) and KYNDER (2014-2015), supported by the "*Laboratoire d'Excellence*" LabexMER (*Agence Nationale de la Recherche* (ANR) -10-LABX-19) and co-funded by the French government under the program "*Investissements d'Avenir*", by the projects of the initiative *ECosphere Continentale et CÔtière* (EC2CO) of the *Institut National des Sciences de l'Univers*/*Centre National de la Recherche Scientifique* (INSU/CNRS): PALMITO (2013-2015), and CA'MOMI (2015-2017) and the ANR HAPAR project (2014-2019). This research was carried out within the framework of K. Klouch's PhD and funded by Ifremer and *Region Bretagne* (Allocation de REcherche Doctorale (ARED) fellowship).

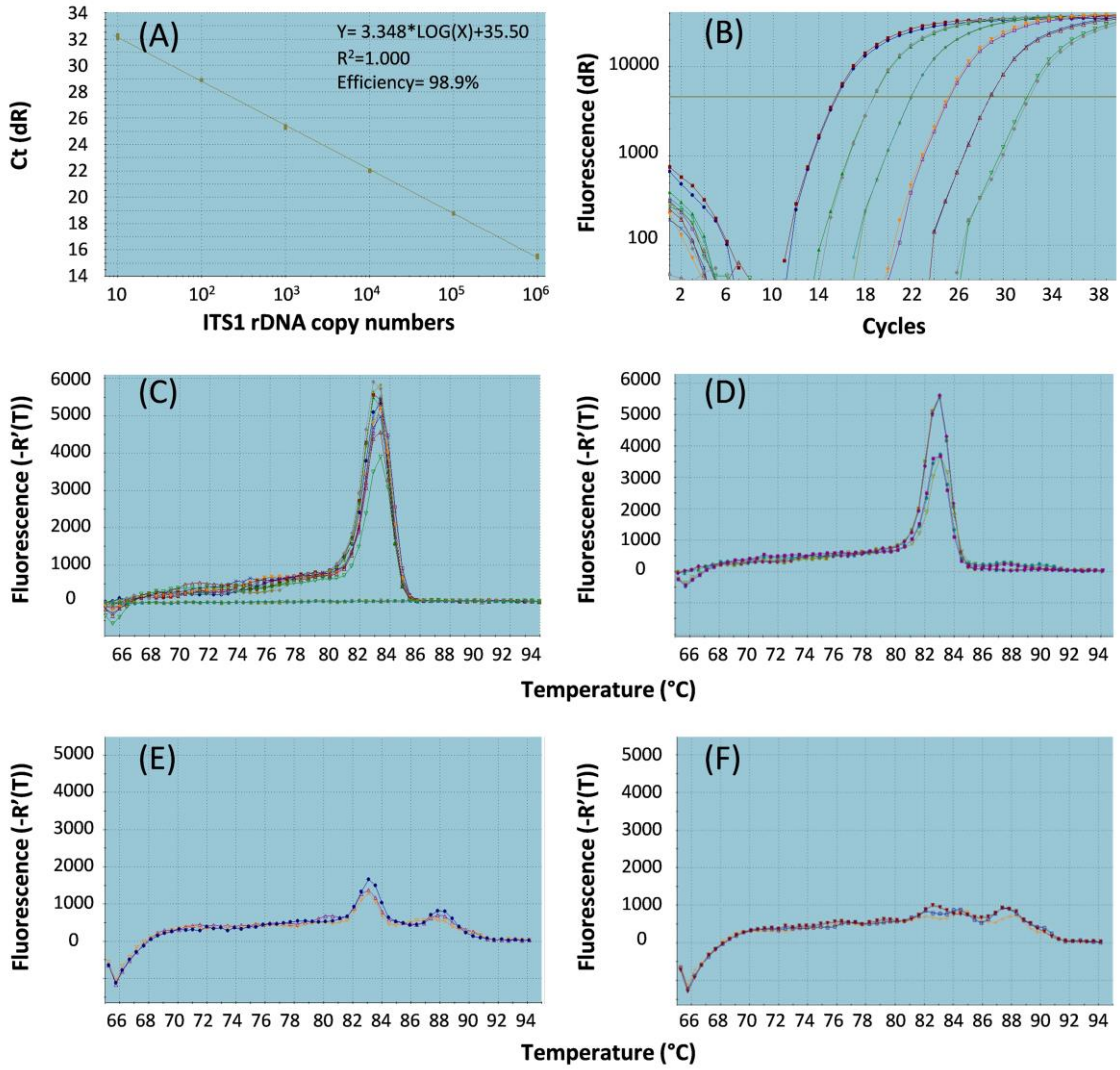
7. Acknowledgements

The authors wish to thank Xavier Caisey and the other scuba divers from Ifremer for sampling the EE core, and Gwendoline Gregoire and Axel Ehrhold from the laboratory GM/LES of Ifremer for allowing the sampling of the DE in the framework of the project SERABEQ ("Laboratoire d'Excellence" LabexMER (ANR-10-LABX-19)). Pierre Bodenes and Tania Hernández-Fariñas are acknowledged for collaborating in the picture developments and Pascale Malestroit for helping with cyst germination and culture maintenance. Pierre Ramond is acknowledged for helping with statistical tests. Finally, we wish to thank the members of the SG2M/LSEM laboratory of Ifremer for access to their laboratory facilities and for all their technical suggestions. This research falls within the scope of the French GDR (*Groupement de Recherche*) Phycotox (http://www.phycotox.fr) (2012-2018) on harmful microalgae and phycotoxins.

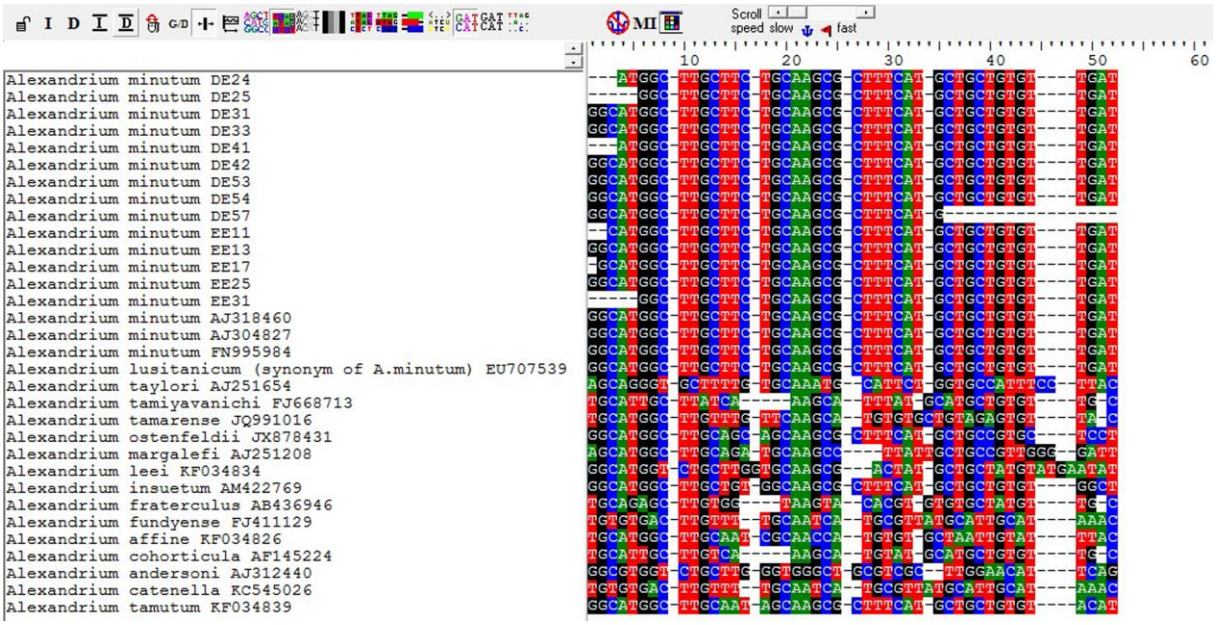
8. Supplementary data



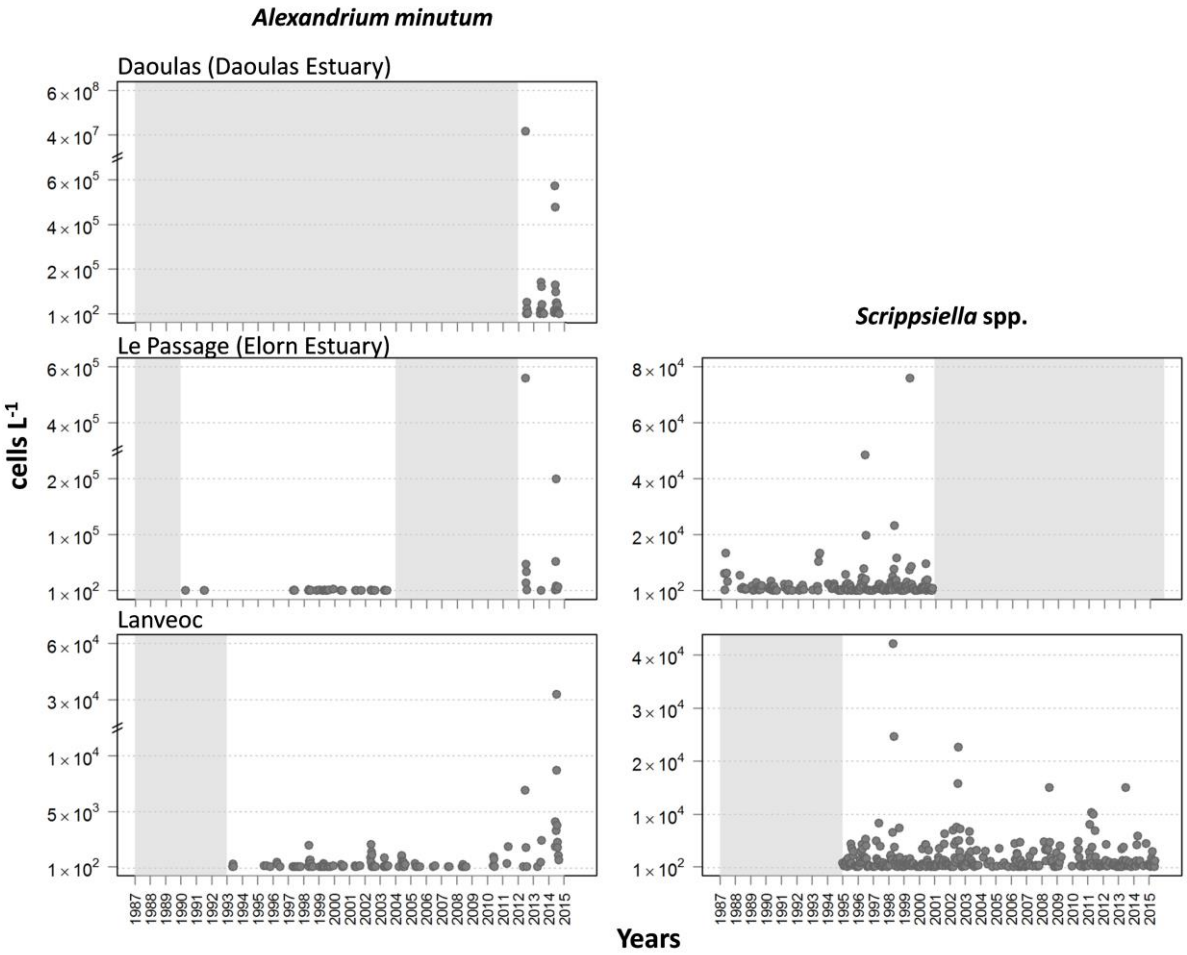
Supplementary Figure 2.1. Total DNA concentration (ng µL^{-1}) from the sediment samples of the Elorn (EE, grey) and Daoulas (DE, black) estuaries.



Supplementary Figure 2.2. Examples of real-time PCR output data for *Alexandrium minutum*. (A) Standard curve constructed from diluted plasmid containing the ITS1 rDNA fragment of *A. minutum.* (B) Amplification plots for standards. Melting curves for: (C) standards and negative control (dark green dotted line), (D) positive and quantifiable amplifications (sediment layer: Daoulas Estuary (DE 4 and DE15)), (E) positive amplification (sediment layer: DE33) for which the quantification was not possible (copies numbers < the Limit of Quantification (= 10 copies)), (F) negative amplification (sediment layer: DE47).



Supplementary Figure 2.3. Screenshot of semi-nested PCR sequences alignment (edited in Bioedit 7.0.9.0) with 3 sequences of *A. minutum* and 15 sequences of other *Alexandrium* species retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank) with their respective accession numbers. The sequences were obtained from the Daoulas Estuary (DE) and Elorn Esutary (EE) cores for sediment layers (numbers = cm depth) indicated with a star symbol in Figure 28.



Supplementary Figure 2.4. Abundances (≥100 cells L^{-1}) of *A. minutum* and *Scrippsiella* spp. in the water column at the three REPHY monitoring stations (*Daoulas, Le Passage, Lanveoc*) of the Bay of Brest. Grey areas indicate the non-monitored periods.

Supplementary Table 2.1. *In silico* specificity of primers used for *Alexandrium minutum* amplification by real-time PCR.

Species	Primer Forward (3'-5')	Primer Reverse (5'-3')
A. minutum[a]	T---GAGCTGTGGTGGGGTTCC	TGCTGCTGTGTTGATGACC
A. lusitanicum[b] (synonym of *A. minutum*)	*---******************	******************
A. taylori[c]	*---A*******A*TCCTGGTT	TGG**CCA*TTCC*TACG*
A. tamiyavanichi[d]	*GG---****CT*CAA*C**G*	TGTATGCA*******T*G*
A. tamarense[e]	*TCT-*ATGA*AT**T**GCAA	T****-*******CA***T
A. ostenfeldii[f]	*---G*GCTG*GG*****T*CC	TGC**CC*****CCCAA**
A. margalefi[g]	G---G*G*TG*GG***T*C*TT	TGC**CC**TGG*GAT*GG
A. leei[h]	A---C*GCTT*GC**---A*T*	TGC**C*A**TGTGAA**T
A. insuetum[i]	*---G*GCTG*GG*****T*CC	TGC**C****TG*CTGA**
A. fraterculus[j]	---TG**CT**G*G*--*C*GT	CACGTG******A**T*G*
A. fundyense[k]	-*ATG**ATGC**G*CAACAGC	TGGCAA**ACAG***ACGG
A. affine[l]	A**GAT**TG*GGGC**T*GCT	C****-******A-AT*GT
A. cohorticula[m]	**ATG***T*C**G*CAAC*GC	TGGCAA**ACAG***ACGG
A. andersoni[n]	*---G*GCT*AGC******GCT	C-T**GAACAT*CA*AACT
A. catenella[o]	*GCT-**********CG-	T****-C**TA**CAT*G*
A. tamutum[p]	*---G*GCTG*GG-****T*CC	TGC**C****TACATGA**

GenBank accession numbers

[a]EU707495, EU707511, EU707595, EU707513, EU707521, EU707523, EU707525, EU707549
[b] EU707539, DQ176667, FJ823507, AY455826
[c]AJ251654, AM296010
[d]AF145224, FJ668713, KP063148
[e]AJ514908, AJ514907
[f] AM238649, AB753843,FJ011435
[g]AJ251208, KJ907372, JQ616827
[h]KF034834
[i]AM422769, JF521630, AB006996
[j]AF20824422, KJ907373, KF034832
[k]JF521647, JF521645
[l]AB565485
[m]AF113935
[n]AJ3112440, HE574399
[o]AJ272120, FM211461, KF924270
[p]KF034839, AM238452

Supplementary Table 2.2. *In silico* specificity of primers used for *Scrippsiella donghaienis* amplification by real-time PCR.

Species	Primer Forward (3'-5')	Primer Reverse (5'-3')
S. donghaeinis[a]	TATTCTGGCAACAC-CTTCCAC	CGCTCAACTTGCTAAGCATCT
S. trochoidea(STR1)[b]	*****C********-*******	***G*******T***A*****
S. trochoidea(STR2)[c]	*****CA*******-*******	***ATT******K**A*****
S. trochoidea(STR3)[d]	*C***CA***R**T-*******	*A*G****K*K****A*G**K
S. lachrymosa[e]	C****C*******TG*CC***T	***A**********
S. rotunda[f]	C**********-**C****	*A*G**********A*****

GenBank accession numbers

[a] AY685008, AY499533, AY676151, AY788357, AY676155, PARALEX147, PARALEX161, PARALEX478, SBR103
[b] HQ658160, AY499524, EU325959, AY676152, AY676157, AY676160, AY676158, AF527093, EU325957
[c] AF527109, AF527069, AY676162, AY676159, AY788358, AY676154, AY499530, RCC1720, PARALEX669
[d] AY676146, AF527074, EU325958, AY499531, AF527079, AF527101
[e] AY788354, AY676150, SBR6
[f] EU325951, EU325952, EU360590, AY788355

Supplementary Table 2.3. Specificity of *Alexandrium minutum* and *Scrippsiella donghaienis* primers controlled by conventional PCR amplification on different dinoflagellate species/strains.

Species/strain	***A. minutum***	***S. donghaienis***
Alexandrium minutum 1097	yes	-
Alexandrium minutum 417	yes	-
Alexandrium minutum 1034	yes	-
Alexandrium tamutum 238	no	-
Alexandrium tamutum 555	no	-
Alexandrium tamarense 187	no	-
Heterocapsa triquetra 31	no	-
Heterocapsa triquetra 61	no	-
Heterocapsa triquetra 382	no	-
Gymnodinium instriatum 400	no	-
Gymnodinium instriatum 1076	no	-
Prorocentrum micans 1081	no	-
Gonyaulax spinifera 1079	no	-
Pentapharsodinium dalei IFR-PDA-Sc44	no	-
Scrippsiella trochoidea (clade STR1) ST147 RCC3047	no	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc36	no	no
Scrippsiella donghaienis IFR-SDO-Sc38	no	yes
Scrippsiella donghaienis SD112	-	yes
Scrippsiella donghaienis SD168	-	yes
Scrippsiella donghaienis SD269	-	yes
Scrippsiella donghaienis SD347	-	yes
Scrippsiella donghaienis IFR-SDO-Sc1	-	yes
Scrippsiella donghaienis IFR-SDO-Sc7	-	yes
Scrippsiella donghaienis IFR-SDO-Sc14	-	yes
Scrippsiella donghaienis IFR-SDO-Sc20	-	yes
Scrippsiella donghaienis IFR-SDO-Sc25	-	yes
Scrippsiella donghaienis IFR-SDO-Sc27	-	yes
Scrippsiella donghaienis IFR-SDO-Sc31	-	yes
Scrippsiella donghaienis IFR-SDO-Sc34	-	yes
Scrippsiella donghaienis IFR-SDO-Sc35	-	yes
Scrippsiella trochoidea (clade STR1) ST161	-	no
Scrippsiella trochoidea (clade STR1) ST156	-	no
Scrippsiella trochoidea (clade STR1) ST478	-	no
Scrippsiella trochoidea (clade STR2) ST653	-	no
Scrippsiella trochoidea (clade STR2) ST659	-	no
Scrippsiella trochoidea (clade STR2) ST110	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc37	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc41	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc43	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc45	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc46	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc47	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc48	-	no
Scrippsiella trochoidea (clade STR2) IFR-STR-Sc49	-	no

CHAPTER 3

Heterogeneous distribution in sediments and dispersal in waters of *Alexandrium minutum* in a semi-enclosed ecosystem

Context of the work

Resting cysts of planktonic species accumulate in the sediment bottom and constitutes the so called "seedbed" or "cyst bank". The amount of time spent in dormancy is determined by several factors, both internal (mandatory dormancy period, endogenous annual clock) and external (temperature, light, oxygen) (Anderson *et al.,* 2014). The germination of resting cyst could occur if they are transported to oxic conditions in the sediment surface or overlying water via physical processes or bioturbation. The cyst banks are more to be found in semi-enclosed ecosystems, such as estuaries, lagoons and harbours (Anderson *et al.,* 2012) and their location was found to be related to the areas of bloom initiation (Genovesi *et al.,* 2009; Ní Rathaille and Raine 2011). Therefore, surveys investigating the distribution and abundance of cysts can be very useful to assess the risk of bloom initiation of harmful microalgae and to take management measures regarding to coastal economic activities.

In the estuarine ecosystems of the Bay of Brest, blooms of *Alexandrium minutum* have been recorded only recently. Indeed, after the intensive toxic bloom of *A. minutum* of July 2012 in Daoulas Estuary (42 million cells L^{-1}), several areas of the Bay of Brest are monitored on a regular basis by the REPHY monitoring network which reports on the *A. minutum* abundance in the water column, blooms and toxicity events. However, little is known about the species cyst distribution in sediments of the Bay of Brest.

In this work, I tried to assess the actual spatial distribution of *A. minutum* in the Bay of Brest and to identify the areas of high accumulation of *Alexandrium minutum* DNA, corresponding potentially to high cyst abundance. The real-time PCR assay developed in the chapter 2 was applied on DNA extract from superficial sediments of 30 stations, to quantify the species abundance (ITS1 rDNA copies), corresponding to potential cyst banks in the area. The presence of viable was confirmed by germination experiments. Using model simulations, the trajectories of passive particles released from different estuaries of the bay were analyzed in order to assess the dispersal potential of *A. minutum* cells.

Author contributions

In this study, I participated to the sediment sampling and performed the genetic analyses (DNA extractions, quantifications, real time PCR analyses) for both 2013 and 2015 data sets. J. Quéré performed cysts germination analyses for the 2013 samples. F. Caradec and S. Schmitt performed sediment analyses (Granulometry, chlorophyll *a*, pheopigment and organic carbon) and provided with the corresponding data. T. Hérnandez-Fariñas performed the statistical analyses and provided

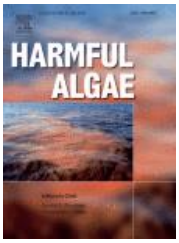
with the corresponding figure; she also participated to the design and interpretation of other figures of this work. L. Pineau-Guillou provided the script for the lagrangian model and the first model simulations. M. Plus optimized and performed the model, he contributed to the data interpretation and provided with the final model outputs and with the corresponding figures and tables. A. Chapelle helped for the lagrangian model interpretations and managed the project Daoulex to which this work contributed. Sediment sampling was performed by almost all manuscript participants. I contributed to the total dataset analyses all along the manuscript elaboration, discussing with different co-authors. I have written parts of the Material and Methods and Result sections. L. Guillou contributed to data interpretation and manuscript elaboration. R. Siano was the research designer and the main writer of the manuscript.

Heterogeneous distribution in sediments and dispersal in waters of *Alexandrium minutum* in a semi-enclosed ecosystem.

Résumé (en français)

Dans le cadre des recherches visant à utiliser des méthodes génétiques pour évaluer la distribution des espèces d'algues nuisibles (HABs) et leur impact sur les écosystèmes côtiers, nous avons amplifié par PCR en temps réel, un fragment de l'ITS1 ADNr d'*Alexandrium minutum* à partir d'extraits d'ADN de sédiments superficiels (1-3 cm) de 30 stations subtidales et intertidales de la Baie de Brest (Bretagne, France), pendant les périodes hivernales de 2013 et 2015. Les amplifications de l'ADN d'*Alexandrium minutum* et les germinations de kystes ont été obtenus pour les sédiments de toutes les stations échantillonnées, ce qui démontre que l'ensemble de la baie est actuellement contaminée par cette espèce toxique. Des estimations précises du nombre de copies d'ITS1 ADNr ont été obtenues pour les deux campagnes d'échantillonnage, soutenant l'hypothèse d'une accumulation régulière du matériel génétique de l'espèce toxique dans la partie sud-est, plus confinées de la zone d'étude, où les sédiments fins et vaseux sont également plus abondants. Des nombres élevés de copies d'ITS1 ADNr ont été détectés dans les sédiments des zones où les efflorescences ont été observées saisonnièrement depuis 2012. Ce résultat suggère que les estimations de matériel génétique spécifique dans les sédiments superficiels de la baie peuvent être un proxy des banques à kyste d'A*. minutum*. La simulation de la trajectoire des particules analysée par un modèle physique lagrangien a montré que les efflorescences qui se produisent dans la partie sud-est de la baie sont déconnectés de ceux de la zone nord-est. La distribution hétérogène d'*A. minutum* déduite de l'eau et des sédiments suggère l'existence de barrières physiques potentielles pour la dispersion de cette espèce dans la Baie de Brest et encourage une analyse plus fine au niveau de la population de cette espèce dans les écosystèmes côtiers semi-fermés.

Article submitted to Harmful Algae Journal



Heterogeneous distribution in sediments and dispersal in waters of *Alexandrium minutum* in a semi-enclosed coastal ecosystem

Klouch K.Z[a,b], Caradec F.[a], Plus M.[a], Hernández-Fariñas T.[a,b], Pineau-Guillou L.[c], Chapelle A.[a], Schmitt S.[a], Quéré J.[a], Guillou L.[b], Siano R.[a]

[a] Ifremer - Centre de Brest, DYNECO PELAGOS, F-29280 Plouzané, France
[b] Sorbonne Universités, UPMC Univ. Paris 6, CNRS, Adaptation et Diversité en Milieu Marin (UMR 7144), équipe DIPO, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff, France
[c] Ifremer - Centre de Brest, LOPS OC, F-29280 Plouzané, France

Abstract

Within the framework of research aimed at using genetic methods to evaluate harmful species distribution and their impact on coastal ecosystems, we amplified by real-time PCR a portion of the ITS1rDNA of *Alexandrium minutum* from DNA extracts of superficial (1-3 cm) sediments of 30 subtidal and intertidal stations of the Bay of Brest (Brittany, France), during the winters of 2013 and 2015. *Alexandrium minutum* rDNA amplifications and cell germinations were obtained for sediments of all sampled stations, demonstrating that the whole bay is currently contaminated by this toxic species. Coherent estimations of ITS1 rDNA copy numbers were obtained for the two sampling cruises, supporting the hypothesis of regular accumulation of genetic material of the toxic species in the south-eastern, more confined embayments of the study area, where fine-muddy sediments are also more abundant. Higher ITS1 rDNA copy numbers were detected in sediments of areas where blooms have been seasonally detected since 2012. This result suggests that specific genetic material estimations in superficial sediments of the bay may be a proxy of the cyst banks of *A. minutum*. The simulation of particle trajectory analyses by a lagrangian physical model showed that blooms occurring in the south-eastern part of the bay are disconnected from those of the north-eastern zone. The heterogeneous distribution of *A. minutum* inferred from both water and sediment suggests the existence of potential barriers for the dispersal of this species in the Bay of Brest and encourages finer analyses at the population level for this species within semi-enclosed coastal ecosystems.

Keywords: molecular ecology, dinoflagellate cyst, spatial distribution, real-time PCR, Lagrangian model, population dynamics

Corresponding author: raffaele.siano@ifremer.fr

1. Introduction

The recovery of resting stages of harmful microalgae in sediment samples is considered a marker of the settlement of a species in a given coastal ecosystem while the identification of accumulation spots indicate potential seeding sources for the initiation of toxic species bloom (Anderson *et al.,* 2012). Cyst bank mapping is therefore particularly useful for the risk assessment of harmful microalgae, since it enables the prediction of blooming areas and the optimization of the management of coastal economic activities.

Alexandrium species distribution in coastal and shelf waters is relatively well known; however comprehensive distributional data, especially for resting stage banks, are still needed (Anderson *et al.,* 2012). On the basis of the available information, some common features in the distribution of *Alexandrium* cysts can be identified. Previous studies have reported that cyst accumulations are favored in fine-muddy rather than sandy sediments (White and Lewis 1982, Kremp 2000, Yamaguchi *et al.,* 1996, Joyce *et al.,* 2005, Gayoso *et al.,* 2001; Matsuoka *et al.,* 2003, Wang *et al.,* 2004, Anderson *et al.,* 2005, Anglès *et al.,* 2010, Horner *et al.,* 2011, Genovesi *et al.,* 2013, Trikia *et al.,* 2014, Fertouna-Bellakhal *et al.,* 2015), supporting the hypothesis that dinoflagellate resting stages behave physically like fine particles (Dale 1983). Given this close association between resting cyst abundance and sediment type, high specific DNA abundances should also be found in the corresponding muddy sediments. However, DNA extracted from sediments can be of both intercellular (resting stages) (Godhe *et al.,* 2002, Erdner *et al.,* 2010) and extracellular origins (Pietramellara *et al.,* 2009), making the relationship between specific DNA traces and sediment type not completely predictable and still barely studied.

Discrete cyst banks may not be found where blooms occur over a large area, but there is evidence of localized cyst accumulation in some estuarine systems (Anderson *et al.,* 2012). A close link between the local distribution of cyst banks and blooms has been documented for some *Alexandrium* species in semi-enclosed, confined ecosystems such as estuaries (Cembella *et al.,* 1988, Crespo *et al.,* 2011, Anderson *et al.,* 2014), lagoons (Genovesi *et al.,* 2009, Genovesi *et al.,* 2013, Trikia *et al.,* 2014, Fertouna-Bellakhal *et al.,* 2015), and harbors (Bravo *et al.,* 2008, Anglès *et al*., 2010). This local distribution has been associated with the hydrodynamic features of the studied ecosystems. Despite occurring in adjacent waters, local blooms of *A. fundyense* Balech were temporally separated, probably due to water retention in the first site where the blooms occurred (Crespo *et al.,* 2011). Cyst densities of *A. tamarense* (Lebour) Balech were influenced by local hydrodynamics, with wind-induced currents causing cyst dispersal in the shallow ecosystem of the Thau lagoon (Genovesi *et al.,* 2013). These examples prove the interest of characterizing discrete, fine spatial scale cyst distributions to deduce local bloom dynamics in semi-enclosed ecosystems.

Traditionally, hot spots of harmful microalgae accumulation in sediments are identified by microscopy counting of their cysts (Genovesi *et al.,* 2013), a method that is time-consuming and suffers from taxonomical limitations in identification due to the lack of distinctive morphological characteristics for the cysts of some species. In contrast, the analysis of specific genetic material in sediment has proved to be a valuable alternative to infer cyst distribution, enabling a large number of samples to be processed objectively in a relatively short time. The amplification of species-specific marker genes from DNA preserved in sediment samples has been used to infer the presence of dinoflagellate cysts (Godhe *et al.,* 2002, Penna *et al.,* 2010). Real-time PCR amplification to quantify DNA genes from sediments has been shown to be a good proxy for cyst abundances, including some *Alexandrium* species (Kamikawa *et al.,* 2007, Erdner *et al.,* 2010, Park and Park, 2010). Lastly, cyst species have been mapped using the fluorescence in situ hybridization (FISH) technique (Hattenrath-Lehmann *et al.,* 2016). Genetic techniques can therefore be used to provide reliable information on accumulation spots of cysts and to infer ecological patterns.

Although *A. minutum* Halim has been present in the Bay of Brest since at least the 19th century (Klouch *et al.,* 2016), the vegetative form of the species was first identified within the framework of the REPHY (National Phytoplankton and Phycotoxin Monitoring Network for French coastal waters) in 1990. The first cyst abundance survey was carried out in the same year and no cyst of *A. minutum* was found in four estuarine stations of the Bay of Brest (Erard-Le Denn 1993, Erard-Le Denn and Boulay, 1995). The species increased over time in the bay, reaching the record concentration of ca. 42 x 10^6 cells L^{-1} in July 2012 (Chapelle *et al.,* 2015, Klouch *et al.,* 2016) in the small, enclosed Daoulas estuary, where bloom occurrences were unsuspected and monitoring was not carried out. In parallel, other blooms of the species were observed in other estuaries on the bay (Elorn River, Aulne River), but these were of minor importance (< 2 x 10^6 cells L^{-1}). After the 2012 event, monitoring of Daoulas Bay was initiated and seasonal blooms of *A. minutum* are observed in the Daoulas estuary along with blooms of lower cell abundance in other monitored estuaries. The development of different intensities in the Bay of Brest raises questions about the distribution of the major cyst banks of the species and the potential connectivity between its different adjacent estuarine ecosystems.

In this study, both genetic analyses of sediments and model simulations in the water column were used to try to explain the heterogeneity of *A. minutum* bloom occurrence in the Bay of Brest. With a recently developed real-time PCR assay (Klouch *et al.,* 2016), the ITS1 rDNA copy number of *A. minutum* from total DNA extracts of superficial sediments was quantified and used to infer the potential distribution of cyst banks in the area. In parallel, the viability of these banks in the sampled stations was determined in order to verify whether DNA genetic data correspond to viable resting stages of the organisms and not only to the amplification of extracellular DNA. By means of a

Lagrangian physical model, passive particle trajectories released from different estuarine zones were simulated in order to study the potential dispersal of *A. minutum* cells of different blooms and the connectivity between different estuaries of the bay. The information gathered on both the benthic and the pelagic habitat contributes to the understanding of *A. minutum* bloom dynamics in the Bay of Brest and provides an example of heterogeneity in the dispersal of toxic microalgae in a semi-enclosed coastal area.

2. Materials and Methods

2.1. Study area

The Bay of Brest (Brittany, France) is a semi-enclosed, marine ecosystem of 180 km^2 connected to the Iroise Sea (Atlantic Ocean) by an opening 1.8 km wide and ~50 m deep (Figure 30). The bay is a shallow (about half of the total surface area is shallower than 5 m) macrotidal coastal system. The semi-diurnal tidal amplitude ranges from 1.2 to 7.3 m (average of 4 m), leading to the presence of extended intertidal flats during low tides. Frequent storms can induce a resuspension of material and a very high turbidity (>100 mg L^{-1}) over a long period of time (Hily *et al.,* 1992). The bay is characterized by fine and coarse sediments in shallow and deep waters, respectively, with a higher proportion of muddy sediments in the upstream part of the estuaries (Hily *et al.,* 1989). The ecosystem hydrology is influenced by 5 different watersheds, with two main rivers, the Aulne (1842 km^2 catchment area, 30 m^3 s^{-1} interannual mean flow) flowing into the south basin and the Elorn (402 km^2 catchment area, 5.63 m^3 s^{-1} interannual mean flow) flowing into the north basin, contributing to about 80% of the total annual freshwater input. The total interannual mean flow has stabilized after a four-decade rise, while the anthropogenic loads of nitrogen and phosphorous have stabilized and decreased, respectively. The ban on washing powders containing orthophosphates in the last two decades has resulted in a decreased phosphorous supply and thus a significant imbalance in the N/P ratio (Chauvaud *et al.,* 2000, Guillaud and Bouriel 2007) which has led to changes in the composition of planktonic and benthic communities (Quéguiner and Tréguer 1984, Del Amo *et al.,* 1997, Chauvaud *et al.,* 2000).

2.2. Sampling strategy

Thirty sites were selected in the Bay of Brest on the basis of available cartographies of sediment typologies and benthic biotopes. Sampled stations correspond to ecosystems where: i) cyst accumulation may be favored by the site geomorphology (estuaries, small bays with low flushing, low

bioturbation rates), ii) genetic material could be better preserved (muddy, anoxic sediments), iii) human activities are developed and/or the impact of Harmful Algal Blooms is higher (harbors, shellfish farming areas). Twenty-three stations were located in intertidal zones and seven in subtidal areas (Table 4). Altogether, the 30 sampled stations cover well the geography of the Bay of Brest (Figure 30). Samples were collected at low tide ± 2 hours during two campaigns of 3-5 days, both carried out during the winter of two consecutive years (December 2013 and January 2015). Subtidal stations were sampled by scuba divers. The top 3 cm of sediments was collected in triplicate using plastic syringes at 1-2 m distance from each other. Sediment samples were carefully preserved in different tubes. DNA samples were immediately frozen in liquid nitrogen then stored at -80°C in the laboratory while samples for cyst germination experiments were preserved in the dark at 4°C. Samples were preserved at 4°C for granulometry, at -80°C for chlorophyll *a* and pheopigments, and at -20° for organic carbon (OC).

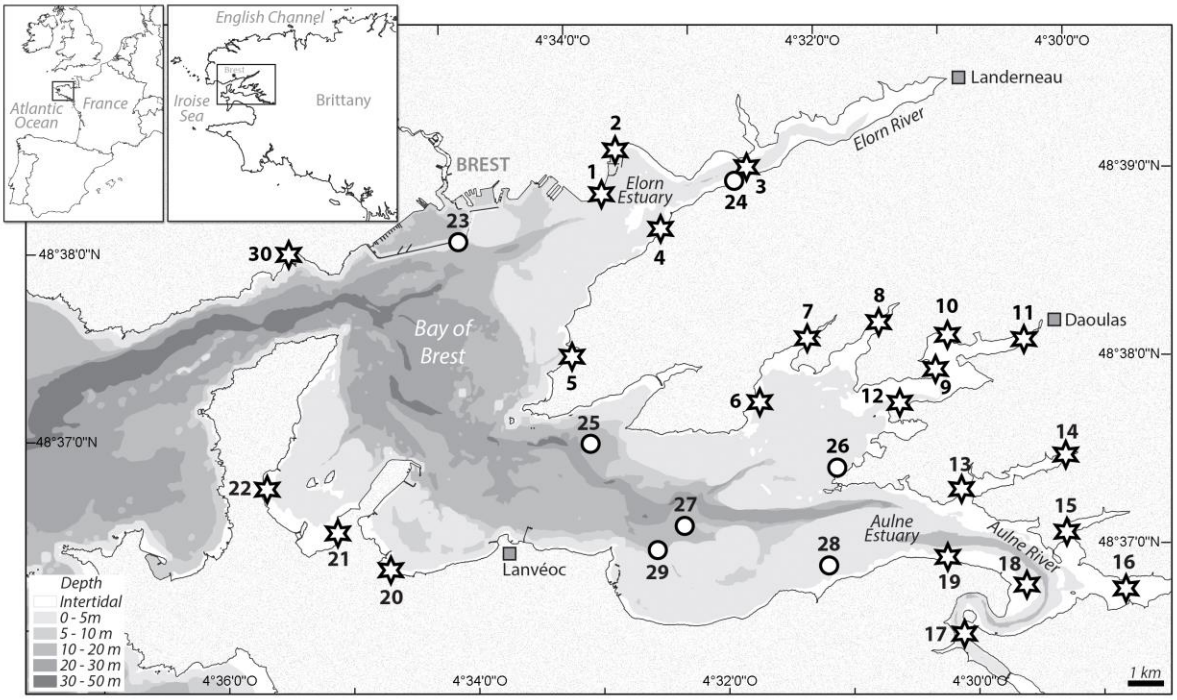


Figure 30. Map of the Bay of Brest indicating intertidal (stars) and subtidal (circles) sampling stations.

Table 4. List and coordinates of sampling stations. Subtidal stations (23-27) are indicated by a circle.

Station ID	Station locality	Coordinates (N/W)
1	Polder	48° 23' 3''/ 4° 26' 6''
2	Moulin Blanc	48° 23' 44''/ 4° 25' 55''
3	Le passage	48° 23' 25''/ 4° 23' 03''
4	Kéraliou	48° 22' 35''/ 4° 24' 41''
5	Caro	48° 20' 28''/ 4° 26' 26''
6	Tinduff	48° 20' 2''/ 4° 22' 03''
7	Moulin Neuf	48° 21' 5''/ 4° 21' 04''
8	Penfoul	48° 21' 25''/ 4° 19' 28''
9	Kersanton	48° 20' 45''/ 4° 18' 2''
10	Lanveur	48° 21' 19''/ 4° 17' 51''
11	Rivière de Daoulas	48° 21' 21''/ 4° 16' 2''
12	Château	48° 20' 11''/ 4° 18' 46''
13	Moulin Mer	48° 18' 56''/ 4° 17'11''
14	Hôpital Camfrout	48° 19' 37''/ 4° 14' 53''
15	Tibidy	48° 18' 25''/ 4°14' 40''
16	Lanvoy	48° 17' 47''/ 4° 13' 29''
17	Térénez	48° 16' 43''/ 4° 16' 49''
18	Landévennec	48° 17' 34''/ 4° 15' 29''
19	Sillon des Anglais	48° 17' 52''/ 4° 17' 22''
20	Fret	48° 16' 54''/ 4° 30' 13''
21	Rostellec	48° 17' 27''/ 4° 31' 30''
22	Persuel	48° 18' 1''/ 4° 33' 17''
23 ○	Port de Commerce	48° 22' 7''/ 4° 29' 18''
24 ○	Le Passage	48° 23' 39''/ 4° 22' 54''
25 ○	Auberlac'h	48° 19' 9''/ 4° 25' 53''
26 ○	Est Tinduff	48° 19' 7''/ 4° 20' 7''
27 ○	Lanveoc-Tinduff	48° 18' 2''/ 4° 23' 31''
28 ○	Quillien	48° 17' 36''/ 4° 20' 6''
29 ○	Ecole Navale	48° 17' 37''/ 4° 24' 6''

2.3. Sediment analyses

Granulometry, chlorophyll *a*, pheopigment and organic carbon (OC) concentrations were determined from samples collected in triplicate at each station. Sediment grain size was analyzed using an LS 200 Beckman Coulter laser granulometer and sediment typologies were classified according to Larsonneur (1977) on the basis of four size classes (0-63, 63-125; 125-500; 500-2000 µm). For chlorophyll and pheopigment concentration measurements, sediment samples were freeze-dried just before extraction and analysis (Reuss and Conley 2005). Before extraction, the sample was homogenized, and gravel and shell debris were removed. Chlorophyll *a* and pheopigments were extracted from 1 g of sediment with 10 mL of 90% acetone for at least 12 h at 4°C. Supernatants

containing the extracted pigments were recovered after sample centrifugation. Chlorophyll *a* and pheopigment concentrations were determined spectrophotometrically at 750 and 665 nm before and after sample acidification with 0.3 N HCl (Lorenzen 1967, Pusceddu *et al.,* 2003). Sediment samples for OC measurement were ground and homogenized. Organic C was measured using a vario EL-III CNS elementary analyzer after decalcification of a subsample of the freeze-dried and ground sediment with phosphoric acid (Cauwet 1975). Concentrations were calculated by comparison with samples of known concentration of organic carbon (acetanilide, sulfanilamide) and analyses were verified with a certified reference sediment sample.

2.4. Genetic analyses

Alexandrium minutum ITS1 rDNA copy numbers were measured directly on DNA extracts from sediments using a newly developed real-time qPCR assay (Klouch *et al.,* 2016). Total DNA was extracted from 10 g of sediment material from each triplicate of all stations using the PowerMax soil isolation kit (Mobio Laboratories Inc., Carlsbad, California, USA), following the manufacturer's instructions. DNA extracts were eluted in a final volume of 5 mL and immediately stored at -80°C. DNA samples were quantified by absorbance measurements using a Take3 trio microplate reader (BioTek, Winooski, Vermont, USA) on 3 µL of DNA extract, and sterile water was used as the blank. DNA quality was checked by 260/280 nm ratio to ensure that no contamination by proteins or other components had occurred during DNA extraction.

Real-time PCR reactions were carried using primers Am_48F (5'-TGAGCTGTGGTGGGGTTCC-3') and Am_148R (5'-GGTCATCAACACAGCAGCA-3') which target a fragment of 100 bp, the optimal amplicon length for real-time PCR efficiency (Klouch *et al.,* 2016). Prior to real-time PCR reactions, a standard curve was constructed by cloning the ITS1 rDNA gene from a local culture of *A. minutum* (A89) into a plasmid (pCR 4) using a TOPO TA cloning kit (Invitrogen, USA). The standard curve was prepared with 10-fold serial dilutions of the plasmid containing the ITS1 rDNA sequence of *A. minutum* and ranged from 10^6 to 10 copies μL^{-1} .Real-time PCR (quantitative PCR or qPCR) reactions were performed using the iTaq Universal SYBR Green supermix kit (Bio-Rad) in a final volume of 20 µL. The reaction mixture was composed of 10 µL of SYBR Green supermix (1X) containing (dNTPs, iTaq DNA polymerase, MgCl2, SYBR Green I), 0.3 µM of the forward primer (Am_48F), 0.2 µM of the reverse primer (Am_148R), sterile water and 2 µL of DNA template. The experiments were conducted in 96-well plates containing the standard curve dilutions in duplicate, the target samples in triplicate and negative controls composed of water instead of DNA in duplicate. The plates were loaded onto a Stratagene Mxpro3000P (Agilent Technologies, Santa Clara, California, USA) thermal cycler with the following cycling conditions: 1 cycle at 95°C for 5 min followed by 40 cycles of 95°C for 5 sec and 62°C

for 30 sec. A melting curve analysis was added at the end of each run to ensure specific *A. minutum* amplification. The optimal annealing temperature of 62°C was initially determined in conventional PCR. The primer combination that yielded the lowest threshold cycle value (Ct) and maximum real-time efficiency (Am_48F; 0.3 µM, Am_148R; 0.2 µM) was retained for further analysis. The reaction efficiency was estimated by the equation $E= 10^{(1/b)}-1$, where b is the slope of the standard curve. To ensure specific amplifications, the melting temperature values (Tm) were systematically checked by analyzing the melting curves. For further details, see Klouch *et al*., (2016). Abundances of *A. minutum* in each sample were expressed (assuming a 100% DNA extraction efficiency) in terms of copy number per g of wet sediment, using the following formula:

Copy number x g^{-1} = copy number μL^{-1} x DNA extraction volume (µL)/sediment wet weight (g)

2.5. Cyst germination experiments

Germination experiments were carried out on samples from the 2013 series. An aliquot of ~5 cm^3 of sediment samples was added to filtered seawater and placed in an ultrasonic bath for 6 min to separate dinoflagellate cysts from inorganic particles. The 20-100 µm fraction of particles was retained for culturing experiments after sample sieving. Some drops of the 20-100 µm sediment fractions were distributed in 12-well plastic plates with K medium (Keller *et al.,* 1987). The plates were placed in a culture room at 16°C, under an irradiance of 60 µmol photons m^{-2} s^{-1} and a light: dark cycle of 12h:12h. The plates were examined qualitatively once every day to check for *A. minutum* cell germination using an inverted microscope (Zeiss Axiovert 135).

2.6. Simulation of *A. minutum* cells: Lagrangian transport

The MARS3D hydrodynamic model (a detailed description is available in Lazure and Dumas (2008)) was used to study planktonic cell trajectories after bloom development. This numerical code solves primitive physics equations (*e.g.* Navier-Stokes under hydrostaticity and Boussinesq assumptions) and is based on a finite difference scheme coupling barotropic and baroclinic modes within a sigma-coordinate framework. For this study, the model was defined for the Bay of Brest with spatial limits ranging between 48.203-48.447 °N and 4.093-4.730 °W, a spatial horizontal resolution of 50 meters and 20 vertical layers. Moreover, the model assumed a wetting and drying capability (intertidal areas), which is mass preserving. The model's bathymetry was provided by the SHOM (French Naval Hydrographic and Oceanographic Service). At its western and southern boundaries, the model was forced for water elevation (tides), water temperature and salinity by another model (Lazure *et al.,* 2009), previously validated for tides and hydrology and simulating the Bay of Biscay and Channel

hydrodynamics. Atmospheric forcing (wind and atmospheric pressure) came from the Météo-France AROME model (Seity *et al.,* 2011) which has a temporal resolution of 1 hour and a spatial resolution of 0.025° (roughly 2.4 km). The three major rivers, the Aulne, the Elorn and the Mignonne, were taken into account, and water flows came from the HYDRO database (Governmental Environment Agency). *Alexandrium minutum* cell trajectories were computed by the ICHTYOP Lagrangian transport tool (Lett *et al.,* 2008) coupled with our hydrodynamic model of the bay. This tool enables offline simulations of bloom dispersion by calculating fictive particle trajectories based on previously calculated currents. Two simulations were run for years 2014 and 2015. Particles were released at the beginning of June, when *Alexandrium* bloom conditions were fulfilled according to Chapelle *et al*., (2015), i.e. when the water temperature was above 15°C and during a neap tide period. Four different starting points were tested: stations 3, 9, 13 and 16 (Figure 30). At each station, a total of 1000 passive particles was released in the surface layer within a square stain of 100 m side length. Each particle position was recorded during 10 days of simulations, which is the approximate period before particles are flushed out of the bay. The level of dropping (surface or bottom) was tested but had no significant influence on the results (not shown here). For these simulations, we also assumed that particles had no buoyancy, no mortality and no growth and that they could not wash up on the coast. Connectivity between the four different release zones was assessed by defining reception areas as the geographical limits of the four different estuarine areas: the Elorn estuary (incorporating station 3), the Daoulas estuary (incorporating station 9), the Camfrout estuary (incorporating station 13), and the Aulne estuary (incorporating station 16). Total numbers of particles released at each station and reaching the other three areas were computed. The model also enabled the calculation of the mean total distance covered by particles starting from the different stations as well as the total number of particles remaining in each area by the end of the simulation as a proxy for confinement.

2.7. Statistical analyses

A Principal Component Analysis (PCA) was used to assess relationships between environmental and biological data along a reduced number of axes (ade4 package for R; Dray and Dufour, 2007). Data used in the PCA include the four sediment size fractions (0-63 µm, 63-125 µm, 125-500 µm, 500-2000 µm) and the three biological variables (Chl *a*, *A. minutum* and total DNA concentration). To reduce the importance of observations with very high values, concentrations of *A. minutum* ITS1rDNA copy number g^{-1} sediment were log10 (x+1)-transformed. The PCA result has the same dimension as the dataset, but the first principal components account as much as possible for the data variability. Thus, only the first two axes explaining most of the variance were retained for later interpretations. For

graphical representation, PCA results were combined with a cluster analysis performed on environmental and biological variables (complete-linkage clustering, vegan package; Oksanen *et al.,* 2015) to highlight further differences between station groups. A single cutting level (Euclidean distance = 8.2) was selected to obtain major groups of samples. Prior to these analyses, all variables were centered and scaled in order to make them dimensionally homogenous. Finally, the Spearman rank correlation coefficients were calculated between all the environmental and biological variables. All statistical analyses were performed using the R software (R Core Team 2015).

3. Results

3.1. Sediment analyses

The relative magnitude of the granulometric size classes analyzed enabled each sampling site to be classified on the basis of their sediment typology (Supplementary Table 3.1) for the two sampling surveys. Of the 30 sampling stations, 13 were classified as sandy-mud, 6 as mud, 1 as muddy-sand, and 2 as fine sand, coherently for both sampling surveys. For the remaining 8 stations, the granulometric classification varied between the two years, more often due to variations in the percentage of either or both 0-63 µm and 125-500 µm sediment size fractions.

3.2. *Alexandrium minutum* quantification in DNA extracts of sediments

Total DNA concentrations extracted from the sediments ranged from 1.95 to 55.20 ng μL^{-1} for 2013 samples and from 1.57 to 36.08 ng μL^{-1} for 2015 samples. According to 260/280 nm ratios, the DNA extracts were of sufficient yield and purity to conduct amplification analyses. The reaction efficiencies of real-time PCR amplifications of about 100 bp of the ITS1rDNA of *A. minutum* ranged from 95 to 99% and the melting temperature values always corresponded to the expected value of 62°C, both results proving the high resolution of our PCR assay. The ITS1rDNA copy number varied from 1.63×10^4 to 5.46×10^7 copies g^{-1} sediment in 2013 and from 2.89×10^4 to 5.47×10^7 copies g^{-1} sediment in 2015. Local *A. minutum* quantification was very variable between replicates of some stations (the ranges of the standard errors between the three replicates were $3.57 \times 10^4 – 2.63 \times 10^7$ and $8.06 \times 10^4 – 2.09 \times 10^7$ copies g^{-1} sediment in 2013 and 2015, respectively) showing a significant spatial variability for some stations at a very fine spatial scale (1-2 m) (Figure 31A, B). At some stations, one replicate differed from the other two in one sampling year and not in another (stations 1-10, 30), while for other stations the data between replicates were coherent for the two years of analyses (stations 11-19). Stations 16-29 were characterized by higher copy numbers in 2015 (Figure

2B). Despite this strong intrasite variability, a coherent pattern of copy numbers of ITS1rDNA of *A. minutum* was identified between the two years. On the whole, both the intertidal (6-19) and subtidal (26-29) stations of the south-eastern part of the Bay of Brest were characterized by higher copy numbers than the subtidal and intertidal stations of the western (20-22, 25, 30) and north-eastern part (1-5, 23-24) of the bay, in both 2013 and 2015. In particular, the intertidal stations within Daoulas Bay (6-12) and the subtidal station outside the bay (26-29) were characterized in both years by higher copy numbers of *A. minutum* ITS1 rDNA (Figure 31A).

3.3. Germination experiments

Alexandrium minutum germinated from all (30/30) 2013 sediment samples within the first 10 days of sediment incubation. No morphological differences (size, number and distribution of chloroplasts, swimming behavior) were observed in light microscopy between specimens of different localities. In the light of the 2013 successful germinations, we considered that cysts of the species had settled in all localities of the Bay of Brest and therefore we did not perform germination experiments on 2015 sediment samples.

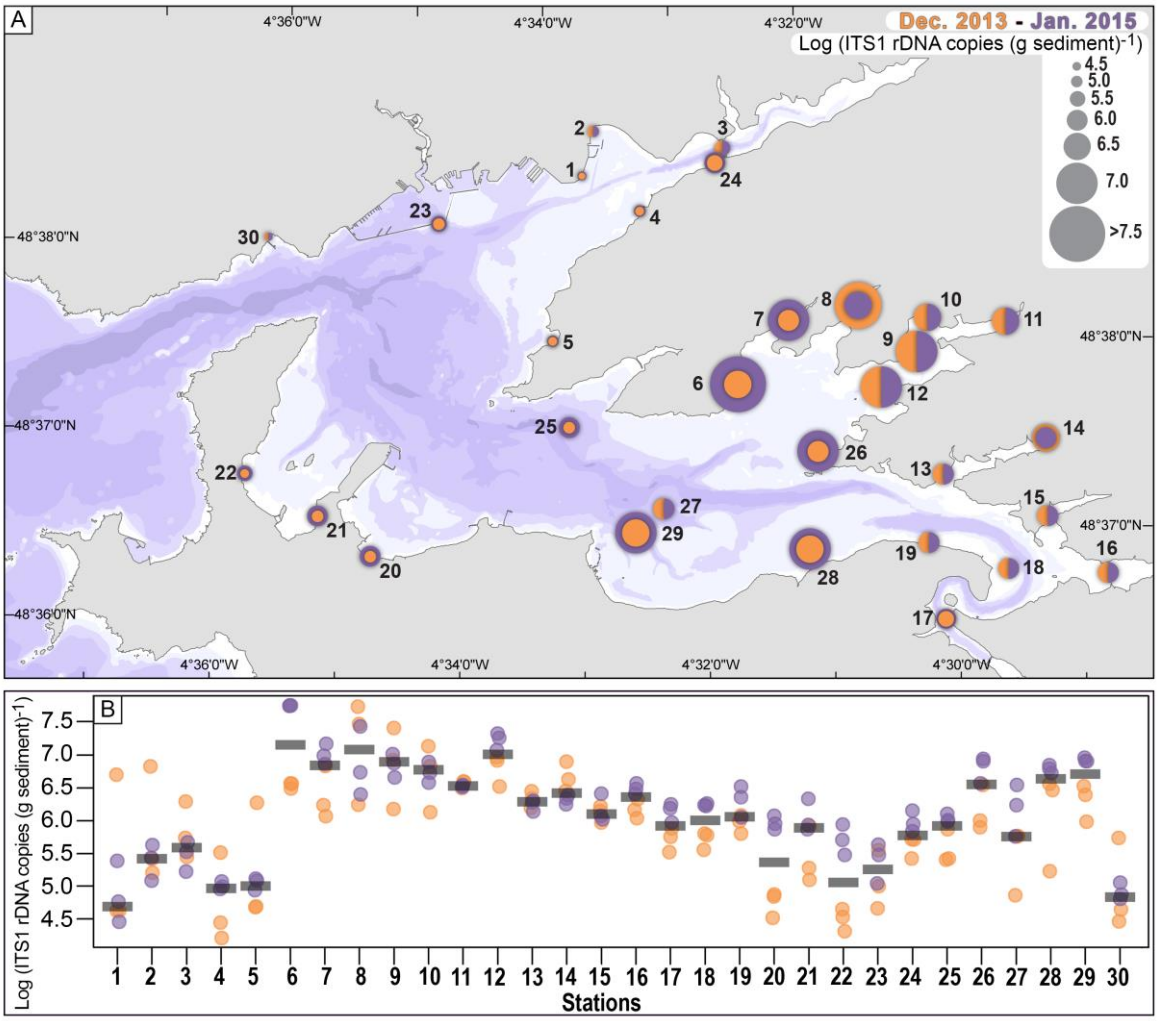


Figure 31. Log_{10} (x+1) transformed real-time PCR data of *Alexandrium minutum* copy number g^{-1} of sediment at the 30 sampled stations during the two surveys (December 2013 and January 2015). A) At each station, colored circles represent the averaged values of copy number concentrations of the three replicates for each annual survey (yellow = December 2013; purple = January 2015). For each station, the highest concentration (larger circle) is in the background and the lowest is represented as a superposed circle. When the concentrations of the two years are of the same order of magnitude, each concentration value is represented by a semi-circle. B) Replicate data for each sampling station and annual survey. The dark gray bar represents the median of the six data values.

3.4. Genetic and environmental data correlations

The PCA performed with genetic, sediment granulometry and biological environmental parameters (Figure 32A, B) as well as Spearman correlations between variables (Figure 32C) showed that *A. minutum* ITS1 rDNA copy numbers (labeled *A. minutum* DNA) and total DNA concentrations (labeled Total DNA) were positively correlated with the fine sediment size fraction (labeled 0-63 µm) and the Organic Carbon (labeled as OC) and chlorophyll *a* (labeled as CHL *a*) concentrations (Figure 32A, C). In particular, *A. minutum* DNA was positively correlated with only fine sediment (0-63 µm) (0.54) and not coarser sediment types. *Alexandrium minutum* DNA and 0-63 µm sediment were both positively

correlated with OC concentration (0.60 and 0.83, respectively) (Figure 32C). Cluster analysis identified two major groups of stations (differentiated in light and dark gray in Figure 3B). Overall, the analysis separates the south-eastern intertidal and subtidal sampling stations of the Bay of Brest (cluster 2: 6-12, 14-19, 26, 28-30) where the highest percentages of the fine sediment size fraction (0-63 µm) were associated with the highest concentrations of OC, CHL *a* and genetic material, from the western and north-eastern stations (cluster 1: 1, 3-5, 20-22, 25, 30) where measured values of these variables were lower. Yet, some exceptions to this geographical separation of sampling stations were highlighted. Eastern stations 13 and 27 were characterized by low correlations and were grouped within cluster 1. On the contrary, the north-eastern station 24 was grouped within cluster 2, showing that the strong association between fine sediment granulometry, OC and CHL *a* and *A. minutum* copy number was not exclusive to a part of the Bay of Brest. Stations 2 and 23 had the same number of replicates in both clusters 1 and 2.

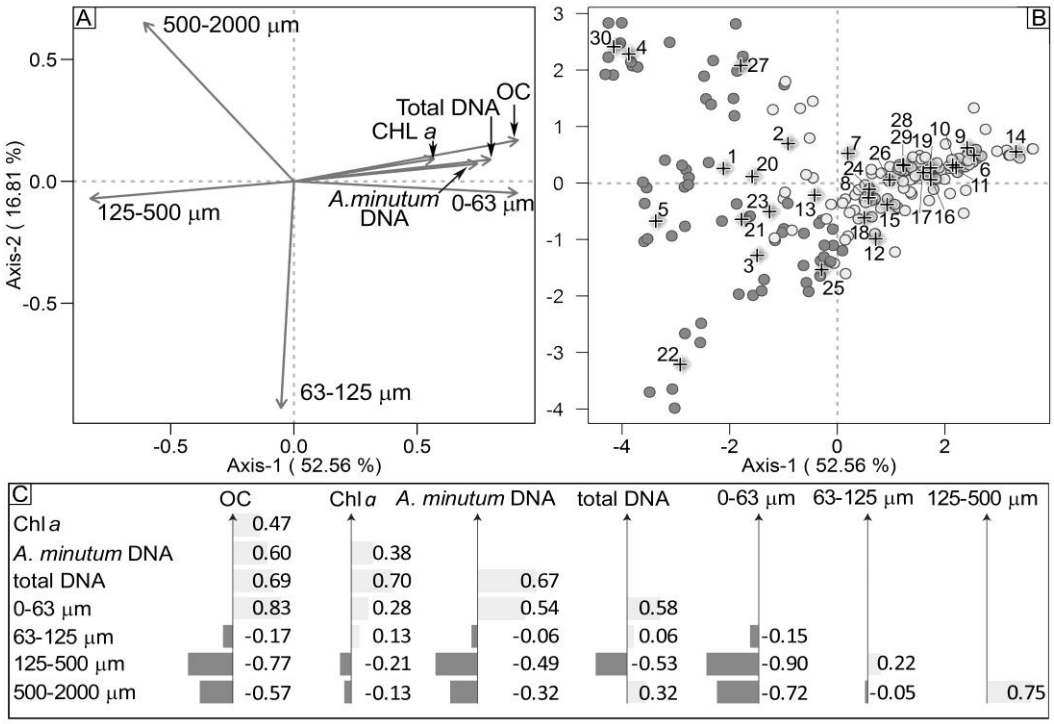


Figure 32. Relationship between environmental parameters (chlorophyll *a*: CHL *a*; organic carbon: OC, sediment size class: 0-63, 63-125, 125-500 and 500-200 µm) and genetic data (ITS1 rDNA copy number concentration g^{-1} of sediment of *Alexandrium minutum*: *A. minutum* DNA; Total DNA concentration (ng/µL) extracted from sediment: Total DNA) analyzed from sediments. (A) Projection of variables on the first two axes of the PCA accounting altogether for 69.37% of the total variance. (B) Samples (site-year-replicate) scores with overlaid clustering results highlighting the two main sample groups detected by data clustering. Each circle represents a sample replicate; the color differentiates the sample clusters: + symbol corresponds to the gravity center of each group of samples belonging to the same sampling station indicated by its corresponding number. (C) Spearman rank correlation coefficients between all variables. Light gray bars indicate positive correlations and dark gray bars negative correlations.

3.5. Simulation of particle trajectories in the water

Simulated trajectories and final particle positions after 10 days as a function of the initial release location and the year are shown in Figure 33A-D as an example for 2015 (2014 simulated trajectories are not shown). Whichever year is considered, 56 to 81% of passive particles remain in the Bay of Brest after 10 days (Table 5). Particles released at Station 9 (Daoulas estuary) show the lowest percentage being flushed out of the Bay of Brest (Figure 33B, Table 5), whilst particles released at station 3 (Elorn estuary) show the highest percentage (Figure 33A, Table 5). The model simulations also show that particles released at stations 13 (Figure 33C) and 16 (Figure 33D) tend to be transported over greater distances than those released at stations 3 or 9. The Daoulas estuary appears to be the most confined area with 26.5-26.2% (depending on years) of the initial number of particles released at station 9 remaining in that area after 10 days (Table 5). The connectivity table between the four release areas (Table 6) shows similar patterns for 2014 and 2015 simulations. As expected, stations 13 and 16 show a high connectivity with the nearby Daoulas estuary (from 29.7 to 30.7% of the initial released particles). Interestingly, this connectivity seems to be rather one-way (from stations 13 or 16 towards the Daoulas estuary) since very few particles starting from station 9 (Daoulas estuary) reach the Camfrout estuary (0-0.4%) or the Aulne estuary (7.8-8.5%). The Daoulas and Elorn estuaries seem particularly hydrodynamically disconnected with very few particles released at station 9 reaching the Elorn estuary (0.2-0.3%) and similarly from station 3 to the Daoulas estuary (1.2-1.4% of particles). Moreover, after 10 days of simulation, no particle released from station 3 reaches the Camfrout estuary. In the center of the Bay of Brest, particles are well-mixed and come from each releasing station. Overall, our simulations demonstrate that the south-east of the bay is quite disconnected from the Elorn estuary, which is the area exporting the highest proportion of particles outside the Bay of Brest, and that the Daoulas estuary is a confined area with little connectivity with the Aulne estuary and even less with the Elorn estuary.

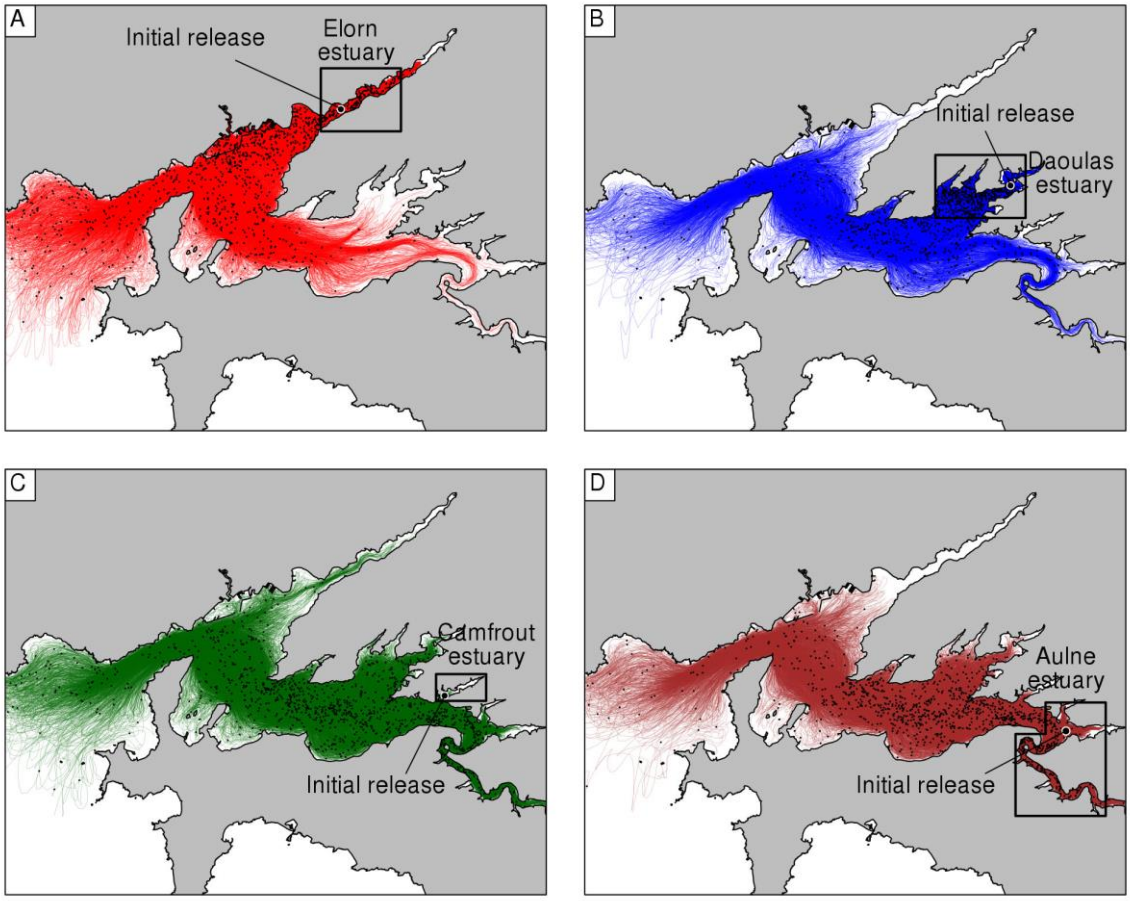


Figure 33. Simulated trajectories of 1000 passive particles coming from 4 releasing estuarine stations and reaching delimited estuarine areas:

A) station 3 (Elorn estuary); B) station 9 (Daoulas estuary); C) station 13 (Camfrout estuary); D) station 16 (Aulne estuary). Simulation duration is 10 days and final particle positions are given as black dots.

Table 5. Summary of simulated particle trajectories initiating from four different areas in the Bay of Brest: F stands for the percentage of particles flushed out of the Bay of Brest at the end of the simulation, D is the mean cumulative distance covered by particles until the end of the simulation and A represents the percentage of particles still located in the estuary of release at the end of the simulation (auto-connectivity). The two numbers stand for years 2014 and 2015, respectively.

Release station	3	9	13	16
F (%)	43.6 – 37.7	9 – 18.7	29.1 – 30.8	23.5 – 24.2
D (km)	129.5 – 127.9	114.0 – 114.3	151.1 – 149.7	152.9 – 153.4
A (%)	9.4 – 8.3	26.5 – 26.2	0 – 0	12.8 – 10.4

Table 6. Connectivity table between releasing stations (3, 9, 13 and 16) and receiving areas (Elorn, Daoulas, Camfrout and Aulne estuaries). Percentage (%) of particles reaching the considered area at least once in 10 days for 2014 and 2015, respectively.

	Releasing stations			
	St. 3	St. 9	St. 13	St. 16
Elorn estuary area	-	0.3 – 0.2	1.6 – 2.0	2.0 – 0
Daoulas estuary area	1.4 – 1.2	-	29.7 – 28.4	30.7 – 28.0
Camfrout estuary area	0 – 0	0.4 – 0	-	6.6 – 6.0
Aulne estuary area	1.3 – 1.5	7.8 – 8.5	58.7 – 57.4	-

4. Discussion

4.1. Genetic mapping of *A. minutum* in sediments

In this study, we have provided a geographically exhaustive survey of the presence of *Alexandrium minutum* in superficial sediment of the Bay of Brest (Brittany, France) during the winter of two consecutive years (December 2013 and January 2015), when cysts of the species are thought to accumulate in sediments. This is not the first screening of *A. minutum* traces in sediment of the study area. In 1990, no cyst of *A. minutum* was found in four estuarine stations of the Bay of Brest (Erard-Le Denn *et al.,* 1993, Erard-Le Denn and Boulay 1995) using cyst identification by light microscopy. Here, real-time PCR amplification of a fragment of the ITS1 rDNA of *A. minutum* from DNA sediment extracts was proposed as an alternative method to cyst quantification. The high proportion of extracellular DNA in the total environmental DNA extracts from sediments and the high and variable number of copies of genomic DNA markers are important issues to take into account when performing specific DNA quantification. These problems may lead to misinterpreting the presence of viable material (resting stages) in sediment and bias estimations of cyst abundances. These issues have been discussed in several studies focusing on the efficiency and limitations of the real-time PCR methodology to analyze cyst banks in sediments. All these studies concluded that DNA amplifications in sediment samples are a good proxy of cyst abundances and that PCR methodologies are valuable methods to infer distribution patterns (Godhe *et al.,* 2002, Penna *et al.,* 2010, Kamikawa *et al.,* 2007, Erdner *et al*., 2010, Klouch *et al.,* 2016). The objective of our study was not to validate the real-time PCR amplifications to estimate exactly the cyst abundances of *A. minutum*, but to map potential accumulation zones in our study area in order to contribute to the understanding of the spatial heterogeneity of bloom dynamics and intensities of *A. minutum* in the estuaries of the Bay of Brest.

Genetic traces of *A. minutum* were found in 30 out of 30 sampled stations of the study area. In parallel, successful germination experiments of *A. minutum* on 2013 sediment samples proved that living resting stages had settled in all stations and that the toxic species currently contaminates the whole Bay of Brest. The reduced interstitial space between fine particles decreases water circulation and can favor the establishment of anoxia in sediments, which are suitable conditions for slowing the digenetic process and preserving organic matter (Genovesi *et al.,* 2013). Therefore, as expected, the organic matter content, chl *a* and total DNA concentrations were higher in stations characterized by fine sand-muddy sediment typology, as proved for instance by the high correlation value (0.83) obtained between the finest sediment fraction (0-63 µm) and the organic carbon content (Figure 32). It is acknowledged that *Alexandrium* cysts behave physically like fine particles and that accumulation spots mostly occur in fine sediment areas (White and Lewis 1982, Kremps 2000, Yamaguch *et al.,* 1996, Joyce *et al.,* 2005, Gayoso *et al.,* 2001, Matsuoka *et al.,* 2003, Wang *et al.,* 2004, Anderson *et al.,* 2005, Anglès *et al.,* 2010, Horner *et al.,* 2011, Genovesi *et al.,* 2013). Consequently, DNA traces of the species should mostly be found in sediments characterized by a large fraction of fine particles. The good positive correlation found between our *A. minutum* ITS1 rDNA amplifications and the 0-63 µm sediment fraction of the sampled stations confirms this hypothesis. Good correlations between ITS1rDNA amplifications and the 0-63 µm sediment fraction were coherent for both 2013 and 2015 samples in stations of the south-eastern part of the Bay of Brest and particularly for stations of the Daoulas estuary, in areas that were confirmed to be of muddy-fine sediment facies in a recent comprehensive, morpho-sedimentological analysis of the Bay of Brest (Gregoire *et al.,* 2016). Seasonal blooms of *A. minutum* have been detected in the Daoulas estuary since July 2012, when a massive toxic bloom event (concentrations of 42 x 10^6 cells L^{-1}) highlighted the Daoulas area as a new risky zone for toxic blooms of *A. minutum* in the Bay of Brest (Chapelle *et al.,* 2015). Before this event, the Daoulas area was not included in the monitored area of the REPHY observation network; in fact, the toxic species has only recently increased in the area as demonstrated by paleogenetic data from ancient sediment cores of the area (Klouch *et al.,* 2016). As well as Daoulas Bay, blooms of the species occur in other zones of the Bay of Brest, but they have always been of minor intensity (Chapelle *et al.,* 2015). In conclusion, we found large numbers of copies of the maker gene of *A. minutum* in sediments or areas where blooms of higher intensity occur in the plankton. This allows us to suggest that the Daoulas estuary is probably an accumulation zone of cysts of *A. minutum* in the Bay of Brest, an area which should be carefully monitored for toxic bloom occurrences.

4.2. Spatial heterogeneity in sediment and in water

The mapping of the potential accumulation spots of *A. minutum* obtained for December 2013 and January 2015 suggests a non-homogenous distribution of banks within the Bay of Brest, with major accumulation areas in the south-eastern part of the bay. In parallel, the simulation of passive particle trajectories performed with real forcings in potential offspring periods of *A. minutum* (June 2014 and 2015, the summer periods after the wintertime cyst accumulation in 2013 and 2015) suggests a differential dispersal of blooms in the bay. Blooms occurring in the south-eastern Daoulas estuary would be relatively disconnected from those in the north-eastern Elorn estuary, as the connectivity between the two areas is very low (<1.4%). Overall, this study reveals a heterogeneous distribution pattern of *A. minutum* in the Bay of Brest in both sediments and the water column. The reasons for this distribution must be found in the hydrology, geomorphology, and hydrodynamics of the bay.

Previous model simulations have shown that the estuaries of the Bay of Brest are preferential ecosystems for dinoflagellate bloom occurrence because of a sustained nitrogen supply from rivers (Menesguen *et al.,* 2006) and significant estuarine nutrient stocks in sediment (Raimonet *et al.,* 2013) that can be resuspended (Tallberg *et al.,* 2006) and due to low flushing rates in estuaries that allow the development of the bloom (Sourisseau *et al.,* accepted). Despite high nutrient loads, the Bay of Brest seems to be resistant to eutrophication problems due to strong semi-diurnal tidal currents that ensure the water exchange with the continental shelf (Le Pape and Menesguen, 1997, Chavaud *et al.,* 2000). In the shallow estuarine ecosystems of the Bay of Brest, the tide and the wind intensity and direction could promote bottom currents that can influence cyst and sediment distributions as shown in other semi-enclosed ecosystems (*e.g.* Genovesi *et al.,* 2013, Trikia *et al.,* 2014). Weak bottom currents favor the settlement of fine-muddy (<0.63 µm) sediment and cysts of *Alexandrium*, which are acknowledged to behave like fine sediment particles (Dale 1983). Therefore, bottom currents in the Bay of Brest may play a role in promoting the sediment movement and creating permanent superficial deposits of *A. minutum* in the shallow, peripheral embayments of the south-eastern zone of the bay, such as the Daoulas estuary. However, the specificity of *A. minutum* distribution in sediments remains to be demonstrated, as it is shown that cysts of different species may have different spatial distributions. (Park and Park 2010, Satta *et al.,* 2013, Fertouna-Bellakhal *et al.,* 2014).

Sediment resuspension and transport in the water column of the Bay of Brest have been analyzed by hydro-sedimentary model simulations. Tide currents would generate higher concentrations of resupended muddy sediments in the south-eastern estuaries of the bay (The Daoualas and Aulne estuaries) than in the north-eastern estuary (Elorn Estuary) (Beudin *et al.,* 2013, Beudin *et al.,* 2014). Tracked suspended mud of the south-eastern estuaries are predicted to be flushed out the bay,

without reaching the Elorn estuary, and the mud of the Elorn estuary are expected to remain and redeposit in the estuarine area without reaching the inner and the south-eastern part of the Bay of Brest (Beudin *et al.,* 2013, Beudin *et al.,* 2014). In parallel, our simulated particle dispersal trajectories suggest tidal currents coupled with river outflows would trigger current trajectories that cause low exchanges between the north-eastern Elorn estuary and the south-eastern Daoulas, Camfrout and Aulne estuaries. Potential blooms developing in the Elorn estuary would be mostly directly exported out of the bay, whereas blooms developing in each of the south-eastern estuaries would be connected to each other but not to the Elorn estuary. Both sediment and particle simulated transports suggest that the hydrodynamics of the bay would create barriers for *A. minutum* dispersal. As a consequence, low interbreeding could exist between populations of the northern and southern zones of the Bay of Brest. Our simulations are based on a physical model that does not include biological variables such as growth, asexual and sexual reproduction and mortality rates. These variables affect the retention time of a bloom and the connectivity between ecosystems and populations of the bay. New model simulations that integrate biological variables and a population genetic approach would complete the information provided by our study, probably supporting the heterogeneity of *A. minutum* dispersal in the Bay of Brest.

5. Conclusions

The successful germination of *A. minutum* from all sampled stations of the Bay of Brest demonstrates that *A. minutum* currently contaminates the whole Bay of Brest. Since in 1990 no cysts were found in estuarine samples of the bay, the information provided in this study contributes to highlighting a relatively recent proliferation of this toxic species in the bay. Higher copy numbers of ITS1rDNA in sediment samples of the Daoulas estuary argue in favor of the possibility that this estuary could be a major accumulation spot of the cysts of *A. minutum*. This distribution pattern could explain the regular occurrence of blooms of higher intensity in this area of the bay. The simulations of particle trajectories demonstrate that the blooms occurring in the north- and south-eastern estuaries of the bay are disconnected and therefore rather independent of each other. This suggests the existence of potential physical barriers to *A. minutum* bloom dispersal and of populations interbreeding in the bay. Overall, a heterogeneous distribution of *A. minutum* in both sediment and the water column emerge from our study, proving that there may be discrete, localized accumulations of cysts even in a semi-enclosed coastal ecosystem.

6. Acknowledgements

This work was financed by the DAOULEX project supported by the Region Bretagne. This research was carried out within the framework of K. Klouch's PhD funded by Ifremer and Region Bretagne (Allocation de REcherche Doctorale (ARED) fellowship) and by the project of the ECosphere Continentale et CÔtière (EC2CO) initiative of the Institut National des Sciences de l'Univers/Centre National de la Recherche Scientifique (INSU/CNRS): PALMITO (2013-2015). The authors wish to thank all colleagues who helped with sample collection and Xavier Caisey for scuba diving sampling assistance. We wish to thank Dominique Hervio-Heath and members of the SG2M/LSEM laboratory of Ifremer for access to their laboratory facilities and all their technical suggestions. Pierre Bodenes is acknowledged for collaborating in the picture developments and Pascale Malestroit for helping with culture maintenance. This research falls within the scope of the French GDR (Groupement de Recherche) Phycotox (http://www.phycotox.fr) (2012-2018) on harmful microalgae and phycotoxins.

7. Supplementary data

Supplementary Table 3.1. Classification of sampling stations based on four sediment size classes (µm) following Larsonneur (1977) for the two sampling surveys (January 2013 and December 2015).

Id St.	0-63		63-125		125-500		500-2000		Classification	
	2013	2015	2013	2015	2013	2015	2013	2015	2013	2015
1	14.3 ± 7.7	53.8 ± 18.2	16.7 ± 0.9	11.9 ± 2.3	47.9 ± 9.4	20.4 ± 5.4	21.0 ± 2.7	13.9 ± 15.0	Muddy sand	Sandy mud
2	63.4 ± 4.7	27.4 ± 4.3	14.8 ± 0.6	12.0 ± 1.2	18.0 ± 2.9	24.7 ± 1.3	3.9 ± 2.5	35.9 ± 5.9	Sandy mud	Sandy mud
3	32.5 ± 0.6	40.8 ± 11.6	28.7 ± 0.3	18.1 ± 1.7	33.2 ± 0.9	33.6 ± 10.9	5.6 ± 0.5	7.5 ± 2.9	Sandy mud	Sandy mud
4	4.1 ± 0.7	5.5 ± 2.8	2.7 ± 0.5	2.2 ± 0.8	61.5 ± 1.5	55.0 ± 3.9	31.6 ± 2.3	37.3 ± 5.6	Fine sand	Sandy mud
5	3.6 ± 0.5	3.5 ± 0.6	17.3 ± 0.3	12.4 ± 4.6	75.6 ± 1.0	81.5 ± 5.3	3.4 ± 1.1	2.5 ± 0.7	Fine sand	Fine sand
6	81.9 ± 2.9	77.7 ± 5.5	11.2 ± 1.0	11.2 ± 1.6	6.3 ± 1.5	10.0 ± 4.0	0.6 ± 0.3	1.1 ± 0.3	Mud	Mud
7	69.6 ± 6.5	35.8 ± 6.6	16.0 ± 2.9	11.9 ± 0.2	12.4 ± 2.5	24.7 ± 4.2	2.0 ± 1.3	27.7 ± 9.9	Sandy mud	Sandy mud
8	61.2 ± 5.4	45.2 ± 6.3	18.9 ± 2.3	15.5 ± 2.1	17.6 ± 3.1	31.0 ± 2.4	2.2 ± 0.8	8.3 ± 3.6	Sandy mud	Sandy mud
9	62.6 ± 5.0	70.0 ± 6.4	11.9 ± 2.0	13.1 ± 1.0	18.9 ± 4.1	15.6 ± 4.7	6.5 ± 3.1	1.4 ± 1.3	Sandy mud	Sandy mud
10	76.0 ± 2.1	70.6 ± 2.2	12.4 ± 0.2	13.5 ± 0	9.8 ± 1.2	14.4 ± 1.7	1.8 ± 0.8	1.5 ± 0.6	Mud	Sandy mud
11	77.2 ± 1.1	76.5 ± 0.5	13.0 ± 0.4	12.8 ± 0.2	9.1 ± 0.6	9.9 ± 0.3	0.7 ± 0.2	0.8 ± 0	Mud	Mud
12	44.2 ± 3.2	52.5 ± 7.7	25.1 ± 3.3	22.2 ± 2.1	24.4 ± 1.6	22.2 ± 5.6	6.3 ± 2.0	3.0 ± 2.5	Sandy mud	Sandy mud
13	51.1 ± 11.1	36.7 ± 31.8	18.4 ± 1.8	12.7 ± 11.0	25.7 ± 9.5	15.1 ± 13.2	4.8 ± 3.2	2.2 ± 2.0	Sandy mud	Sandy mud
14	80.1 ± 1.4	74.1 ± 1.8	13.0 ± 0.5	13.5 ± 0.2	6.5 ± 1.0	11.6 ± 1.7	0.5 ± 0.2	0.9 ± 0.7	Mud	Sandy mud
15	69.6 ± 2.4	65.2 ± 3.5	16.5 ± 1.8	16.9 ± 1.2	12.2 ± 0.5	15.8 ± 2.0	1.6 ± 0.6	2.1 ± 0.8	Sandy mud	Sandy mud
16	70.0 ± 12.7	76.6 ± 0.4	16.2 ± 4.0	12.0 ± 0.5	12.4 ± 7.8	10.4 ± 0.6	1.4 ± 0.9	1.0 ± 0.3	Sandy mud	Mud
17	65.3 ± 6.1	64.2 ± 5.7	15.8 ± 1.0	13.5 ± 1.2	14.9 ± 2.9	18.6 ± 5.3	4.0 ± 2.7	3.6 ± 1.3	Sandy mud	Sandy mud
18	54.3 ± 8.5	70.0 ± 3.5	21.8 ± 1.6	15.6 ± 0.4	19.9 ± 6.9	12.9 ± 2.9	3.9 ± 3.2	1.5 ± 0.8	Sandy mud	Sandy mud
19	77.7 ± 2.3	82.1 ± 2.3	12.9 ± 0.4	10.8 ± 0.9	8.4 ± 1.5	6.8 ± 1.1	1.0 ± 0.4	0.3 ± 0.3	Mud	Mud
20	15.8 ± 7.8	67.4 ± 4.7	9.0 ± 2.0	17.5 ± 2.9	54.0 ± 11.1	14.1 ± 2.1	21.3 ±16.6	1.0 ± 0.3	Muddy sand	Sandy mud
21	49.8 ± 10.7	10.9 ± 0.9	23.7 ± 2.8	10.1 ± 0.5	23.9 ± 6.8	72.2 ± 0.8	2.5 ±1.1	6.8 ± 2.2	Sandy mud	Muddy sand
22	7.2 ± 1.1	10.4 ± 1.6	40.5 ± 1.8	31.9 ± 1.1	52.0 ± 2.7	56.5 ± 1.5	0.3 ± 0.1	1.2 ± 1.2	Muddy sand	Muddy sand
23	57.3 ± 6.8	27.5 ± 24.8	18.2 ± 1.1	12.4 ± 10.8	22.2 ± 3.8	23.3 ± 20.7	2.3 ±2.0	3.5 ± 4.5	Sandy mud	Sandy mud
24	69.1 ± 4.8	81.6 ± 1.1	15.9 ± 0.6	10.7 ± 1.0	12.9 ± 3.0	7.3 ± 0.2	2.1 ±1.4	0.3 ± 0.6	Sandy mud	Mud
25	63.3 ± 1.0	62.5 ± 1.5	24.9 ± 2.2	23.3 ± 1.6	10.4 ± 0.6	13.1 ± 0.6	1.4 ±1.1	1.1 ± 0.1	Sandy mud	Sandy mud
26	77.4 ± 3.3	81.5 ± 2.6	12.6 ±0.7	11.0 ± 1.5	9.4 ± 2.7	7.5 ± 1.2	0.6 ±0.1	0	Mud	Mud
27	35.1 ±2.8	32.1 ± 6.6	7.4 ±0.7	7.7 ± 1.4	19.4 ±2.1	25.9 ± 5.2	38.1 ±1.6	34.3 ± 10.5	Sandy mud	Sandy mud
28	77.7 ± 1.1	81.7 ± 4.0	10.4 ± 0.2	9.8 ± 1.2	11.1 ± 0.7	8.2 ± 2.3	0.9 ± 0.9	0.3 ± 0.6	Mud	Mud
29	79.4 ± 3.3	84.6 ± 0.7	10.9 ± 1.3	8.9 ± 0.4	8.9± 1.4	6.5 ± 0.6	0.8 ±0.7	0	Mud	Mud
30		2.6 ± 1.3		4.1 ± 0.9		51.9 ± 6.2		41.4 ± 7.3	-	Fine sand

CHAPTER 4

Phenotypic responses of dinoflagellate strains of different ages in phosphorus-depleted conditions

Context of the work

Marine organisms constantly undergo pressures brought on by changes in their habitat conditions. In consequence, they can manifest physiological response as adaptation, change of the geographic range, invasions and extinctions (Brierley and Kingsford 2009). The Bay of Brest (Brittany, France) has experienced several changes in hydrological conditions since the 50', particularly by important fluctuations in the nutrient concentrations leading to an imbalance of the N/P ratio. Indeed, Phosphorous concentration has diminished markedly in the water after the modification of agriculture policy. Although the consequences of such changes has been studied in the past, revealing changes in dominant species composition and blooms dynamics (Quéguiner and Tréguer 1984), one approach is to take advantage of the fact that some phytoplankton species are able to produce resting stages as part of their life-cycle, which are deposited in the sediment record and can remain viable for long-time periods. These resting stages are physiologically dormant and can be viewed as "time capsules", as they allow for the preservation of biological material through time (Ribeiro *et al.,* 2013). The germination of resting stages formed in the past provides a novel possibility to directly test the response of past living populations to inferred changes in the environment at a multidecadal scale.

In this study, we used dinoflagellate culture strains obtained from cyst germination experiments in the chapter 2 to investigate their ecophysiological responses through time in two Brittany ecosystems (the Bay of Brest and The Bay of Morlaix). Specifically, we investigated the growth rate of populations of *Scrippsiella donghaienis* and *Alexandrium minutum* of different ages in two culture conditions contrasted by their phosphorous (P) concentrations in order to investigate if population of different ages would respond differently to a P limitation. These conditions were supposed to mimic the environmental changes which have occurred in the estuaries of Brittany.

Author contributions

In this work, I performed and optimized the cyst germination experiments, and I established the monoclonal cultures. I performed the genetic identification of strains: DNA extraction from cultures, PCR and gel verifications, sequence analyses and phylogeny for the taxonomic assignation. During my Master 2 internship in 2013, before the beginning of the Ph.D, I set up ecophysiological experiments and carried out preliminary experiments. These outcomes have been used to formulate the hypotheses of this part of the Ph.D. work which have been mainly carried out in the frame on the Master 2 internship of M. Georges des Aulnois, whom I supervised in collaboration with R. Siano. M. Georges des Aulnois performed ecophysiological experiments and data analyses. C. Labry helped in

designing the ecophysiological experiments and the interpretation. L. Guillou, isolated and cultivated strains from revived cysts from the Bay of Morlaix (Penzé Estuary) which have been used for this part of the Ph.D work. M. Latimier, J. Quéré, A. Youenou (technicians) helped in conceiving the experiments, they also performed the analyses on experimental strains (cell counting, fluorescence measurement, cytometer counting). I followed the data analyses and interpretation and I participated to text writing in French (Master 2 report), which has been mainly performed by M. Georges des Aulnois. I performed the English translation of the final manuscript. R. Siano was the research designer.

Phenolotypic responses of dinoflagellate strains of different ages in phosphorus-depleted conditions

Résumé (en français)

Les réponses phénotypiques des protistes marins aux perturbations de l'environnement côtier ont été peu étudiées à l'échelle multidécennale. Un cycle de vie court associé à une diversité génétique et phénotypique pouvant s'exprimer au niveau d'une même population, leur permettent de répondre rapidement aux variations de l'environnement. Nous avons étudié, les différentes caractéristiques physiologiques de populations de dinoflagellés anciennes et modernes, lors d'une modification du ratio N/P. Ces populations de dinoflagellés ont été obtenues à partir de kystes anciens préservés dans les sédiments de l'estuaire de l'Elorn (rade de Brest) et de Penzé (Baie de Morlaix). Le taux de croissance, les paramètres intracellulaires (contenu en chlorophylle *a*, taille et complexité des cellules) de 20 souches de *Scrippsiella donghaienis* et d'*Alexandrium minutum* datant des années 70', 80', 90' et 2000 ont été analysées à partir de deux milieux de culture différents censés représenter l'évolution du rapport N/P depuis une soixantaine d'années dans les estuaires bretons (N/P=25 et N/P=100). Nos résultats montrent une variabilité intraspécifique de la croissance des souches et de leur consommation du phosphore, conduisant au rejet de l'hypothèse initiale d'une séparation des populations de dinoflagellés en fonction de leur âge. Ces résultats suggèrent l'existence d'une plasticité phénotypique des populations de dinoflagellés à l'échelle de plusieurs décennies.

Phenolotypic responses of dinoflagellate strains of different ages in phosphorus-depleted conditions

Klouch K.Z, Georges des Aulnois M., Labry C., Latimier M., Quéré J., Youenou A., Guillou L., Siano R.

Abstract

The phenotypic responses of marine protist to environmental perturbations of the coastal ecosystem have been rarely studied across a decadal time-scale. Given their rapid life cycles and their genetic and phenotypic intraspecific diversity, marine protists can rapidly respond to environmental changes even at the population level. Here, we studied if ancient-to-modern populations of marine dinoflagellates revived from up to 50 years old cysts buried in sediments of the Elorn Estuary (Bay of Brest) and Penzé Estuary (Bay of Morlaix), might have developed different physiological characteristic in relation to the changes in the N/P (25 to 100 (Nitrogen/Phosphorous)) ratio occurred over the last century in the Brittany estuaries. In particular, we analysed the growth rates and intracellular parameters (chlorophyll *a*, cell size and complexity) of 20 strains of *Scrippsiella donghaienis* and 6 strains of *Alexandrium minutum* of the years '70, '80, '90 and 2000 when cultivated in a dystrophic (100) and balanced (25) N/P ratio which mimic phosphorous limitation occurred in Britanny estuaries. Our results showed an intraspecific variability in the strain growth, and phosphorous assimilation leading to discard the initial hypothesis of the separation of population according to their ages. Furthermore, these results suggest a phenotypic plasticity of dinoflagellate populations which can be expressed over a multidecal time-scale.

1. Introduction

Marine ecosystems are influenced by ecological changes at different temporal and spatial s scales. At the global scale, during the last century, the phytoplankton biomass decreased (Boyce *et al.,* 2010) and areas of low chlorophyll *a* concentration have expanded (Polovina *et al.,* 2008). During the last 200 years, the coastal ecosystems, suffered from eutrophication events (Cloern 2001) and from spatial-temporal variations of phytoplankton communities (Hallegraeff 2010) as a consequence of climate changes and human population development. In particular, these changes can be attributed to the nutrient enrichment (Nixon 2005) and/or the deregulation of the trophic balance of the ecosystem (Vilmi *et al.,* 2015). In fact, the nutrient concentration, their ratios C/N/P (Redfiled 1958) and the competition for nutritive resources (Lagus *et al.,* 2004) are important factor affecting the structure of marine protist communities.

In Europe, the French coasts experienced important hydrological variations during the last century. The development of human and farming activities led to fertilization of the coastal waters. As a consequence, the nutrient balance has been disturbed (Delmas and Tréguer 1983). The Bay of Brest suffered from those perturbations as documented by investigations carried out in this ecosystem over the past 50 years (Chauvaud *et al.,* 2000; Hafsaoui *et al.,* 1985; Le Pape *et al.,* 1996). The bay receives high nutrient inputs from 5 watersheds and is strongly influenced by the tidal cycle. Studies of the consequences of these two types of physical forcings have shown that the Bay of Brest is a highly nutrient-enriched area (Le Pape *et al.,* 1996). The development of the urban area contributed to the increase of phosphorus inputs in the bay, before the ban of washing powders containing orthophosphates leading to a decrease of phosphorous concentrations in the environment (Guillaud and Bourriel 2007). At the same time, the watersheds of the bay were marked by an increase of nitrogen inputs due to the intensive farming activities on the lands (Cann 1995).

In almost all of the rivers of Brittany, the nitrogen concentration doubled between 1970 and 1990 and increased tenfold since the beginning of the century (Le Pape *et al*., 1996). As a consequence, the period of minimal nitrogen concentrations was reduced from 5 months in 1970 to 1 month in 1990 (Daniel 1995). From 1990, the change of the agricultural policy of the region, led to a significant reduction of 80% of nitrogen concentration in the watershed of Britanny (Guillaud and Bourriel 2007).

In view of the fact that that the nutrient ratio (Si/C/N/P) drastically changed in the Bay of Brest since 1950, numerous studies have aimed at investigating the risk of eutrophication and the possible consequences on the phytoplankton dynamics (Delmas and Tréguer 1983; Quéguiner and Tréguer 1984; Hafsaoui *et al.,* 1985; Le Pape *et al.,* 1996; Del Amo 1997; Guillaud and Bourriel 2007; Trommer *et al.,* 2013). During the 1970s, nitrogen was considered as the limiting factor for phytoplankton

growth in the summer (Quéguiner and Tréguer 1984). Conversely, phosphorus concentration remained low and stable, being controlled by adsorption-desorbption phenomenon at the interface water-sediment (Hafsaoui *et al.,* 1985). Recently, Trommer *et al*., (2013) also showed that the inorganic phosphate is limiting from March to June in the Bay of Brest. The perturbation of the ratio N/P affected bloom dynamics (Quéguiner and Tréguer 1984). The intensity of the spring blooms decreased in favour of summer blooms (Chauvaud *et al.,* 2000). In terms of species composition, Quéguiner and Tréguer (1984) showed that diatoms dominated the blooms between 1981 and 1982. A few years later, the blooms were dominated both by diatoms and dinoflagellates (Del Amo *et al.,* 1997). These variations in the specific composition of phytoplankton community were supported by Beucher *et al*., (2004) who showed that the phosphorus limitation can favour dinoflagellate blooms. Modelling studies and *in vitro* experiments demonstrated that an increase of dinoflagellate biomass may be favoured by nitrogen enrichment and/or low phosphorus concentration (Chapelle *et al.,* 2010). Finally, mesocosm experiments on natural samples confirmed that nitrogen enrichment associated to silica limitation favoured dinoflagellate growth (Claquin *et al.,* 2010).

The importance of phosphorous for dinoflagellate dynamics was confirmed by ecophysiological experiences. Dinoflagellates showed a better growth in inorganic phosphate-limited medium than other phytoplankton organisms like diatoms (Lin *et al.,* 2016). Moreover, different phosphorus concentrations induced different strategies of growth between the two dinoflagellate species, *Alexandrium minutum* (toxic species) and *Heterocapsa triquetera* (nontoxic species) (Labry *et al.,* 2008). However, such experiments did not consider the degree of variability between different strains because of the limited number of strains considered simultaneously (Langer *et al.,* 2009; Alpermann *et al.,* 2010). Yet, modeling analyses showed an important intraspecific variability in the phosphorus cell content of different strains of the diatom, *Thalassiosira pseudonana* (Fredrick *et al.,* 2013).

Because of their rapid generation time and longevity, many phytoplankton species are expected to respond to environmental changes at short time-scales (Hallegraeff 2010). Therefore, in experimental *in vitro* approaches, it is relatively easy to study the potential niche and the potential adaptation of modern phytoplankton strains to environmental changes, mimicking the natural environment conditions. For example, Collins and Bells (2004) have studied the adaptive responses of *Chlamydomonas* in response to increasing CO_2 concentration over 1000 generations. The cells acclimatized to these changes but no genetic changes could be described. This experiment demonstrated the abilities of acclimatization rather than adaptation (Collins and Bells 2004). Another approach is possible to understand the evolution history of marine protist, especially for dinoflagellate. Indeed, some representatives of this group can form resting stages (cysts) whenever

the environmental conditions become unsuitable for their development in the water column. Resting cysts settle at the sea floor and accumulate in the sediments. The resting cysts in the sediment ensure the survival of a population in the ecosystem and provide the inoculum for the initiation of seasonal blooms (Anderson and Wall 1978). The existence of these forms allows the populations to resist to environmental fluctuations even at a long time-scale (Tyler *et al.,* 1982) in the absence of sediment resuspension and if favourable physical-chemical conditions (*e.g.* anoxia) occur. Resting stages deposited in marine sediments can remain viable for many decades and be revived in the laboratory (Ribeiro *et al.,* 2011; Lundholm *et al.,* 2011; Miyazono *et al.,* 2012). The use of strains revived from ancient cysts is an alternative source of information on population's evolution of a species. For example, Ribeiro *et al*., (2013) reported on ecophysiological variability of the marine *Pentapharsodinium dalei* populations over a 100-year period of experienced shifts of salinity in a Swedish fjord.

Are the changes in phytoplankton community observed during the last 50 years in the Bay of Brest due to the ability of dinoflagellate populations to adapt to nutrientsfluctuations? In order to contribute to this debate, in this study we used the living dinoflagellate sedimentary record of the Bay of Brest and the Bay of Morlaix. We studied the physiological responses of populations of different ages of the dinoflagellates *Scrippsiella donghaienis* and *Alexandrium minutum* when cultured in dystrophic N/P ratios with P-depleted conditions. The culture conditions used here, tried to mimic the environmental changes experienced by the dinoflagellate populations in the estuaries of Brittany. We tried to investigate on potential different physiological responses of populations of different ages using the P limitation as a potential factor differentiating the populations studied.

To the best of our knowledge this study represents the first attempt to understand the ability of marine dinoflagellate species to adapt to nutrient changes of the environment using revived populations from sediments.

2. Material and methods

Here we used the terms of "strain age" to refer to the time period in which cyst are supposed to accumulate according to sediment layer dating provided for the Bay of Brest (Elorn Estuary, EE) in Klouch *et al.,* (2016) and for the Bay of Morlaix (Penzé estuary, EP) (Figure 34, Table 7). Sediment dating methods are provided in Klouch *et al.,* (2016).

Figure 34. Sampling sites of sediment cores.

Table 7. Culture stains selected for experimentations.

Elorn Estuary (EE)	-	***S. donghaienis***	
Sediment layer dating (years)	**Layer depth (cm)**	**Strain ID**	
1993 ±1	10	Sc 51, Sc 53, Sc 150	
1986 ± 2	13	Sc 14, Sc 15, Sc 20	
1978 ± 2	17	Sc 27, Sc 28, Sc 29	
Penzé Estuary (PE)		***S. donghaienis***	***A. minutum***
Sediment layer dating (years)	**Layer depth (cm)**	**Strain ID**	**Strain ID**
2006 ± 2.3	15	Sc 21, Sc 22, Sc 65	Am 76, Am 77
1998 ± 4.6	30	Sc 129, Sc 130, Sc 142	-
1997 ± 4.9	31	-	Am 53, Am 55, Am 60

Cyst germination experiments were performed similarly to previous paleoecological studies on diatoms (Härnström *et al*., 2010) and dinoflagellates (Miyazono *et al*., 2012). Sediment samples (~5 cm^3) were added to filtered seawater and placed in an ultrasonic bath for 6 min to separate dinoflagellate cysts from inorganic particles. The sediments were sieved with filtered seawater through a 100-µm onto a 20-µm sieve to separate the sediment size fraction containing the majority of dinoflagellate cysts. Different media (F/2, K, K/2, site-specific seawater) and temperature (16 and 18°C) conditions were tested to optimize cyst germination. Irradiance (60 µmol photons m^{-2} s^{-1}) and photoperiod (12h:12h) were kept constant during this optimization. Finally, the combination of 16°C

and K medium (Keller *et al*., 1987) was retained. A few drops of the 20-100 µm fraction were distributed onto a 12-well plastic plate containing K medium (2 mL per well). The plates were placed in a culture room at 16°C, under an irradiance of 60 µmol photons m^{-2} s^{-1} and a light:dark cycle of 12h:12h. The plates were examined for vegetative cell germination once to three times per week using an inverted microscope (Zeiss Axiovert 135). *Alexandrium minutum* cells were identified by light microscopy and 13 culture strains were established from 3 sediment layers of the EP. For *Scrippsiella* spp., 82 cultures strains were established from 12 and 5 sediment layers of the EE and EP cores, respectively. In addition, 9 other strains of *S. donghaienis* were obtained from the Daoulas Estuary core (DE) but these were not used in this study due to the limited number of strains per layer (<3), a perquisite we imposed in this study.

The cultured strains were identified genetically. DNA was extracted from the strain cultures using the DNeasy plant mini kit (QIAGEN, Germany) following the manufacturer's instructions. The LSU rDNA region was amplified by PCR using dinoflagellate-specific primers (Ldino6, Ldino1) from Probert *et al*., (2014) and the following PCR program: 35 cycles composed of an initial denaturation at 95°C for 2 min, a denaturation at 95°C for 30 sec, a hybridization at 56°C for 30 sec, an elongation at 72°C for 1 min and a final elongation at 72°C for 5 min. After purification by the QIAquick PCR purification kit (QIAGEN, Germany), the PCR products were sent to GATC Biotech (http://www.gatcbiotech.com/en/products/sanger-services) for Sanger sequencing.

2.1. Ecophysiological experiments in variable N/P ratio conditions

Among the 95 strains of EE and EP (82 for *S. donghaienis* and 13 for *A. minutum*), Twenty strains were randomly selected based on the number of strains (≥ 3 per layer). We finally considered 3 and 2 periods of time, respectively in the Elorn estuary (EE) and the Penzé estuary (PE) (Table 7). These strains were maintained during 3 years in the laboratory on K medium and 34.5 salinity, with a photoperiod 12h:12h under an irradiance of 60 µMol photons m^{-2} s^{-1} before the beginning of ecophysiological experiments.

In our experimental conditions, batch cultures were conducted in triplicates for each strain and nutrient condition. In order to study the responses of each strain to a variation of N/P and in particular to inorganic phosphate, we used the F/2 culture medium (Guillard and Ryther 1962) which contains only one source of inorganic phosphate (PO_4^{3-}) and N/P ratio=25. The P-depleted culture medium named as ($F/2_{\ P/4}$) was prepared from the original recipe of the F/2 culture medium by reducing the concentration of inorganic phosphate by a factor of four to get a final N/P ratio=100. The concentration of each component is summarized in Table 8. Natural autoclaved and filtered water (0.22 µm) from the Biological Station of Argenton (IFREMER) was used to prepare the growth

media in sterile conditions. Twenty four hours after an enrichment of the sea water, a second filtration was conducted in order to discard the silica flocculants.

Table 8. Nutrient composition of the culture media used in this study.

Compounds	F/2 medium concentration	F/2 $_{P/4}$ medium concentration
$NaNO_3$	8.83×10^{-4}	8.83×10^{-4}
NaH_2PO_4, H_2O	3.63×10^{-5}	9.08×10^{-5}
Na_2SiO_3, $9H_2O$	1.07×10^{-4}	1.07×10^{-4}
N/P ratio	25	100

Before the experiment, the strains were acclimatized for each experimental condition during 10 days. For the acclimatization phase as well as for the experimental phase, the salinity, irradiance and photoperiod conditions were kept the same as for the maintenance conditions. The temperature was increased from 16 to 18°C in order to stimulate the growth activity during both the acclimatization phase and experimental phase.

An inoculum of 500-1000 cells mL^{-1} in the exponential growth phase has been used for the 20 strains and the two media conditions. The volume required to obtain the selected inoculum was calculated by counting a subsample of 1 mL of the original cultures on Nageotte counting cell under a light microscope. For each sample, a minimum of 40 stripes was counted. Each experimental culture was completed to a final volume of 180 mL in plastic, non-ventilated flasks (250 mL volume). A total of 120 cultures were launched simultaneously and followed on a daily basis during 12 days (from t_0 to t_{11}). Each day, 5 mL of culture were sampled for the measurement of different parameters, following the same protocol: i) 2 mL were used for fluorescence analyses, performed on a microplate fluorescence reader to estimate the cell growth daily, ii) 1 mL was fixed, in two replicates, in a mix of glutharaldéhyde (50%) and poloxamer (Pluronic) (10%) for flow-cytometry analyses (Marie *et al.,* in Andersen, 2005), iii) An aliquot of 1 mL was fixed with Lugol solution (4%) for counting in light microscopy. In order, to verify nutrient, and in particular P limitation of the cultures, the concentration of inorganic phosphate and nitrate were monitored at the beginning of the experiment (t_0) and the end of the exponential growth phase and estimated by *in vivo* fluorescence measurements (t_{lim}). For nutrient analyses, 20 mL of culture were filtered on 0.45 µm and stored in plastic falcon before freezing (Aminot and Kérouel 2004). Only one replicate was analysed at t_0, and all the replicates were analysed At t_{lim}. All nutrients were analysed by automatic methods according to Aminot and Kérouel (2007). Nitrate and Inorganic phosphate concentrations were analysed for all the strains of the EE but only for the strain SC 142 of the PE, chosen on the basis of growth dynamic analyses. Measurements of pH were performed on 10 mL of cultures at t_0 and $t_{lim,}$using an electronic

pH-meter, Mettler Toledo SevenEasy, calibrated with two buffer solutions at pH 7 and 9.2 (Aminot and Kérouel, 2004).

2.2. Cell growth analyzed using in vivo fluorescence

In order to estimate in real-time, the cell growth chl *a* fluorescence was recorded every day on a Biotek FLX 800 plate reader. The analyses were conducted on a multiwell plate (24 wells). The fluorescence was recorded after submitting 2 mL of culture to an excitation at 480 nm and an emission at 680 nm. For each well, 81 reads were collected. These analyses were carried out every day from t_0 to t_{lim} at the same hour at least 3 hours after the lighting of culture chambers.

2.3. Cell growth analyzed by flow cytometry

The cell concentration of the 120 cultures was estimated every day by flow cytometry measurements using a BD FACSAria cytometer and a red laser (488 nm), at the Biological Station of Roscoff (Roscoff, France).Three parameters were estimated using 3 scatters (chl *a*, Side Scatter (SSC) and Forward Scatter (FSC)). The chl *a* scatter allowed to get an estimation of cell growth and to analyse the evolution of chl *a* per cell. The SSC and FSC are proxies for the length and the complexity of the cells, respectively. Each analysis was conducted during 1 min with a cell flow comprised between 80 and 100 µL min^{-1}. In order to calibrate our data, 10 µL of a bead solution (3 µm) were added to each sample before the measurements (Marie *et al*., in Andersen 2005). The analysis was performed with an emitting laser at a wavelength of 488 nm and using a green fluorescence scatter (525 ± 20 nm) and a chl *a* scatter (680 ± 40 nm). Bacteria counting (one replicate per strain and per nutrient condition) were conducted after the analysis of dinoflagellate cells on a BD Accuri C6 cytometer. Before the analysis, samples were diluted by 100 in a tris buffer solution (pH = 8) containing SYBR Green (1/10 000) and incubated for a minimum of 15 minutes before the analysis.

2.4. Growth rate calculation

Strain specific exponential growth rates (µ) were determined from flow cytometric counts. Growth rates were estimated for a minimum of 4 consecutive points. Cell concentrations were linearized by a logarithm transformation. In an exponentially growing culture, the growth rate corresponds to the slope of the straight line (Wood *et al*., in Andersen 2005). The growth rate µ (d^{-1}) was calculated as follows:

$$\mu = \frac{\ln (N) - \ln (N_0)}{t} \quad => \quad \ln (N) = \mu t + \ln (N_0)$$

with:

μ: growth rate in day^{-1}

t: number of days used in the regression

N: cell concentration cells mL^{-1} at the last sample of the regression period

N_0: cell concentration at t_0

2.5. Statistical analyses

In order to reveal differences between populations of different ages and between the culturing conditions, the data of the strains were grouped according to their age and the growth medium. Each age was studied using data obtained with 3 strains cultured in triplicate (except for *A. minutum* from 1997 ± 4.9 in PE for which only 2 strains were available). The growth dynamic of each strain was analysed by using the mean cell concentration (3 replicates) for each experimental day.

The differences between periods were analysed using the mean growth rate (3 replicates) and the maximal cell concentration for each strain and each culture medium. The statistical analyses were performed using R software (R Development Core Team 2012). Shapiro-Wilk test was used to verify the normality of the data distribution. Considering the low number of strains, non-parametric statistical tests were chosen for analysing all of the measured parameters. For every culture, differences between F/2 and F/2 $_{P/4}$ medium in terms of growth rates and maximal cell concentration were tested using a Wilcoxon-Mann Whitney test (n=3) (level of significance 5%).

In order to verify the homogeneity of the strains triplicates for each age and culture condition, a Kruskall-Wallis (K-W) (level of significance 5%) was conducted on growth rate and maximal cell concentration data. If the K-W test was significant, a *post hoc* Nemenyi test (level of significance 5%) was applied. The same tests were used in order to verify a possible age effect for each culture condition both in growth and maximal cell concentration data.

3. Results

3.1. Initial (t_0) experimental conditions

At t_0, cell concentrations ranged between 269 and 1114 cells mL^{-1}. Inorganic phosphate (Pi) concentrations ranged between 31.25 and 33.65 µM for F/2 and between 6.21 and 8.00 µM for F/2 $_{P/4}$. Nitrate concentrations for F/2 and F/2 $_{P/4}$ ranged between 901 and 932 µM. The N/P ratio for F/2 medium ranged between 27 and 29 whereas for F/2 $_{P/4}$ it ranged between 114 and 146.

3.2. Cell concentration dynamics

Cell concentration increased exponentially as a function of time for almost all strains of *Scrippsiella donghaienis* from the EE (Figure 35) and the EP (Figure 36) for both nutrient conditions. Low variation between replicatesfor each strain was observed as indicated by low standard errors.

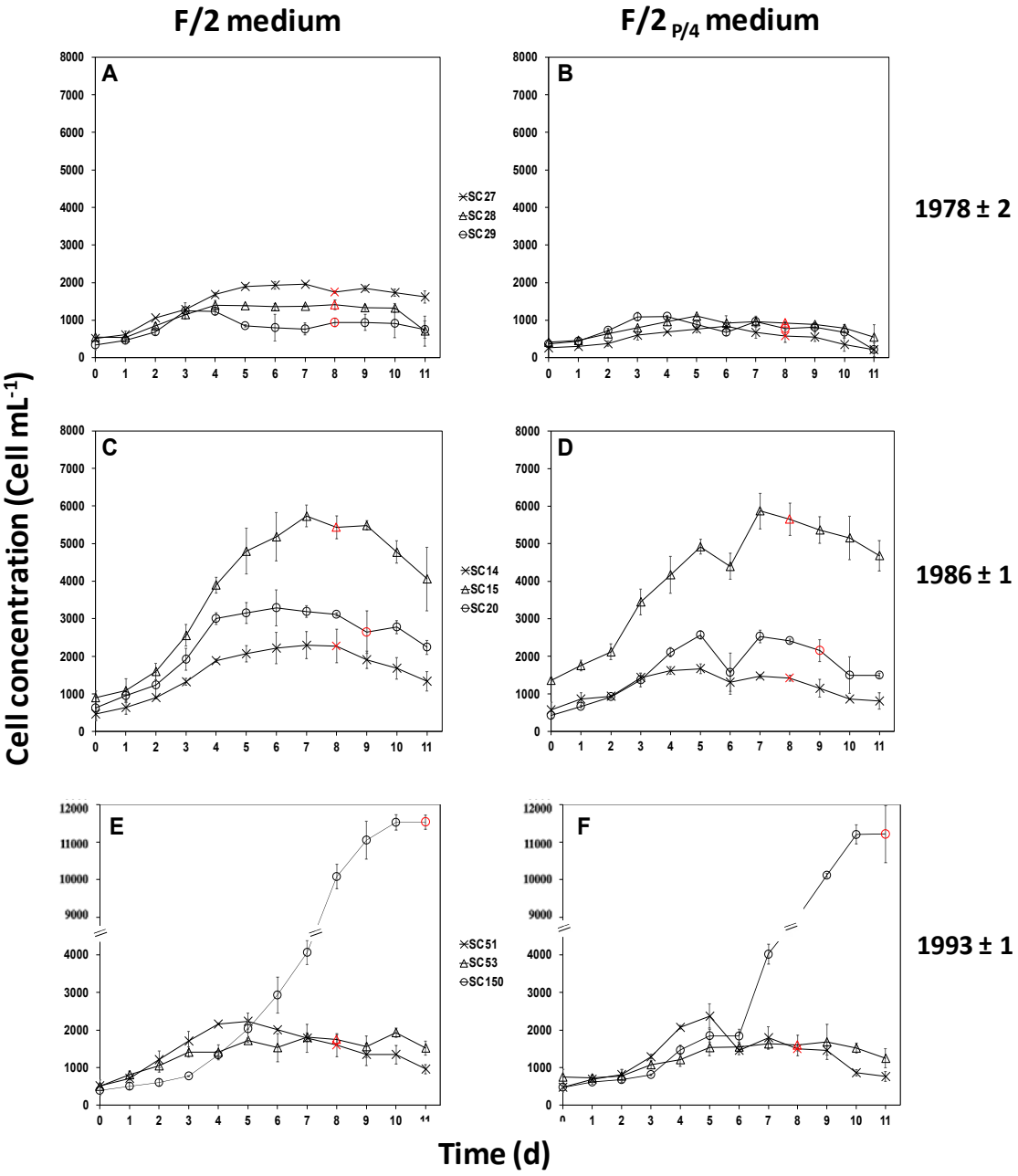


Figure 35. Cell concentration as a function of time in the two culture conditions for *S. donghaienis* strains (three ages) of the EE. Each point of the mean value of the three replicates and errors bars represent the standard error of the means. For each strain the sampling of nutrient and pH, chosen on the basis of the fluorescence data is represented in red.

For the EE, growth dynamics differed between strains for each period. Strains from 1978 ± 2 seemed to be more homogenous with a cell concentration of all strains <ca. 2000 cells mL^{-1}. Strains SC14, SC15, SC20 (1986 ±1) reached maximal cell concentration (F/2: 2136 ± 112, 5737 ± 279, 3419 ± 273 cells mL^{-1}; F/2 $_{P/4}$: 1701 ± 100, 6030 ± 382, 2648 ± 81 cells mL^{-1}). The duration of exponential phase was different among strains and for both growth media. (Figure 35-C,D).

The period 1993 ± 1 was marked by the growth dynamic of the strain SC150 (maximal cell concentration reached F/2: 11581 ± 291 cells mL^{-1}; F/2 $_{P/4}$: 11269 ± 1192 cells mL^{-1}) 6 fold higher than those of the strains SC51 and SC53. No differences was observed in growth between the two media (Figure 35-E,F).

For the EP, growth dynamics were more homogeneous for the strains of 1998 ± 4.6 than those of 2006 ± 2.3 (Figure 36). Little differences were obtained between growth media for the strains SC129 and SC130. For SC142, no growth limitation was observed in F/2 medium until $t_{9,}$ whereas a limited growth occurred from t_7 in the F/2 P/4 medium. Maximal cell concentration for SC142 in F/2 $_{P/4}$ (6076 ± 54 cells mL^{-1}) was lower than in F/2 medium (10054 ± 819 cells mL^{-1}). As for the period 2006 ± 2.3 in F/2, SC 22 showed a better growth and maximum cell concentration than strains SC21 and SC65. SC21 and SC22 showed a lag phase from t_0 until t_7 in F/2 $_{P/4}$ (Figure 36-C,D).

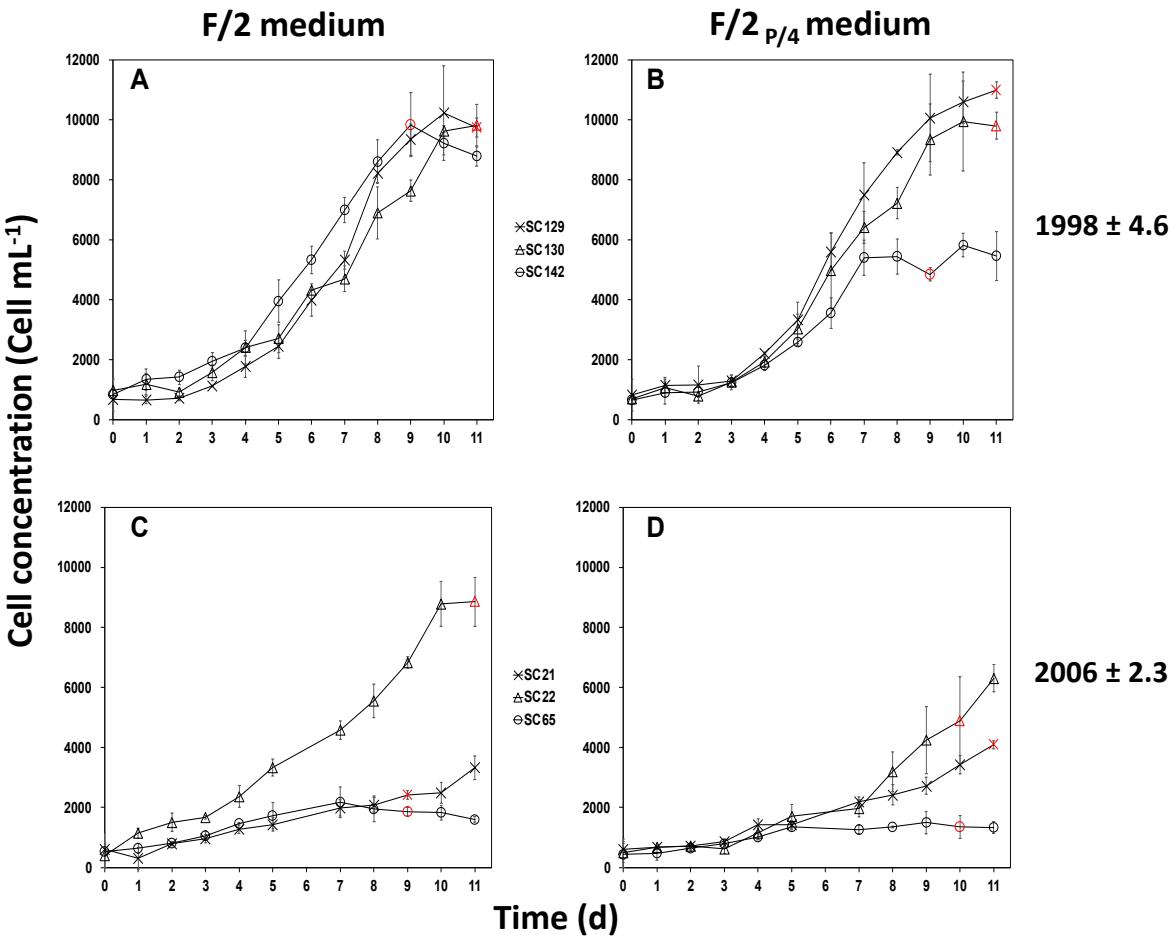


Figure 36. Cell concentration as a function of time in the two culture conditions for *S. donghaienis* strains (three ages) of the EP. Each point of the mean value of the three replicates and errors bars represent the standard error of the means. For each strain the sampling of nutrient and pH, chosen on the basis of the fluorescence data is represented in red.

Maximal cell concentration of of *A. minutum* strains isolated from PE were < ca. 2500 cells mL^{-1} except for the strains Am55 (F/2: 17809 ± 958 cells mL^{-1}; $F/2_{P/4}$: 12947 ± 362 cells mL^{-1} (Figure 37C,D). Growth dynamics for Am76 and Am77 of 1997 ± 4.9 are similar (Figure 37A,B). No difference in terms of maximal cell concentration was evidenced between the two culture media for both strains of 1997 ± 4.9 and 2006 ± 2.3.

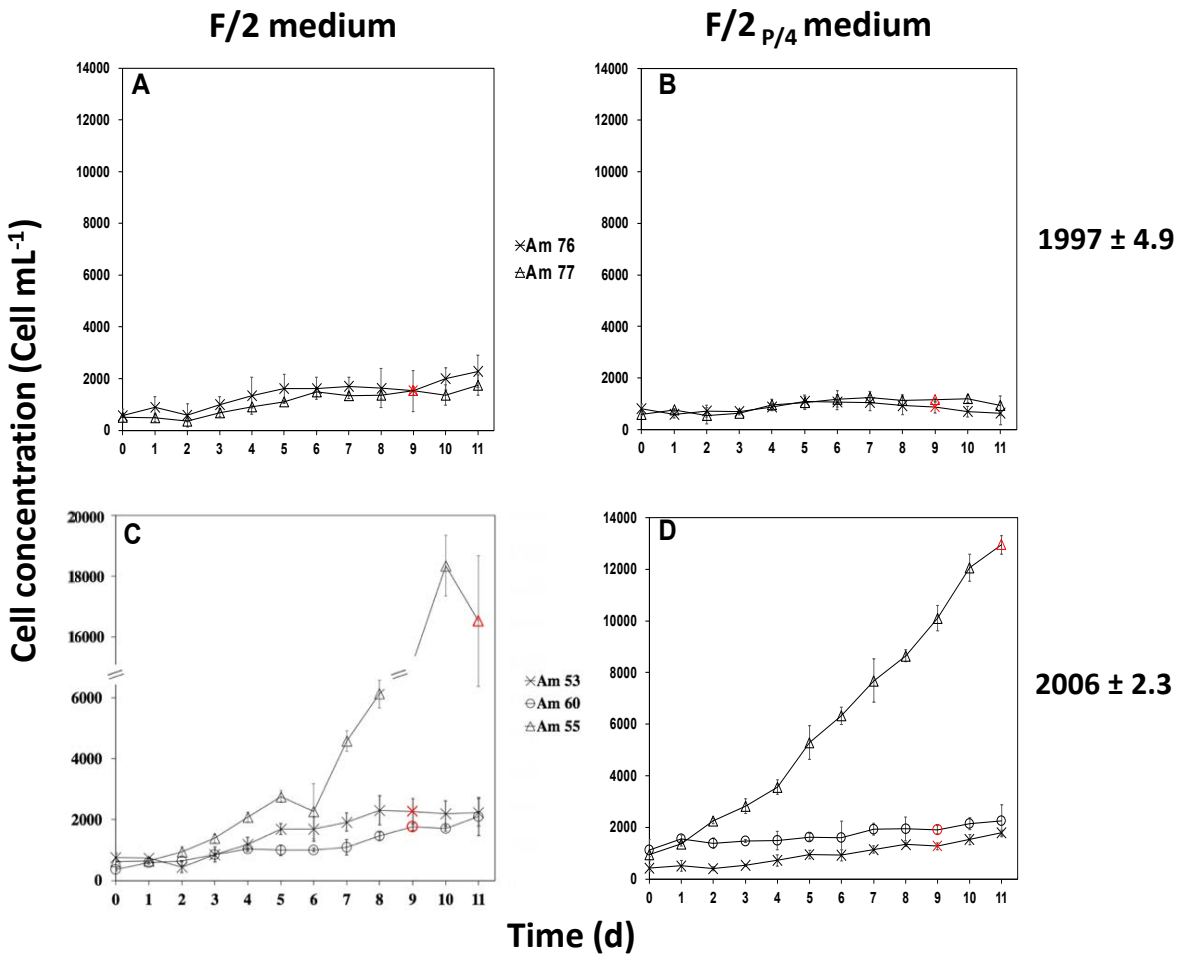


Figure 37. Cell concentration as a function of time in the two culture conditions for *A. minutum* strains (two ages) of the EP. Each point of the mean value of the three replicates and errors bars represent the standard error of the means. For each strain the sampling of nutrient and pH, chosen on the basis of the fluorescence data is represented in red.

3.3. Growth rate analyses

For all the strains, the growth dynamics were obtained through an analysis of the growth rate μ (d^{-1}) and the maximal cell concentration (cells mL^{-1}) in order to seek for significant differences between populations of different ages. The calculated growth rate (μ) on a minimum of 4 successive samples were positive. The growth rate ranged between 0.05 ± 0.01 d^{-1} (Am 60 (F/2 $_{P/4}$)) and 0.46 ± 0.05 d^{-1} (SC 150 (F/2)) (Tables 9,10).

At EE, significant differences between the growth rates of the strains SC51 and SC53 (1993 ± 1) cultivated in F/2 $_{P/4}$ were evidenced, demonstrating a heterogeneity within the populations of this age. For both species, no significant differences were observed in the mean growth rate values according to the strain age, within the different media (Figure 38, Table 3).

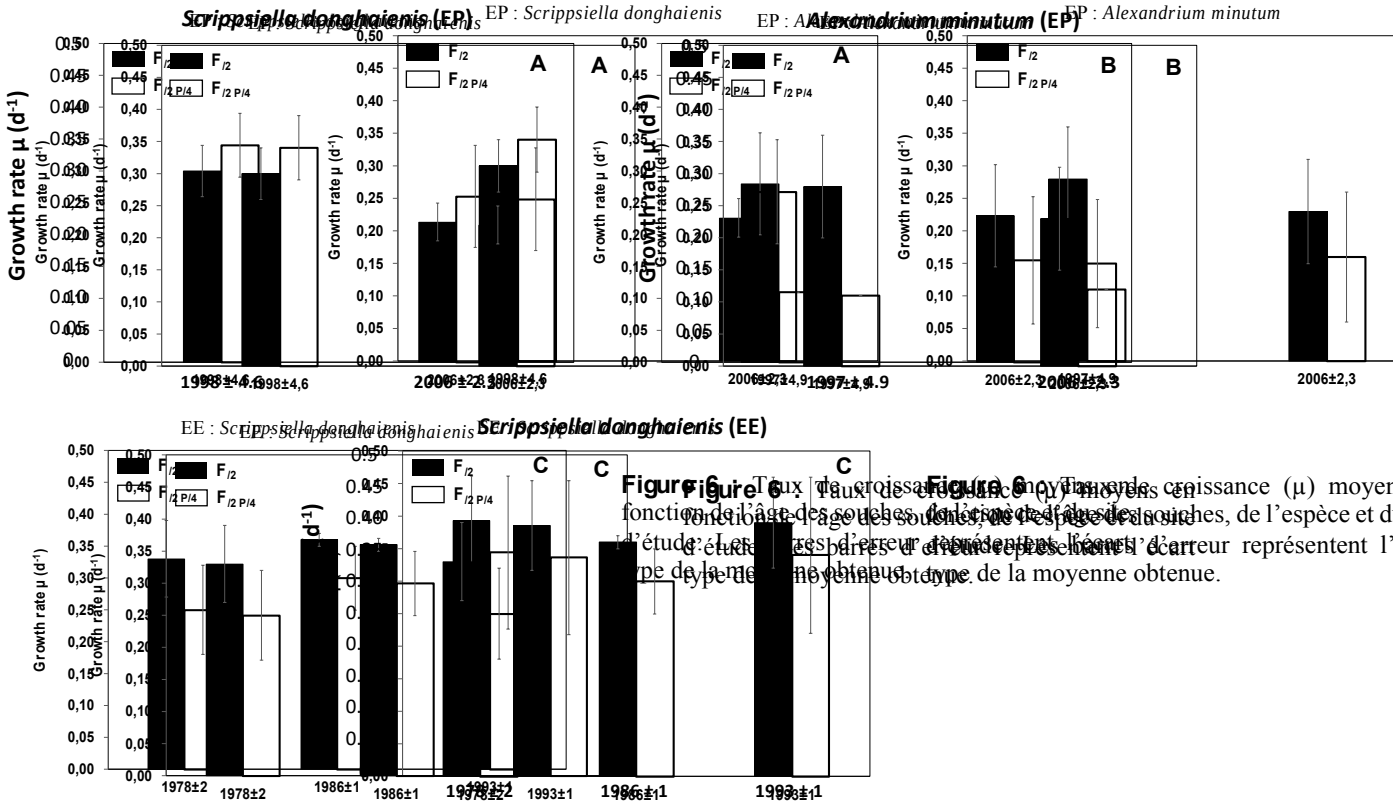


Figure 38. Average growth rate (μ) for *S. donghaienis* and *A. minutum* in the two culture conditions, according to the strain age and the study site.

Table 9. Statistical tests applied on the growth rates for all *S. donghaienis* strains of the EE.
The mean growth rate for the three replicates and the corresponding standard error are reported for each strain. The p values < 0.05 indicating the significance of the test are represented in bold.

		F/2		F/2 P/4		Wilcoxon	
Strain age (year)	**Strain**	**μ (d^{-1})**	**Standard Error**	**μ (d^{-1})**	**Standard Error**	**p-value < 0.05**	**p-value**
1978 ± 2	SC 27	0.28	0.02	0.21	0.02	-	0.0722
	SC 28	0.3	0.05	0.22	0.03	-	0.2
	SC 29	0.4	0.06	0.33	0.03	-	0.2683
	Mean	0.33	0.06	0.25	0.07	-	0.4
		p= 0.0972	K-W	p= 0.0571	K-W		
1986 ± 1	SC 14	0.35	0.04	0.26	0.09	-	0.3737
	SC 15	0.37	0.05	0.28	0.01	-	0.1
	SC 20	0.37	0.06	0.37	0.01	-	1
	Mean	0.36	0.01	0.30	0.05	-	0.3537
		= 0.71	K-W	p= 0.1472	K-W		
1993 ± 1	SC 51	0.38	0.01	0.48	0.05	-	0.1
	SC 53	0.33	0.05	0.24	0.07	-	0.2683
	SC 150	0.46	0.05	0.31	0.02	-	0.1
	Mean	0.39	0.07	0.34	0.12	-	0.7
		p= 0.0549	K-W	**p= 0.0438**	K-W		
				*** : 51-53**	Post hoc		

Table 10. Statistical tests applied on the growth rates for *S. donghaienis* and *A. minutum* strains of the EP. The mean growth rate for the three replicates and the corresponding standard error are reported for each strain. The p values < 0.05 indicating the significance of the test are represented in bold.

		F/2		F/2 $_{P/4}$		Wilcoxon	
Strain age (year)	**Strain**	**μ (d^{-1})**	**Standard Error**	**μ (d^{-1})**	**Standard Error**	**p-value< 0.05**	**p-value**
1998 ± 4.6	SC 129	0.34	0.04	0.38	0.05	-	0.3758
	SC 130	0.26	0.03	0.28	0.01	-	0.3458
	SC 142	0.29	0.07	0.36	0.03	-	0.4
	Mean	0.30	0.04	0.34	0.05	-	0.4
		p= 0.1716	KW	p= 0.0614	KW		
2006 ± 2.3	SC 21	0.19	0.04	0.18	0.02	-	0.8248
	SC 22	0.25	0.04	0.34	0.06	-	0.2878
	SC 65	0.22	0.11	0.27	0.03	-	0.7
	Mean	0.22	0.03	0.26	0.08	-	0.7
		p=0.5501	KW	**p= 0.0390**	KW		
				*** : 21-22**	Post hoc		
1997 ± 4.9	Am 76	0.22	0.06	0.11	0.02	-	0.1
	Am 77	0.33	0.02	0.11	0.06	-	0.1
	Mean	0.275	0.08	0.11	0	-	0.2207
	P= 0.1		Wilcoxon	P= 0.7	Wilcoxon		
2006 ± 2.3	Am 53	0.25	0.06	0.2	0.05	-	0.3758
	Am 55	0.29	0.03	0.23	0.01	-	0.0765
	Am 60	0.14	0	0.05	0.01	-	0.0636
	Mean	0.23	0.08	0.16	0.10	-	0.4
		p= 0.0459	K-W	**p= 0.0580**	K-W		
				*** : 51-53**	Post hoc		

At the EP, significant differences between the growth rates of strains SC21, SC22 and SC65 (2006 ± 2.3) for the F/2 $_{P/4}$ medium have been evidenced, showing the heterogeneity of the growth within this group (Table 10). For both species, no significant differences in the growth rate were observed according to the strain age, within the different media. In summary, the analysis of the growth rate did not allow differentiating the populations according to their age or their culture medium. However, significant differences of the growth rate values were observed within populations of the same age, showing the heterogeneity of these groups.

3.4. Maximum cell concentrations analyses

No significant difference has been evidenced between maximum cell concentrations of strains of different ages for both media. At EE, significant differences were observed among the maximum cell concentration values of the strains of the same age for the F/2 medium. For the F/2 $_{P/4}$ medium, significant differences occurred for the 1986 ± 1 and 1993 ± 1 (Table 11).

At the EP, significant differences were evidenced between the maximum cell concentration of strains SC21, SC22 and SC65 (2006 ± 2.3) for both F/2 and F/2 $_{P/4}$ media and for strains of *A. minutum* for the

F/2 $_{P/4}$ (Table 12). These results highlight the heterogeneity of the maximum cell concentrations reached by strains of the same age (Figure 39).

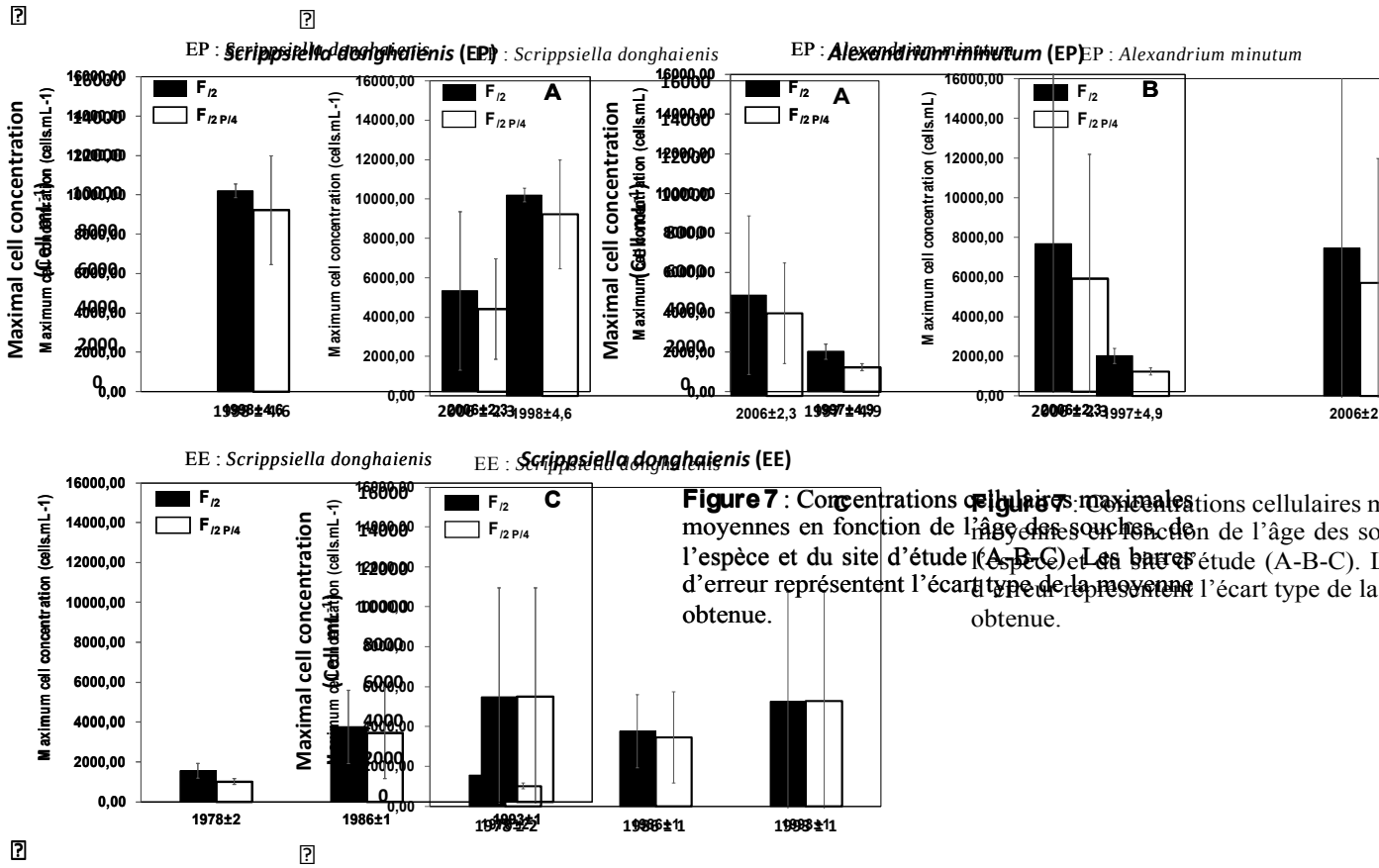


Figure 7 : Concentrations cellulaires maximales moyennes en fonction de l'âge des souches, de l'espèce et du site d'étude (A-B-C). Les barres d'erreur représentent l'écart type de la moyenne obtenue.

Figure 7 : Concentrations cellulaires m
moyennes en fonction de l'âge des so
l'espèce et du site d'étude (A-B-C). L
d'erreur représentent l'écart type de la
obtenue.

Figure 39. Average cell maximal concentration for *S. donghaienis* and *A. minutum* in the two culture conditions, according to the strain age and the study site.

Table 11. Statistical tests applied on the maximum cell concentration for all *S. donghaienis* strains of the EE. The mean growth rate for the three replicates and the corresponding standard error are reported for each strain. The p values < 0.05 indicating the significance of the test are represented in bold.

		F/2		**F/2 $_{P/4}$**		**Wilcoxon**	
Strain age (year)	**Strain**	**Maximal cell concentration (cells mL^{-1})**	**Standard Error**	**Maximal cell concentration (cells mL^{-1})**	**Standard Error**	**p-value< 0.05**	**p-value**
1978±2	SC 27	1970	27	837	87	-	0.1
	SC 28	1476	78	1114	33	-	0.1
	SC 29	1264	111	1101	25	-	0.0765
	Mean	1570	362	1017	156	-	0.1
		p= 0.0273	KW	p= 0.065	KW		
		*** : 29-27**	Post hoc				
1986±1	SC 14	2136	112	1701	100	-	0.1
	SC 15	5737	279	6030	382	-	0.7
	SC 20	3419	273	2648	81	-	0.1
	Mean	3764	1825	3460	2276	-	1
		p= 0.0273	KW	**p= 0.0273**	KW		
		*** : 14-15**	Post hoc	*** : 14-15**	Post hoc		
1993±1	SC 51	2268	180	2412	250	-	0.7

	SC 53	1969	97	1861	196	-	0.4
	SC 150	11568	316	11565	936	-	0.6759
	Mean	5268	5458	5279	5451	-	1
		p = 0.0265	KW	**p = 0.0273**	KW		
		*** : 53 - 150**	Post hoc	*** : 53 - 150**	Post hoc		

Table 12. Statistical tests applied on the maximum cell concentration for *S. donghaienis* and *A. minutum* strains of the EP. The mean growth rate for the three replicates and the corresponding standard error are reported for each strain. The p values < 0.05 indicating the significance of the test are represented in bold.

		F/2		**F/2 $_{P/4}$**		**Wilcoxon**	
Strain age (year)	**Strain**	**Maximal cell concentration (cells mL^{-1})**	**Standard Error**	**Maximal cell concentration (cells mL^{-1})**	**Standard Error**	**p-value< 0.05**	**p-value**
1998 ± 4.6	SC 129	10594	1082	11240	128	-	0.7
	SC 130	9954	459	10338	1270	-	1
	SC 142	10054	819	6076	54	-	0.1
	Mean	10201	344	9218	2758	-	1
		p= 0.7326	KW	p= 0.0608	KW		
2006 ± 2.3	SC 21	3325	397	4091	122	-	0.0765
	SC 22	9718	700	6791	1268	-	0.1
	SC 65	2314	368	1681	284	-	0.1212
	Mean	5119	4015	4188	2556	-	1
		p= 0.0273	KW	**p= 0.0265**	KW		
		*** : 22-65**	Post hoc	*** : 22-65**	Post hoc		
1997 ± 4.9	Am 76	2282	632	1105	273	-	0.1
	Am 77	1752	393	1352	77	-	0.1
	Mean	2017	375	1229	175	-	0.33
		p= 0.4	Wilcoxon	p= 0.4	Wilcoxon		
2006 ± 2.3	Am 53	2407	400	1788	113	-	0.1
	Am 55	17809	958	12947	362	-	0.1
	Am 60	2189	526	2388	464	-	0.7
	Mean	7468	8956	5708	6277	-	0.7
		p= 0.0509	KW	**p= 0.0273**	KW		
			Post hoc	***: 53-55**			

3.5. Analysis of P limitation

The analysis of P concentration at t_{lim} has been performed only for the strains which clearly showed a growth dynamic affected by a phosphorous limitation and different growth dynamic between the media used (Table 13). The P concentration analyses were performed for strains SC14, SC27 and SC142 which showed a limited growth in the F/2 $_{P/4}$ medium compared to the growth in F/2 medium. For comparison, the P concentrations have been measured for the strain SC150 which did not show growth difference between the media. For all the strains analysed, P assimilation per day (µM cell^{-1}.day^{-1}) was higher for the F/2 medium, but the assimilation was variable between the different

strains (F/2: 0.8 x 10^{-5} – 4.0 x 10^{-4} µM cell^{-1} day^{-1}; F/2 $_{P/4}$: 5.7 x 10^{-5} – 1.2 x 10^{-3} µM cell^{-1} day^{-1}). The bacterial growth dynamic was similar between the strains.

Table 13. Inorganic phosphate concentration for the strains displaying a Pi limitation. $\mu_{[PO43-]}$ (day^{-1}) : the rate of PO_4^{3-} assimilation per day.

Medium/strain	$t_0[PO_4^{3-}]$ (µM)	t_{lim} $[PO_4^{3-}]$ (µM)	$[PO_4^{3-}]$: t_0-t_{lim} (µM)	$\mu_{[PO4^{3-}]}$ (µM cell day^{-1})	$[Bacteria]_{max}$ (cells mL^{-1})
F/2 Sc 150	33.65	23.40	10.25	8.1 x 10^{-5}	3 x 10^7
F/2 $_{P/4}$ Sc 150	7.17	0.09	7.07	5.7 x 10^{-5}	3 x 10^7
F/2 Sc 27	31.25	27.03	4.22	3.8 x 10^{-4}	-
F/2 $_{P/4}$ Sc 27	6.21	3.39	2.82	1.2 x 10-3	5 x 10^7
F/2 Sc 14	32.88	26.81	6.07	4.0 x 10^{-4}	6 x 10^7
F/2 $_{P/4}$ Sc 14	7.53	2.41	5.12	6.9 x 10^{-4}	5.5 x 10^7
F/2 Sc 142	32.42	22.25	10.17	1.2 x 10^{-4}	2 x 10^7
F/2 $_{P/4}$ Sc 142	7.72	0.05	7.66	1.8 x 10^{-4}	5 x 10^7

4. Discussion

The originality of this work consisted in using strains of two species revived from ancient sediment samples in order to compare the phenotype of populations of different ages, over a time-scale of about 40 years. To the best of our knowledge this work is the first attempt of phenotypic comparison of the species of *S. donghaienis* and *A. minutum* using ancient populations. This work also allowed getting the first data of the growth rate of *S. donghaienis* for which no ecophysiological information are available so far. The major result of this study, carried out on strains cultivated in different inorganic phosphate (P) concentrations, is the evidence of a high intraspecific variability of the phenotypes, and in particular of growth rates and P assimilation, for *S. donghaienis* and *A. minutum*. This result allowed us to discard the initial hypothesis of a possible separation of the populations of these species on the basis of their ages and on the tested parameters.

4.1. Experimental conditions

The major limitation of ecophysiological analyses *in vitro* is the reproduction of natural conditions and the integration of all environmental parameters that trigger the cellular growth in the natural environment. For example, the patchy and spiky concentrations of nutrients in the natural environment are not reproducible in culture, as far as the seasonal and daily variability of abiotic parameters (Lakeman *et al.,* 2009). The use of clonal, non axenic cultures does not allow the characterisation of the biotic interactions that a natural population may experience (Fukami *et al.,* 1997). Finally, the majority of the *in vitro* ecophysiological studies are performed on a limited

number of strains, thus overlooking the intraspecific variability of marine protists (Burkholder and Gilbert, 2006). For example, strains of *Karenia brevis*, genetically identical on the basis on their 18S and ITS1 rDNA, showed an important intraspecific variability of their growth rates and toxin production (Loiret *et al.,* 2002). The long-lasting maintenance of the strains in the laboratory has been also studied. It has been suggested that the long-lasting maintenance in the laboratory may induce homogeneity of the strains if the number of generations produced are sufficient to trigger an adaptation or an evolution of the strains to the culture conditions (Lakeman *et al.,* 2009). It has also been showed that a long-lasting maintenance of the cultures might not induce changes on the physiological functions of the organisms (Berge *et al.,* 2010).

The approach proposed in this study tried to take into account the biological variability existing between different strains of the same age and between different ages. The use of revived strains allowed taking into consideration, the life history of the different populations preserved in the sediment samples upon the form of resting stages. The dinoflagellate cysts are physiologically dormant cells; they ensure the preservation of the physiological adaptation of the vegetative cell at the moment of their formation. Working with strains revived from ancient strains allowed analysing the physiology of ancient populations (Ribeiro *et al.,* 2013). In our experimental work, the use of dystrophic N/P ratios allowed to ensure that the inorganic P would be the first limiting nutrient. The use of the F/2 medium, chosen as the base to create the dystrophic medium F/2 $_{P/4}$, reduced the use of P to only one phosphorous source, the inorganic phosphate (PO_4^{3-}). The use of different strains (20) and replicates (3), different ecosystems (Penzé Estuary and Elorn Estuary) and ages (’70, ’80, ’90, and 2000), allowed the best possible characterisation of the growth dynamic of the species.

4.2. Growth dynamic and intraspecific variability

The growth dynamic of the different strains of *S. donghaienis* and *A. minutum* was variable among strains of the same age, and did not allowed the grouping of the strains according to their age. The unexpected variability of the inoculum concentrations (ca. 500-1000 cells mL^{-1}) at t_0 may be the factor at the origin of this different growth dynamics. Yet, this factor cannot completely explain the high variability observed of an important number of strains.

The most ancient strains of *S. donghaienis* (EE-1978 ± 1: SC27, SC28, SC29; EP-1998 ± 4.9: SC129, SC130, SC142) and *A. minutum* showed the most homogenous growth dynamics within an age. This homogeneity could be likely linked to the limited revivification success of the ancient strains. Similarly, Ribeiro *et al*., (2013), showed low variability of the growth parameters of the most ancient strains of *Penthapharsodinium dalei*. They also demonstrated that the germination success of the most ancient cysts was lower than that of the modern cysts. The germination and isolation protocol might have induced the homogeneity of our most ancient cysts. The strains that are able to

germinate from ancient cysts may have similar physiological properties which led to homogeneity of the phenotypes observed. In order to optimize the germination success of different species, we revived cysts directly from sediment samples and not from isolated cysts. Therefore we cannot determine the germination success of each species and populations, and the hypothesis of a potential bias in the selection of our ancient populations remains to be proved.

Globally, the studied strains showed similar growth dynamics between the two experimental media. The P concentrations at t_{lim} of the F2 $_{P/4}$ medium were not sufficiently low to induce a limitation of the growth. Despite, the initial P concentrations at (t_0) were similar (6.21-7.72 µM) for the two media, the daily P assimilation rates were variable between the strains. This suggests an intraspecific variability of P assimilation of the analysed species which can be explained by an intracellular differential stock of the P by the different strains. Fredrick *et al.,* (2013) showed by a modelling approach that different mechanisms of using P may play a role on the variability of the intracellular P stocks.

Recently, a high genotypic and genetic diversity has been evidenced in populations within a phytoplankton bloom but also in populations from two distinct blooms (Lebret *et al.,* 2012; Dia *et al.,* 2014). Our study showed the variability in the growth of different strains of the same populations over contrasting nutrient concentrations, corroborating previous evidences of heterogeneity within a microalgal population. The number of strains per age (3 strains), did not integrate the whole variability of a population, but even the use of 6 strains proved a strong variability within populations of different ages (Ribeiro *et al.,* 2013). The low germination success of ancient cysts seems to limit the number of old revivable strains, thus the integration of the intraspecific variability of a microalgal population is still a challenge for the ecophysiological studies using ancient populations.

5. Conclusions

This study has showed the potential of an ecophysiological study on revived populations and its limits in integrating the complexity of diversity of microalgal populations. On the basis of our experimental approach and the number of strains per age used, it is not possible to separate the populations of different ages of *S. donghaienis* and *A. minutum* in function of the P assimilation. This work showed a strong phenotypic variability of the two tested dinoflagellate species over a multiannual time-scale and across different ecosystems. Our experimental conditions did not allow to adequately evaluate the effect of a phosphorous limitation for the two species. Therefore, stronger P-depleted conditions need to be tested on the populations analysed here. The variability of the phenotypes observed may correspond to a genetic difference among the populations; this may be tested using appropriate

genetic markers, such as the microsatellites. Finally a proteomic and metabolomic approaches would probably allow a larger screening of other potential physiological markers which could likely help in discriminating populations according to their age.

DISCUSSION GÉNÉRALE ET PERSPECTIVES

Au cours de ce travail de paléoécologie, en utilisant des archives biologiques sédimentaires, j'ai pu identifier des dynamiques et des variations de communautés, d'espèces et de populations des protistes marins de l'écosystème côtier. Bien que l'approche de paléoécologie sédimentaire ait déjà été employée pour l'étude des protistes dans différents environnements (marins, lacustres, fjords, permafrosts), celle-ci n'a pas été utilisée pour répondre à des questions écologiques en milieu estuarien. En effet, peu d'informations existent sur le potentiel d'un tel écosystème à garder en mémoire des traces biologiques anciennes. Notre étude est donc pionnière pour la reconstruction de patrons paléoécologiques en milieu estuarien. Par ailleurs, elle montre l'importance du choix du site pour effectuer des analyses paléoécologiques. En effet, l'absence de résultats exploitables sur les carottes de la Baie de Morlaix et de la Baie de Vilaine, montre la difficulté de trouver un site adéquat. Les sites non remaniés par l'hydrologie (phénomène des marées, forts remaniements des sédiments saisonniers, ...) ou par l'action de l'homme (dragage des zones fluviales, pêcheries, aménagement, …), et avec une bioturbation limitée sont sans doute rares dans ce type d'écosystèmes. Il semble nécessaire de produire une typologie des sites pouvant être exploités, afin d'en dresser l'inventaire futur, avant d'évaluer l'intérêt de ces approches en milieux estuariens. Mon étude qui au départ ciblait une échelle géographique plus large et une analyse écologique intrasite, a dû se limiter à la rade de Brest, qui finalement est apparue comme un site atelier très adapté aux études de paléoécologie.

Travailler sur des sédiments considérés comme peu exploitables pour les études paléoécologique a impliqué de prendre en considération des paramètres supplémentaires. Par exemple, la migration verticale de l'ADN à travers les différentes couches de sédiment d'une carotte à cause du déplacement de l'eau interstitielle peut provoquer une contamination de l'ADN ancien par de l'ADN moderne de surface. Ce facteur ne concerne habituellement pas les environnements froids ou gelés tels que les permafrosts, mais il pourrait avoir un impact dans un écosystème humide de type estuarien. Toutefois, conjointement à cette étude, une analyse de l'eau interstitielle des différentes couches sédimentaires (résultats non montrés ici) a permis de montrer que l'eau interstitielle était présente uniquement dans les 6 premiers centimètres de la carotte de l'Elorn et les 8 premiers centimètres de la carotte de Daoulas. Par conséquent, l'impact de ce facteur sur l'authenticité des résultats des carottes exploitées devrait être minime. Ce travail a concerné également des sédiments récents (> 1866), ce qui est relativement court en paléoécologie. Les dépôts sédimentaires en zones productives sont tels qu'il est possible de travailler sur une échelle de temps relativement fine, très appropriée à l'étude des changements récents globaux. Ces milieux, du fait de leur dynamisme, sont sans doute moins bien adaptés à l'étude des communautés sur une plus longue échelle de temps.

Compte tenu de l'historique des variations écologiques ayant caractérisé les écosystèmes côtiers bretons au cours du siècle dernier et le manque de séries temporelles suffisamment longues dans la région, j'ai appliqué mes recherches en paléoécologie dans deux écosystèmes estuariens de la rade de Brest. Les deux carottes sédimentaires analysées ont servi comme modèle pour essayer de répondre à plusieurs questions concernant : i) la diversité des protistes préservée dans les sédiments anciens, ii) la dynamique multidécennale et spatiale d'une espèce phytoplanctonique toxique à caractère potentiellement invasif, et enfin iii) la réponse phénologique de populations revivifiées de dinoflagellés de différentes époques à des variations en nutriments en mimant les variations hydrologiques s'étant produit dans l'écosystème côtier.

Potentiel de préservation des protistes dans les sédiments anciens de l'écosystème estuarien

Dans un premier temps, je me suis intéressée à la diversité des protistes pouvant être archivée dans notre écosystème modèle de la rade de Brest à partir de deux carottes de sédiment couvrant des archives biologiques d'environ 150 ans. Notre approche d'analyse de la diversité basée sur le metabarcoding de la région V4 du 18S ADNr a permis d'avoir une vision générale et la plus exhaustive possible actuellement de la diversité des protistes dans les sédiments. Cette approche a été jusqu'à présent très rarement utilisée pour l'analyse de l'ADN ancien (e.g. Coolen *et al*., 2013; Lejzerowicz *et al*., 2013; Capo *et al*., 2015).

Nos résultats montrent que tous les grands super-groupes d'eucaryotes sont présents dans les sédiments et que similairement à d'autres études effectuées dans le milieu pélagique (de Vargas *et al*., 2015), le groupe des alvéolés (Alveolata) est le plus diversifié et le plus abondant retrouvé par la technique du metabarcoding. Cependant, de façon générale, plus les sédiments sont profonds (anciens), moins d'OTUs (Operational Taxonomic Units) de protistes sont représentés. Le résultat le plus étonnant mis en évidence est que quelque soit l'écosystème étudié de la rade de Brest (estuaire de Daoulas et estuaire de l'Elorn), il y a une proportion plus ou moins stable (max 25% d'OTUs) de la diversité des protistes de sédiments superficiels qui reste dans les couches profondes. Cette diminution de la richesse spécifique (en termes d'OTUs) est très similaire à celle de la diminution de la concentration d'ADN total observée dans les sédiments (discuté dans le chapitre 2) et à un profil type de dégradation de la matière organique dans le sédiment. Cette observation est en faveyr d'une dégradation régulière de l'ADN dans les sédiments. Cependant, il est possible que cette dégradation ne concerne que certains groupes de protistes. En effet, la majeure partie des protistes retrouvés dans les sédiments anciens sont capables de former des formes de résistance, tels que les dinoflagellés et les ciliés (Alveolata), les diatomées (Stramenopiles). L'ADN que l'on retrouve dans

des couches profondes de sédiment pourrait donc provenir proportionnellement plus de protistes formant des formes de résistance dont les structures extérieures de protection favorisent la préservation de l'ADN.

Des méthodes pour séparer l'ADN extracellulaire de l'ADN intracellulaire ont été développées (e.g. Corinaldesi *et al*., 2005, Alawi *et al*., 2014, Lever *et al*., 2015). Elles consistent globalement en une étape de traitement de l'échantillon de sédiment avec une solution alcaline de phosphate, et une étape de centrifugation afin de séparer la phase aqueuse (contenant l'ADN extracellulaire) de la phase solide (contenant l'ADN dans des cellules vivantes, mortes). Grâce à ce traitement, l'adsorption de l'ADN est censée diminuer en présence du phosphate à cause d'une compétition entre les ions phosphates et les groupes phosphates présents dans la molécule d'ADN. Cette méthode peut être intéressante lorsque l'on veut analyser un seul type d'ADN séparément (extracellulaire ou intracellulaire à l'intérieur des kystes) mais cela devient plus compliqué lorsqu'il s'agit d'analyser simultanément plusieurs taxa présents dans un échantillon environnemental (metabarcoding), y compris ceux qui ne forment pas de forme de résistance. De plus, si l'on veut étudier les deux types d'ADN simultanément, les analyses doivent être réalisées immédiatement après l'échantillonnage des sédiments étant donné les conditions de stockage différentes des échantillons destinés aux analyses d'ADN (-80°C) et ceux destinés pour l'analyse des kystes (2°C). Le choix de ne pas appliquer un traitement sur les échantillons d'ADN pour séparer l'ADN environnemental de l'ADN intracellulaire a été donc volontaire dans le cadre de mon étude, puisque celle-ci visait à réaliser aussi une analyse de la biodiversité générale des protistes de sédiments anciens.

Certains facteurs ont pu induire une sous-estimation de la diversité obtenue dans notre étude. Les bases de données utilisées pour les annotations taxinomiques des OTUs sont incomplètes et ne permettent pas d'identifier certains taxa tels que les dinoflagellés benthiques. Aussi, les primers utilisés dans cette étude ne permettent pas d'amplifier les foraminifères (Rhizaria) pélagiques et benthiques et certains représentants des excavates (Excavata). Compte tenu de la dégradation par fragmentation de l'ADN ancien, des fragments de taille inférieure à celle du V4-18S ADNr (390 paires de bases) n'ont probablement pas pu être amplifiés et identifiés induisant donc une sous-estimation de la diversité de certains groupes. Etant consciente de cette dernière limite avant d'entamer cette étude de diversité, et voulant optimiser l'identification même des fragments d'ADN dégradés et fragmentés, j'ai voulu utiliser le marqueur V9 du 18S ADNr, plus court (130 paires de base) à la fois sur les deux estuaires de la rade de Brest mais également sur d'autres carottes de sédiments prélevées en Baie de Morlaix et en Baie de Vilaine (Annexe 1). Cependant, les résultats de ces analyses n'ont pas été concluants en raison de l'abondance élevée de procaryotes (bactéries et

archées) ainsi que la grande proportion de diversité n'ayant pas pu être déterminée taxonomiquement. Par conséquent, j'ai dû abandonner la comparaison entre fragments d'ADN de diffèrent taille pour l'analyse de la diversité. Par ailleurs, Domaizon *et al*., (2011), n'ont noté aucune différence significative sur la diversité identifiée en amplifiant à partir d'un même échantillon de sédiment à la fois un fragment long (1500 bp) et un fragment court (150 bp). Bien que sans doute partielle, notre approche a le mérite de donner une première image de la diversité des protistes retrouvés dans les sédiments anciens en milieu estuarien, et pose les premiers jalons de leur utilisation pour la reconstruction des communautés pélagiques anciennes.

Changements de la composition des communautés de protistes

La composition des communautés de protistes en rade de Brest diffère à travers le temps sur une échelle de 150 ans, tout en suivant un double patron ; 1) à petite échelle temporelle : plus les échantillons sédimentaires sont proches (de par leur âge) et plus leurs communautés se ressemblent, 2) à grande échelle temporelle, la composition des communautés d'avant 1950 était différente de celle d'après 1950, constituant ainsi deux paléocommunautés distinctes. Ces résultats indiqueraient d'une certaine manière que chaque période de temps est marquée par une biodiversité de protistes spécifique. Probablement une dégradation progressive et spécifique de l'ADN ancien de certains groupes a contribué à structurer différemment les paleocommunautés en fonction du temps, mais il est aussi vrai que la rade de Brest a subi plusieurs variations des conditions hydrologiques au cours du dernier siècle qui ont pu contribuer à un changement des communautés. En effet, depuis plus de 60 ans, les activités humaines exercées près des côtes ont induit un déséquilibre des apports en nutriment qui n'ont cessé de fluctuer considérablement. Cela s'est répercuté sur les assemblages biologiques, notamment, sur les variations de dominance des principaux groupes de phytoplancton durant les efflorescences, basculant entre les diatomées et les dinoflagellés (Quéguiner and Tréguer 1984 ; Del Amo *et al*., 1997). Etant donné que les études sur les changements des communautés ont été réalisées sur un nombre restreint de groupes de protistes, notamment le phytoplancton, et que ceux-ci représentent la base de la chaine trophique, Il est fort probable que ces changements se soient produits également sur d'autres protistes. Avec l'approche de metabarcoding, j'ai pu donc avoir une vision plus complète des changements des communautés de protistes des derniers 150 ans, sans me restreindre seulement au phytoplancton.

Sur la base des résultats obtenus, les dinoflagellés apparaissaient comme le groupe dominant, et ce à toutes les périodes de temps et quel que soit l'estuaire étudié. L'importance relative des dinoflagellés diminue vers les années récentes, avec un profile opposé à celui de la dégradation observée de l'ADN total. Le fait de retrouver une importance relative majeure des dinoflagellés dans

les couches plus profondes de sédiments peut dériver de la disparition (à cause de la dégradation de leur ADN) de certains groupes dans les sédiments anciens. Cependant, cette diminution de l'importance de dinoflagellés vers les années récentes est en accord avec les données d'abondance dans l'eau (cells L^{-1}) acquise par réseau de surveillance (REPHY). Cette corrélation permet de formuler l'hypothèse que la diminution des dinoflagellés dans le temps est réelle et pas un biais de notre analyse paléogénétique. Au niveau des genres de dinoflagellés, nous avons pu également constater des patrons intéressants pour 2 taxons producteurs de kystes, se traduisant par une tendance à l'augmentation des abondances d'*Alexandrium* vers les années récentes, avec une tendance inverse pour le genre *Gonyaulax*. Ces changement des communautés de dinoflagellés (global et au niveau de la proportion relative des différents genres) est donc très probablement attribuable aux changements hydrologiques que la rade de Brest a subi pendant le dernier siècle. Les raisons spécifiques de ces changements des communautés et la relation cause-effet entre les changements hydrologiques et les variations des communautés restent encore à approfondir, mon travail restant encore très descriptif.

Alexandrium minutum, *espèce envahissante en rade de Brest*

Les analyses de la diversité des paléocommunautés (chapitre 1) ont permis de révéler l'importance des dinoflagellés en tant que groupe dominant des protistes et ont confirmé le potentiel de l'utilisation de l'ADN pour identifier des patrons écologiques intéressants même au niveau du genre. Cela m'a conforté dans le choix des dinoflagellés comme principal groupe d'étude pour la suite de ces travaux de thèse. De cette manière, dans le chapitre 2, je me suis intéressée à étudier la dynamique de l'espèce *Alexandrium minutum* Halim, potentiellement toxique et considérée officiellement comme espèce invasive en Europe (http://www.europe-aliens.org) à partir des traces biologiques dans les sédiments et sur une échelle de temps relativement longue. En effet, selon les données de surveillance dans l'eau acquises par le réseau local (REPHY), la première observation de cette espèce en rade de Brest remonte à l'année 1990, six ans après le début de surveillance dans la zone. De plus, en 1990, l'inspection de sédiments superficiels n'a pas révélé de présence de kystes dans 4 stations de la rade de Brest (Erard-Le Denn *et al.,* 1991). C'est donc naturellement que notre hypothèse de départ soutenait une arrivée récente et une invasion de cette espèce dans notre écosystème modèle. J'ai voulu donc remonter, avec des quantifications de l'ADN ancien spécifique d'*A. minutum*, aux premières traces potentielles du passage de l'espèce dans nos écosystèmes d'étude et valider que *A. minutum* était bien une espèce invasive en rade de Brest.

Malgré la double limite rencontrée dans cette étude (technique : liée à la sensibilité du test de PCR en temps réel, et biologique : liée aux faibles concentrations d'ADN dans les couches de sédiment

profondes à cause des processus de dégradation), nos résultats paléogénétiques montrent clairement que *A. minutum* est présente dans la rade de Brest depuis au moins l'année 1873 ± 7, puisque des traces génétiques de l'espèce ont été retrouvé même dans l'avant dernière couche de sédiments de la plus longue carotte analysée dans le cadre de cette étude. Les abondances d'*A. minutum* (copies d'ITS1 rDNA g^{-1} sédiment) sont en augmentation ces dernières années dans les 2 sites étudiés, et ce en accord avec les données d'abondances (cells L^{-1}) dans l'eau (données REPHY). Les résultats obtenus suggèrent donc que sur une échelle de temps d'environ 150 ans, *A. minutum* ne serait pas une espèce invasive mais plutôt une espèce envahissante, c'est-à-dire une espèce indigène qui prolifère soudainement du fait de l'action de l'homme et/ou du changement de l'écosystème. Il n'est pas à exclure que l'analyse de carottes plus longues aurait peut-être permis de détecter des traces de la présence d'*A. minutum* avant 1873 ± 7. Aussi une carotte plus longue aurait pu mettre en évidence l'absence de traces d'ADN de manière consistante sur plusieurs couches de sédiments, et de confirmer que l'invasion de *A. minutum* en rade de Brest aurait pu se produire bien avant 150 ans. De façon intéressante, les résultats génétiques du chapitre 1, montrent également une augmentation des abondances relatives des OTUs du genre *Alexandrium* dans les années récentes. Cependant, il est difficile aujourd'hui de savoir à quelles espèces d'*Alexandrium* appartiennent ces OTUs ; une méthode d'assignation taxonomique plus fine des séquences devant être encore réalisée. Les quantifications des abondances des copies de l'ITS1 ADNr d'*A. minutum* dans les couches profondes n'a pas été possible principalement à cause de la faible concentration de l'ADN ancien de l'espèce mais également à cause de la sensibilité du test (limites de quantification). Les faibles concentrations d'ADN observées dans des couches profondes sont sans doute dues à la dégradation de l'ADN par divers processus biotiques et abiotiques. L'utilisation d'une procédure de pré-amplification préalablement à la PCR en temps réel, telle que préconisée récemment par (Andersson *et al*., 2015) aurait probablement permis d'augmenter la sensibilité de la PCR en temps réel et permettre une meilleure quantification de l'ADN ancien dégradé.

Pour valider les données paléogénétiques et valider la présence d'*A. minutum* dans les sédiments anciens sous forme de kystes, j'ai essayé de revivifier l'espèce directement à partir des sédiments avec des expériences de germination en condition de culture contrôlée. La germination des kystes d'*Alexandrium minutum* confirme l'existence de kystes viables de l'espèce dans les sédiments anciens. Cependant je n'ai pas pu mettre en évidence une corrélation claire entre les données de PCR en temps réel et la germination d'*A. minutum*. Bien que différentes conditions de germination (température, irradiance, milieu de culture) aient été testées, peu de kystes ont germé à partir des couches profondes et anciennes de sédiment. La modification du protocole initial aurait peut-être amélioré ces résultats. En effet, notre protocole a consisté à mettre directement en culture une

fraction de sédiment tamisée, or selon la littérature (Lundholm *et al*., 2011, Erdner *et al*., 2010), une autre méthode consiste à d'abord séparer la fraction des kystes de celle du sédiment par un gradient de densité à l'aide d'une solution de sodium metatungstate monohydrate (Bolch, 1997) puis de soumettre cette fraction à des conditions expérimentales similaires aux nôtres.

*Accumulation de l'ADN d'***A. minutum** *dans les sédiments*

Des efflorescences toxiques d'*A.minutum* se produisent saisonnièrement, et sont plus ou moins importantes en rade de Brest depuis l'événement de 2012 (42 x 10^6 cells L^{-1}) dans l'estuaire de Daoulas. Bien que d'autres efflorescences de l'espèce soient également signalées dans d'autres estuaires de la rade, celles-ci sont d'une moindre intensité (2 x 10^6 cells L^{-1}). En dehors de ces lieux d'efflorescence habituels, les zones occupées actuellement par l'espèce sont inconnues. Dans cette optique, et en considérant la prolifération récente de cette espèce dans la rade de Brest (chapitre 2) que je me suis interrogée dans le chapitre 3 sur la distribution spatiale actuelle d' *A. minutum* en utilisant les données quantitatives de traces d'ADN dans le sédiment comme proxy d'éventuelles zones d'accumulation des kystes en rade de Brest. Si l'on considère que ces kystes sont majoritairement responsables du signal génétique dans les sédiments les plus anciens en milieu estuarien, ce type d'informations est également crucial pour comprendre la dynamique temporelle à plus large échelle ou pour guider les prochaines études dans le choix des sites.

La quantification des kystes dans le sédiment s'effectue le plus couramment par un comptage microscopique des kystes après isolement du sédiment. Cette méthode, bien qu'efficace, est coûteuse en temps et nécessite une expertise taxinomique considérable pour l'identification des kystes. L'utilisation d'outils moléculaires pour la quantification des kystes apparaît comme une approche alternative rapide, plus précise et moins coûteuse. Il existe une méthode qui a déjà été appliquée pour des échantillons de sédiments (exemple Erdner *et al*., 2010) et qui concilie l'approche microscopique et moléculaire. Elle consiste à isoler les kystes à partir des sédiments, en effectuer le comptage, en extraire l'ADN et construire une gamme étalon de dilution d'ADN permettant ainsi de faire la correspondance entre la concentration d'ADN et le nombre de kystes par PCR en temps réel. Cette approche fournit une mesure plus pertinente de l'abondance des kystes. Cependant, cette gamme étalon ne permet pas une quantification exclusive des kystes directement dans les sédiments, où l'ADN quantifié appartient à la fois à de l'ADN contenus dans des kystes et à l'ADN existant à l'état extracellulaire. En effet, la principale difficulté rencontrée lors de l'utilisation d'outils moléculaires est de pouvoir distinguer entre les différents types d'ADN présents. Une solution consisterait à appliquer un traitement chimique sur le sédiment afin de séparer la fraction de l'ADN extracellulaire de celui contenu dans les kystes. Récemment, une méthode a été décrite pouvoir

quantifier exclusivement de l'ADN intracellulaire des kystes encore intègres, consistant à dénaturer l'ADN extracellulaire par la chaleur (75°C) (Kim *et al*., 2016). De cette manière, l'élimination de l'ADN extracellulaire devrait permettre une quantification précise et exclusive des kystes en PCR en temps réel, mais ne permettrait plus la quantification de l'ADN préservé dans les kystes non intègres, dans les couches les plus profondes. D'autres études, ont montré que la méthode de PCR en temps réel était une alternative au comptage des kystes en microscopie optique (Kamikawa *et al*., 2007; Erdner *et al*., 2010). Dans mon étude, je ne visais pas à quantifier de manière précise les kystes dans les sédiments ou à valider la technique de PCR en temps réel pour l'analyse des kystes d'*A. minutum* dans les sédiments, mais plutôt à étudier les traces génétiques de cette espèce, quelque soit son origine afin d'évaluer sa distribution dans le temps et dans l'espace. L'approche de biologie moléculaire utilisée m'a donc semblé suffisante pour cet objectif.

A l'aide du protocole de PCR en temps réel développé dans le chapitre 2, j'ai quantifié l'abondance d'ADN d'*A. minutum* dans les sédiments superficiels de 30 stations de la rade de Brest sur 2 années consécutives. J'ai effectué des expériences de germination pour valider la présence des kystes viables dans les échantillons de sédiments et corroborer les analyses génétiques. L'observation de la germination d'*A. minutum* dans toutes les stations analysées a permis de confirmer que l'espèce toxique est actuellement présente partout dans la rade de Brest. Les données de quantification par PCR en temps réel suggèrent une distribution hétérogène de cette espèce dans les sédiments de surface de la rade, avec de fortes abondances localisées dans la partie sud-est, et particulièrement dans l'estuaire de Daoulas, lieu d'efflorescence habituelle. Ceci montre que même dans un écosystème côtier semi-fermé, il peut y avoir des zones d'accumulation localisées. Cette distribution peut sans doute être expliquée par les conditions hydrodynamiques de la rade de Brest, la présence de faibles courants de fond permettraient probablement des accumulations localisées des kystes d'*A. minutum*. Cependant, l'absence de telles données sur l'hydrodynamisme de fond n'a pas permis d'établir cette relation. Cette étude suggère que les kystes d'*A. minutum* semblent s'accumuler préférentiellement dans des zones riches en matière organique où les sédiments sont fins (< 63 µm) et de type sablo-vaseux. Cette relation entre abondance plus importante de kystes et sédiment de typologie vaseuse a déjà été mise en évidence pour d'autres espèces d'*Alexandrium* et dans d'autres écosystèmes. C'est le cas pour *A. catenella et A. tamarense* dans le lagon de Thau, (France) (Genovesi *et al*., 2013). Cette distribution hétérogène et préférentielle a soulevé la question de savoir si celle-ci était spécifique aux kystes d'*A. minutum* ou similaire à celles d'autres espèces formant des kystes telles que *Gonyaulax* spp. ou *Scrippsiella* spp. présentes également dans la rade de Brest. Cette question est d'autant plus intéressante car elle permettrait de mieux comprendre les facteurs physiques et hydrodynamiques agissant sur la dispersion et la distribution des kystes et de leur

enfouissement au cours du temps. Les simulations des trajectoires de particules dispersées dans la colonne d'eau ont permis de mettre en évidence, là encore, une hétérogénéité des échanges de cellules d'*A. minutum* entre différents estuaires de la rade. Les échanges de masses d'eau entre les estuaires du sud-est (Aulne, Daoulas et Camfrout) et celui du nord de la rade (Elorn) sont très limités, ce qui permet d'avancer l'hypothèse de la présence de barrières physiques pour la dispersion et les échanges génétiques entre populations de la rade. Il serait donc intéressant d'aborder cette question d'un autre point de vue, en utilisant des approches de génétique de populations afin de vérifier si les populations d'*A. minutum* de l'Elorn et des estuaires du sud de la rade sont effectivement isolées. Pour une représentation plus réaliste de la dispersion d'*A. minutum* en rade de Brest, il apparait nécessaire d'améliorer le modèle physique utilisé en intégrant des paramètres biologiques tels que la reproduction et la mortalité dont les rôles dans la dispersion des cellules sont non négligeables.

Variabilité intraspécifique des populations de dinoflagellés de différentes époques

Dans le chapitre 4, je me suis interrogée sur les réponses de populations des deux dinoflagellés *A. minutum* et *S. donghaienis* face au changement des conditions environnementales en terme de nutriments. Ces deux espèces ont été ciblées car elles ont germé en grand nombre dans les différentes couches sédimentaires et, pour *S. donghaienis,* dans des sédiments relativement anciens. Pour cela, les performances physiologiques en terme de taux de croissance et de biomasse maximale des souches de différentes époques ont été analysées *in vitro* en condition de culture avec diffèrents rapports N/P (en variant les concentrations de P), censé mimer des changements de la concentration de nutriments s'étant produits en rade de Brest pendant le siècle dernier.

Contrairement à notre hypothèse initiale soutenant des performances physiologiques différentes en fonction des populations de différente époques, ni l'âge des souches, ni le milieu de culture n'ont eut d'influence sur le taux de croissance. En revanche, nous avons mis en évidence une variabilité intraspécifique importante pour les deux espèces étudiées, à la fois en terme de taux de croissance et en terme de consommation en phosphore inorganique. Habituellement dans les études d'écophysiologie, le nombre de souches étudié est souvent un critère limitant à la fois pour reproduire des conditions environnementales réelles que pour obtenir des résultats fiables. Dans cette étude, cela a été d'autant problématique puisque le nombre de souches analysé dépendait directement du succès de germination des kystes à partir des sédiments. En effet la germination des kystes a été particulièrement difficile et plusieurs tentatives ont été nécessaires pour augmenter le succès de germination, notamment dans les couches profondes. Cependant, bien que l'on ait observé plusieurs espèces de diatomées et de dinoflagellés dans des couches de sédiments récentes, seule *S. donghaienis* a germé à partir de sédiments profonds daté de 1978 ± 2. Les résultats des

expériences d'écophysiologie montrent une variabilité intraspécifique plus faible des souches anciennes par rapport à celle des souches modernes. Ces résultats pourraient s'expliquer par une sélection plus importante qui interviendrait au niveau de la germination des souches anciennes, le milieu et le temps agissant comme un filtre. La mise en culture de souches anciennes est sans doute loin d'être représentatif des populations de l'époque, et notre capacité à faire germer un maximum d'individus représentatifs de cette diversité est donc un verrou encore à résoudre pour les études physiologie en paléoécologie.

Considérations globales sur les avantages et limites de l'approche paléoécologique

Dans ce travail, l'approche paléoécologique utilisée a impliqué l'utilisation de différents outils, sédimentaires, moléculaires, microscopiques et écophysiologiques. Ces outils, bien que perfectibles, sont apparus adaptés aux questions que je me suis posées. Ainsi, il a été possible de reconstruire à la fois la diversité des communautés des protistes mais aussi l'histoire d'une espèce en particulier. A l'aide de marqueurs génétiques adaptés, notre approche de paléogénétique a permis d'identifier quasiment tous les organismes anciens préservés dans les sédiments y compris ceux qui ne laissent pas de traces fossilisables, sur une échelle de temps de 150 ans. L'approche paléoécologique basée sur la germination des kystes a permis non seulement de vérifier la présence de kystes viables afin de valider les données moléculaires mais aussi d'examiner leur viabilité dans un écosystème très peu étudié. De plus, cette approche nous a permis d'obtenir des souches de différentes époques et d'étudier leurs réponses physiologiques dans des conditions reflétant des changements passés de l'écosystème.

Quelques limites générales à cette approche paléoécologique ont été identifiées pendant mes travaux, certaines connues avant d'entamer ce travail, d'autres identifiées pendant le parcours de ma thèse. En effet, l'analyse de carottes de sédiments non adéquates (Baie de Morlaix et Baie de Vilaine) a prouvé l'importance du choix du site pour toute analyse paléoécologique. De plus, tout au long de ce travail, je me suis confrontée au fait de ne pas pouvoir distinguer entre la proportion de l'ADN amplifié appartenant à des organismes vivants tels que les kystes et celle correspondant à l'ADN extracellulaire. Cet aspect est particulièrement important et à tenir en compte lors des analyses paléogénétiques et doit être adapté à la question de l'étude. Une autre limite qui peut fortement jouer sur la pertinence des résultats paléoécologiques est liée au taux de préservation de l'ADN qui est variable au sein d'un même sédiment (en fonction de la profondeur) mais aussi d'un environnement à l'autre. Nous n'avons pas une estimation du taux de préservation de l'ADN extracellulaire dans nos sédiments. L'analyse d'autres paramètres physicochimiques (oxygène, pH) sur les carottes de sédiment étudiées aurait probablement permis d'améliorer ce point. Malgré ces

limites et incertitudes dans mes analyses paléogénétiques, je considère que les résultats produits de cette thèse contribuent au débat international sur le type d'ADN extrait et analysé à partir des sédiments anciens. Dans ce travail, nous avons suggéré que la plus grande partie de l'ADN des protistes détecté dans les sédiments anciens appartenait à des taxa connues pour former des kystes, il est donc probable que la plupart de l'ADN ancien ait été de l'ADN intracellulaire contenu dans des formes de résistance.

Conclusions générales

Cette première étude paléoécologique de l'écosystème côtier de la rade de Brest nous a permis de contribuer à l'amélioration des connaissances concernant la dynamique des protistes et des espèces produisant des efflorescences toxiques. De plus, ce travail est une contribution à l'avancement des études de paléoécologie en milieu estuarien, montrant que:

- La richesse spécifique des protistes est plus ou moins stable dans les sédiments anciens, et majoritairement attribuables aux taxa formant des kystes de résistance.
- L'approche de metabarcoding basée sur l'utilisation du barcode V4 du 18S ADNr permet de caractériser la diversité des protistes même dans des sédiments anciens. Toutefois, il apparait nécessaire d'améliorer les amorces pour atteindre une vision plus exhaustive de la diversité. Aussi, il y a une nécessité d'améliorer les bases de données d'annotation des séquences pour atteindre une caractérisation taxinomique plus satisfaisante, en identifiant certains taxa non représentés aujourd'hui dans nos analyses de metabarcoding tels que les dinoflagellés benthiques).
- Le développement de l'approche de metabarcoding de protistes marins sur de fragment de l'ADN plus court que le V4 est nécessaire en paléogénétique pour analyser la biodiversité de certains taxa dont l'ADN est dégradé dans les sédiments anciens.
- Le caractère invasif ou envahissant d'une espèce de protiste peut être établi grâce l'approche paléoécologique, en choisissant des sites d'études adéquats, ce qui contribue à l'analyse et la compréhension des phénomènes d'invasions biologiques.
- L'application des analyses de PCR en temps réel, est adaptée à la fois pour des études temporelles de paléoécologie (détection de fragments anciens dégradés) et des études de distribution spatiale en écologie. Cependant des limites à la quantification de l'ADN ancien existent.
- La revivification des populations anciennes est possible mais plus difficile dans les sédiments anciens. En outre, cette germination peut concerner uniquement un nombre restreint

d'espèces et d'individus, ce qui suggère en quelque sorte une double sélection à la fois en termes de cellules capables de germer et en termes d'âge maximal des espèces revivifiées.

Perspectives

Dans un écosystème estuarien, autant que dans d'autres environnements, les perspectives de recherches sur les protistes anciens sont nombreuses. Les résultats obtenus au cours de cette thèse nous permettent de mieux comprendre le potentiel de préservation des protistes dans des sédiments estuariens et d'évaluer le potentiel d'utilisation des approches paléoécologiques pour reconstruire des patrons anciens, à la fois au niveau des communautés, de l'espèce et des populations. De plus, cette approche paléoécologique peut être étendue à d'autres environnements.

Afin de mieux caractériser la diversité des protistes, l'utilisation d'un barcode de taille plus petite permettrait probablement d'obtenir une vue plus générale de la diversité en identifiant la part de la diversité dégradée. Il serait également intéressant de comparer la diversité de protistes dans l'eau avec celle identifiée dans les sédiments superficiels comme cela a été fait en eau douce (Capo *et al*., 2015).

Il serait intéressant de valider le statut d'*A. minutum* en tant qu'espèce envahissante en adoptant la même approche paléoécologique dans d'autres écosystèmes bretons où l'espèce a été détectée avant 1990. Des études complémentaires de génétique des populations à l'aide de marqueurs microsatellites permettraient de déterminer si les traces d'ADN d'*A. minutum* dans le sédiment appartiennent de point de vue génétique à la même population ou à des populations différentes récemment introduites en rade de Brest. Aussi, il serait intéressant de voir si l'on peut améliorer la connaissance du caractère envahissant d'*A. minutum* et comprendre les raisons de sa prolifération récente en croisant nos données avec des données d'analyses de pollens et d'autres microfossiles qui pourraient renseigner sur les conditions hydrologiques passées.

Le suivi de la distribution d'*A. minutum* dans les sédiments de la rade de Brest doit être poursuivi dans les années futures afin d'acquérir des données de distribution sur plusieurs années, ce qui aiderait à la fois à mieux comprendre et prédire les évènements d'efflorescences toxiques et à améliorer la gestion des activités économiques impactées par les efflorescences algales toxiques. Toutefois, dans le cas d'une approche spécifique sur la distribution d'une espèce, des améliorations techniques doivent être apportées sur les quantifications des kystes, notamment en utilisant des techniques (chimiques, thermiques) permettant d'éliminer l'ADN extracellulaire des échantillons à analyser avant l'application de la PCR en temps réel. En complément, des comptages des kystes à partir des sédiments devraient également être incorporées afin de valider les analyses. Aussi, un suivi

sur d'autres espèces formant des kystes en rade de Brest permettrait d'améliorer les connaissances sur les facteurs physiques influençant leur dispersion.

Afin d'explorer la variabilité entre souches de différentes époques, il conviendrait d'avoir une vision la plus large possible des différences physiologiques existantes entre populations. Une analyse de la diversité des métabolites produits en milieux de culture contrastés permettrait probablement de mettre en évidence des traits phénotypiques différents entre populations de différentes époques. Une étude de la génétique des populations à l'aide de microsatellites associée à une analyse par métabolomique permettrait de déterminer si la variabilité entre populations correspond également à une différence significative au niveau génétique.

RÉFÉRENCES BIBLIOGRAPHIQUES

A

Abdenadher, M., Hamza, A., Fekih, W., Hannachi, I., Zouari Bellaaj, A., Bradai, M. N., & Aleya, L. (2012). Factors determining the dynamics of toxic blooms of Alexandrium minutum during a 10-year study along the shallow southwestern Mediterranean coasts. *Estuarine, Coastal and Shelf Science*, *106*, 102–111.

Adler, C. J., Dobney, K., Weyrich, L. S., Kaidonis, J., Walker, A. W., Haak, W., … Cooper, A. (2013). Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions. *Nature Genetics*, *45*, 450–455.

Alawi, M., Schneider, B., & Kallmeyer, J. (2014). A procedure for separate recovery of extra- and intracellular DNA from a single marine sediment sample. Journal of Microbiological Methods, 104, 36–42.

Allentoft, M. E., Collins, M., Harker, D., Haile, J., Oskam, C. L., Hale, M. L., … Bunce, M. (2012). The half-life of DNA in bone: measuring decay kinetics in 158 dated fossils. *Proceedings of The Royal Society B*, *279*, 4724–4733.

Allentoft, M., Collins, M., D, H., & Haile, J. (2012). Ancient DNA. *Proceedings of the Royal Society*, *2*, 4724–4733.

Alpermann, T. J., Tillmann, U., Beszteri, B., Cembella, A. D., & John, U. (2010). Phenotypic variation and genotypic diversity in a planktonic population of the toxigenic marine dinoflagellate alexandrium tamarense (Dinophyceae). *Journal of Phycology*, *46*, 18–32.

Amaral-Zettler, L. a, McCliment, E. a, Ducklow, H. W., & Huse, S. M. (2009). A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA genes. *PloS One*, *4*, e6372.

Aminot, A., & Kérouel, R. (2004). Hydrologie des écosystèmes marins. Paramètres et analyses. Ifremer.

Aminot, A., & Kérouel, R. (2007). Dosage automatique des nutriments dans les eaux marines : méthodes en flux continu. Ifremer.

Andersen, R. A. (2005). *Algal Culturing Techniques*. Elsevier/Academic Press.

Anderson, D. M. (1998). Physiology and bloom dynamics of toxic Alexandrium species, with emphasis on life cycle transitions. *Physiological Ecology of Harmful Algal Blooms. NATO ASI Series Vol. G41.*

Anderson, D. M., & Wall, D. (1978). Potential importance of benthic cysts of *Gonyaulax tamarensis* and *G.excavata* in initiating toxic dinoflagellate blooms. *Journal of Phycology*, *14*, 224-234.

Anderson, D. M., Keafer, B. a, Kleindinst, J. L., McGillicuddy, D. J., Martin, J. L., Norton, K., … Butman, B. (2014). Alexandrium fundyense cysts in the Gulf of Maine: long-term time series of abundance and distribution, and linkages to past and future blooms. *Deep-Sea Research. Part II, Topical Studies in Oceanography*, *103*, 6–26.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., & Montresor, M. (2012). The globally distributed genus *Alexandrium*: Multifaceted roles in marine ecosystems and impacts on human health. *Harmful Algae, 14*, 10-35.

Anderson, D.M., Stock, C.A., Keafer, B.A., Nelson, A.B., Thompson, B., McGillicuddy, D.J. Jr., Keller, M., Matrai, P.A., & Martin, J. (2005). *Alexandrium fundyense* cyst dynamics in the Gulf of Maine. *Deep Sea Research Part II: Topical Studies in Oceanography, 52*, 2522–2542.

Anderson-Carpenter, L. L., McLachlan, J. S., Jackson, S. T., Kuch, M., Lumibao, C. Y., & Poinar, H. N. (2011). Ancient DNA from lake sediments: bridging the gap between paleoecology and genetics. *BMC Evolutionary Biology*, *11*, 30.

Andersson, D., Akrap, N., Svec, D., Godfrey, T. E., Kubista, M., Landberg, G., & Ståhlberg, A. (2015). Properties of targeted preamplification in DNA and cDNA quantification. Expert Review of Molecular Diagnostics, 15, 1085–100.

Andresen, C.S., Schmidt, S., Seidenkrantz, M. S., Marit-Solveig, S., Fiammetta S., Grycel, A., Hass, C. H., Henrik Kjær, K., Nørgaard-Pedersen, N., Dyke, L. M., Olsen, J. , Kuijpers, A. (2014). A 100-year record of changes in water renewal rate in Sermilik Fjord and its influence on calving of Helheim Glacier, Southeast Greenland. *Continental Shelf Research*, *85*, 21–29.

Anglès, S., Jordi, A., Garcés, E., Basterretxea, G., & Palanques, A. (2010). Alexandrium minutum resting cyst distribution dynamics in a confined site. *Deep Sea Research Part II: Topical Studies in Oceanography*, *57*, 210–221.

Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., & Arya, N. (2005). Basic principles of real-time quantitative PCR. *Expert Rev. Mol. Diagn*, *5*, 1–11.

Auffret, G. (1981), Dynamique sédimentaire de la marge continentale celtique. (Thèse de doctorat), Université de Bordeaux I, France.

Avşar, U., Hubert-Ferrari, A., De Batist., M, Schmidt, S., & Fagel, N. (2015). Sedimentary records of past earthquakes in Boraboy Lake during the last ca 600 years (North Anatolian Fault, Turkey). *Palaeogeography, Palaeoclimatology, Palaeoecology, 433*, 1-9.

B

Baldauf, S. L. (2003). The deep roots of eukaryotes. *Science (New York, N.Y.)*, *300*, 1703–6.

Batten, S. D., & Burkill, P. H. (2010). The continuous plankton recorder: Towards a global perspective. *Journal of Plankton Research*, *32*, 1619–1621.

Bazzoni, A. M., Caddeo, T., Pulina, S., Padedda, B. M., Satta, C. T., Sechi, N., & Lugliè, A. (2015). Spatial distribution and multiannual trends of potentially toxic microalgae in shellfish farms along the Sardinian coast (NW Mediterranean Sea). *Environmental Monitoring and Assessment*, *187*, 86.

Belin, C. Distribution of *Dinophysis* spp. and *Alexandrium minutum* along French coasts since 1984 and their DSP and PSP toxicity levels (1993). In: *Toxic Phytoplankton Blooms in the Sea*. Amsterdam: Elsevier, 469–474.

Bellemain, E., Davey, M. L., Kauserud, H., Epp, L. S., Boessenkool, S., Coissac, E., … Brochmann, C. (2013). Fungal palaeodiversity revealed using high-throughput metabarcoding of ancient DNA from arctic permafrost. *Environmental Microbiology*, *15*, 1176–89.

Berge, T., Daugbjerg, N., Balling Andersen, B., & Hansen, P. (2010). Effect of lowered pH on marine phytoplankton growth rates. *Marine Ecology Progress Series*, *416*, 79–91.

Beucher, C., Tréguer, P., Corvaisier, R., Hapette, A. M., & Elskens, M. (2004). Production and dissolution of biosilica, and changing microphytoplankton dominance in the Bay of Brest (France). *Marine Ecology Progress Series*, *267*, 57–69.

Beudin, A., Chapalin, G., & Guillou, N. (2013). Suspended sediment modeling in the Bay of Brest impacted by the slipper limpet *Crepidula fornicata. Proceedings of the 7th international Conference on Coastal Dynamics*, 193-202.

Beudin, A., Chapalin, G., & Guillou, N. (2014). Modelling dynamics and exchanges of fine sediments in the Bay of Brest. *La Houille Blanche*, *6*, 47-53

Bickert, T., & Mackensen, A. (2004). Last glacial Holocene changes in South Atlantic deep water circulation. In: The South Atlantic in the Late Quaternary: Reconstruction of material budgets and current systems. Springer-Verlag, Berlin Heidelberg, New York Tokyo: 671- 695.

Bigler, C., & Hall, R. I. (2002). Diatoms as indicators of climatic and limnological change in Swedish lapland: A 100-lake calibration set and its validation for paleoecological reconstructions. *Journal of Paleolimnology*, *27*, 97–115.

Bik, H. M., Sung, W., De Ley, P., Baldwin, J. G., Sharma, J., Rocha-Olivares, A., & Thomas, W. K. (2012). Metagenetic community analysis of microbial eukaryotes illuminates biogeographic patterns in deep-sea and shallow water sediments. *Molecular Ecology*, *21*, 1048–1059.

Birks, H. H., & Birks, H. J. B. (2000). Future uses of pollen analysis must include plant macrofossils. *Journal of Biogeography*, *27*, 31–35.

Birks, H. J. B., & Birks, H. H. (1980) Quaternary Palaeoecology. Edward Arnold, London.

Bissett, A., Gibson, J. A. E., Jarman, S. N., Swadling, K. M., & Cromer, L. (2005). Isolation , amplification , and identification of ancient copepod DNA from lake sediments. *Limnology and Oceanography*, *3*, 533–542.

Blanco, E. P., Lewis, J., & Aldridge, J. (2009). The germination characteristics of Alexandrium minutum (Dinophyceae), a toxic dinoflagellate from the Fal estuary (UK). *Harmful Algae*, *8*, 518–522.

Boenigk, J., Pfandl, K., Garstecki, T., Novarino, G., & Chatzinotas, A. (2006). Evidence for geographic isolation and signs of endemism within a protistan morphospecies. *Applied Environmental Microbiology, 72*, 5159–5164.

Boere, A. (2010). Validation and application of fossil DNA as a recorder of past marine ecosystems and environmental conditions (Thèse de doctorat). Netherlands: 175.

Boere, A. C., Sinninghe Damsté, J. S., Rijpstra, W. I. C., Volkman, J. K., & Coolen, M. J. L. (2011). Source-specific variability in post-depositional DNA preservation with potential implications for DNA based paleoecological records. *Organic Geochemistry*, *42*, 1216–1225.

Boissière, A., Tchioffo, M. T., Bachar, D., Abate, L., Marie, A., Nsango, S. E., Shahbazkia, H. R., Awono-Ambene, P. H., Levashina, E. A., Christen, R., & Morlais, I. (2012). Midgut microbiota of the malaria mosquito vector Anopheles gambiae and interactions with Plasmodium falciparum infection. *PLoS Pathogens*, *8*, 1–12.

Boudouresque, C. F. (2012). Les invasions et transferts biologiques, avec une attention spéciale au milieu marin. GIS Posidonie publ, Marseille : 248.

Boyce, D. G., Lewis, M. R. & Worm, B. (2010). Global phytoplankton decline over the past century. *Nature*, *466*, 591–596.

Brassel, S. C. (1993). Applications of biomarkers for delineating marine paleoclimatic fluctuations during the Pleistocene. *Organic Geochemistry, 11*, 699–738.

Bravo, I., Fraga, S., Isabel Figueroa, R., Pazos, Y., Massanet, A., & Ramilo, I. (2010). Bloom dynamics and life cycle strategies of two toxic dinoflagellates in a coastal upwelling system (NW Iberian Peninsula). *Deep Sea Research Part II: Topical Studies in Oceanography*, *57*, 222–234.

Bravo, I., Vila, M., Maso, M., Figueroa, R.I., & Ramilo, I. (2008). *Alexandrium catenella* and *Alexandrium minutum* blooms in the Mediterranean Sea: toward the identification of ecological niches. Harmful *Algae, 7*, 515-522.

Brierley, A. S., & Kingsford, M. J. (2009). Impacts of Climate Change on Marine Organisms and Ecosystems. *Current Biology, 19*, R602–R614.

Brocks, J. J., & Pearson, A. (2005). Building the Biomarker Tree of Life. *Reviews in Mineralogy and Geochemistry*, *59*, 233–258.

Burgin, A. J., & Hamilton, S. K. (2007). Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. *Frontiers in Ecology and the Environment, 5*, 89–96.

Burkholder, J., & Glibert, P. (2006). Intraspecific variability: an important consideration in forming generalisations about toxigenic algal species. *African Journal of Marine Science*, *28*, 177–180.

Burki, F., Lane, N., Mcfadden, G. I., Gray, M. W., & Douglas, A. E. (2014). The eukaryotic tree of life from a global phylogenomic perspective. *Cold Spring Harbor Perspective Biology*, 6:a016147.

C

Caffrey, J. M. (2004). Factors controlling net ecosystem metabolism in US estuaries. *Estuaries, 27*, 90–101.

Cameron, W. M., & Pritchard D., (1963). Estuaries. In: The sea Volume 2, John Wiley and Sons, New York: 306-324.

Canfield, D. E., Jorgensen, B. B., Fossing, H., Glud, R., Gundersen, J., Ramsing, N. B., Thamdrup, B., Hansen, J. W., Nielsen, L. P., & Hall P. O. J. (1993). Pathways of organic carbon oxidation in three continental margin sediments. *Marine Geology, 113*, 27-40.

Cann, C. (1995). Flux de nutriments d'origine agricole vers la rade de Brest. *Ingénieries CEMAGREF Hors série*, 37-44.

Capo, E., Debroas, D., Arnaud, F., & Domaizon, I. (2015). Is Planktonic Diversity Well Recorded in Sedimentary DNA? Toward the Reconstruction of Past Protistan Diversity. *Microbial Ecology*, *70*, 865–75.

Caporaso, J. G., Lauber, C. L., Walters, W. a, Berg-Lyons, D., Huntley, J., Fierer, N., … Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, *6*, 1621–1624.

Caron, D. A., Countway, P. D., Jones, A. C., Kim, D. Y., & Schnetzer, A. (2012). Marine Protistan Diversity. *Annual Review of Marine Science*, *4*, 467–493.

Castel, J. (1993). Long-Term distribution of zooplankton in the Gironde estuary and its relation with river flow and suspended matter. *Cahiers de Biologie Marine, 34*, 145-163.

Cauwet, G. (1975). Optimization of an analytical technique for evaluation of organic carbon in sediments. *Chemical Geology, 16*, 59-63.

Cembella, A.D., Turgeon, J., Therriault, J.C., & Beland, P. (1988). Spatial distribution of *Protogonyaulax tamarensis* resting cysts in nearshore sediments along the north coast of the lower St. Lawrence estuary. *Journal of Shellfish Research*, 7, 597–610.

Center for advanced marine core research (Kochi University). (2001). Understanding the Earth's Environmental System. (http://www.kochi-u.ac.jp/marine-core/research/index_e.html)

Chang FH, Garthwaite I, Anderson DM,. Towers, N., Stewart, R., & MacKenzie, L. (1999) Immunofluorescent detection of a PSP-producing dinoflagellate, *Alexandrium minutum*, from Bay of Plenty, New Zealand. *New Zealand Journal of Marine Freshwater Research, 33*, 533– 43.

Chapelle, A., Gac, M. Le, Labry, C., Siano, R., Quere, J., Caradec, F., … Gouriou, J. (2015). The Bay of Brest (France), a new risky site for toxic *Alexandrium minutum* blooms and PSP shellfish contamination. *HARMFUL ALGAE NEWS*, *51*, 4–5.

Chapelle, A., Labry, C., Sourisseau, M., Lebreton, C., Youenou, A., & Crassous, M. P. (2010). *Alexandrium minutum* growth controlled by phosphorus. An applied model. *Journal of Marine Systems*, *83*, 181–191.

Chariton, A. A, Stephenson, S., Morgan, M. J., Steven, A. D. L., Colloff, M. J., Court, L. N., & Hardy, C. M. (2015). Metabarcoding of benthic eukaryote communities predicts the ecological condition of estuaries. *Environmental Pollution (Barking, Essex : 1987)*, *203*, 165–74.

Chariton, A. A., Court, L. N., Hartley, D. M., Colloff, M. J., & Hardy, C. M. (2010). Ecological assessment of estuarine sediments by pyrosequencing eukaryotic ribosomal DNA. *Frontiers in Ecology and the Environment*, *8*, 233–238.

Chauvaud, L., Jean, F., Ragueneau, O., & Thouzeau, G. (2000). Long-term variation of the Bay of Brest ecosystem: Benthic-pelagic coupling revisited. *Marine Ecology Progress Series*, *200*, 35–48.

Chen, L., Zonneveld, K. A. F., & Versteegh, G. J. M. (2011). Short term climate variability during " Roman Classical Period" in the eastern Mediterranean. *Quaternary Science Reviews*, *30*, 3880–3891.

Chertensky, A. (2012). Trapping of sediment in tidal estuaries. (Thèse de doctorat). Netherlands : 163.

Claquin, P., Longphuirt, S. N., Fouillaron, P., Huonnic, P., Ragueneau, O., Klein, C., & Leynaert, A. (2010). Effects of simulated benthic fluxes on phytoplankton dynamic and photosynthetic parameters in a mesocosm experiment (Bay of Brest, France). *Estuarine, Coastal and Shelf Science*, *86*, 93-101.

Cloern, J. E. (1996). Phytoplankton bloom dynamics in coastal ecosystems: A review with some general lessons from sustained investigation of San Francisco Bay, California, *Reviews of Geophysics, 34,* 127–168.

Cloern, J. E. (2001). Our evolving conceptual model of the coastal eutrophication problem. *Marine Ecology Progress Series*, *210*, 223–253.

Collins, S., & Bell, G. (2004). Phenotypic consequences of 1,000 generations of selection at elevated CO2 in a green alga. *Nature*, *431*(7008), 566–569.

Coolen, M. J. L., & Shtereva, G. (2009). Vertical distribution of metabolically active eukaryotes in the water column and sediments of the Black Sea. *FEMS Microbiology Ecology*, *70*, 525–39.

Coolen, M. J. L. (2011). 7000 Years of Emiliania huxleyi Viruses in the Black Sea. *Science*, *333*, 451–452.

Coolen, M. J. L., & Overmann, J. (2007). 217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. *Environmental Microbiology*, *9*, 238–49.

Coolen, M. J. L., Boere, A., Abbas, B., Baas, M., Wakenham, S. G., & Sinninghe Damsté, J. S. (2006). Ancient DNA derived from alkenone-biosynthesizing haptophytes and other algae in Holocene sediments from the Black Sea. *Paleoceanography*, *21*, 1–17.

Coolen, M. J. L., Orsi, W. D., Balkema, C., Quince, C., Harris, K., Sylva, S. P., … Giosan, L. (2013). Evolution of the plankton paleome in the Black Sea from the Deglacial to Anthropocene. *Proceedings of the National Academy of Sciences of the United States of America*, *110*. doi:10.1073/pnas.1219283110

Coolen, M. J. L., Saenz, J. P., Giosan, L., Trowbridge, N. Y., Dimitrov, P., Dimitrov, D., & Eglinton, T. I. (2009). DNA and lipid molecular stratigraphic records of haptophyte succession in the Black Sea during the Holocene. *Earth and Planetary Science Letters*, *284*, 610–621.

Corinaldesi, C., Barucca, M., Luna, G. M., & Dell'Anno, A. (2011). Preservation, origin and genetic imprint of extracellular DNA in permanently anoxic deep-sea sediments. *Molecular Ecology*, *20*, 642–654.

Costanza, R., D'Arge, R., De Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K. Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P. & Van Den Belt, M. (1997).The value of the world's ecosystem services and natural capital. *Nature, 387*, 253-260.

Cowart, D. a, Pinheiro, M., Mouchel, O., Maguer, M., Grall, J., Miné, J., & Arnaud-Haond, S. (2015). Metabarcoding is powerful yet still blind: a comparative analysis of morphological and molecular surveys of seagrass communities. *PloS One*, *10*, e0117562.

Crespo, B.G., Keafer, B.A., Ralston, D.K., Lind, H., Farber, D., & Anderson, D.M. (2011). Dynamics of *Alexandrium fundyense* blooms and shellfish toxicity in the Nauset Marsh System of Cape Cod (Massachusetts, USA). *Harmful Algae*, 12, 26-38.

Crutzen, P. J (2002). Geology of mankind. *Nature, 415*, 23.

Curry, W. B., & Oppo, D. W. (2005). Glacial water mass geometry and the distribution of $\delta^{13}C$ of ΣCO_2 in the western Atlantic Ocean. *Paleoceanography,* 20, PA1017.

D

D'Andrea, W. J., Lage, M., Martiny, J. B. H., Laatsch, A. D., Amaral-Zettler, L. a., Sogin, M. L., & Huang, Y. (2006). Alkenone producers inferred from well-preserved 18S rDNA in Greenland lake sediments. *Journal of Geophysical Research*, *111*, G03013.

d'Abbadie, M., Hofreiter, M., Vaisman A., Loakes, D., Gasparutto, D., Cadet, J., Woodgate, R., Pääbo, S., & Holliger, P. (2007). Molecular breeding of polymerases for amplification of ancient DNA. *Nature Biotechnology, 25*, 939–43.

Dale, B. (2009). Eutrophication signals in the sedimentary record of dinoflagellate cysts in coastal waters. *Journal of Sea Research*, *61*, 103–113.

Dale, B., (1983). Dinoflagellate resting cysts: benthic plankton. In: Survival Strategies of the Algae, Cambridge University Press, Cambridge: 69–136.

Daniel, A. (1995). Le cycle de l'azote en écosystème côtier : développement d'un analyseur submersible pour la mesure in situ des nitrates et nitrites; variations saisonnières de la production azotée phytoplanctonique. (Thèse de doctorat), Université de Bretagne Occidentale, Brest, France.

Danovaro, R. Corinaldesi, C. Luna, G. Dell'Anno, A. (2006). Molecular tools for the analysis of DNA in marine environments. In: Marine Organic Matter: Biomarkers, Isotopes and DNA, Springer, Berlin: 105–126.

Davies, J. H. (1964). A morphological approach to the world of shorelines. *Zeitschrift für geomorphology*, 8, 127- 142.

Davies, S. J., Metcalfe, S. E., Caballero, M. E., & Juggins, S. (2002). Developing diatom-based transfer functions for Central Mexican lakes. *Hydrobiologia*, *467*, 199–213.

Davis, J. R., & Koop, K. (2006). Eutrophication in Australian rivers, reservoirs and estuaries - A southern hemisphere perspective on the science and its implications. *Hydrobiologia*, *559*, 23–76.

De Bruyn, M., Hoelzel, A. R., Carvalho, G. R., & Hofreiter, M. (2011). Faunal histories from Holocene ancient DNA. *Trends in Ecology and Evolution*, *26*, 405–413.

de Groot, A. G., Laros, I., & Geisen, S. (2016). Molecular Identification of Soil Eukaryotes and Focused Approaches Targeting Protist and Faunal Groups Using High-Throughput Metabarcoding *Microb Environ Genomics*, *1399*, 125-140.

de Vargas, C. De, Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., … Romac, S. (2015). Eukaryotic plankton diversity in the sunlit ocean, *348*, 1–12.

de Vernal, A. de, Henry, M., Matthiessen, J., Mudie, P. J., Rochon, A., Boessenkool, K. P., … Voronina, E. (2001). Dinoflagellate cyst assemblages as tracers of sea-surface conditions in the Northern North Atlantic, Arctic and sub-Arctic seas: The new “n = 677” data base and its application for quantitative palaeoceanographic reconstruction. *Journal of Quaternary Science*, *16*, 681–698.

de Vernal, A., Eynaud, F., Henry, M., Hillaire-Marcel, C., Londeix, L., Mangin, S., Matthiessen, J., Marret, F., Radi, T., Rochon, A., Solignac, S. & Turon, J. L. (2005). Reconstruction of sea-surface conditions at middle to high latitudes of the Northern Hemisphere during the Last Glacial Maximum (LGM) based on dinoflagellate cyst assemblages. *Quaternary Science Review, 24*, 897-924.

Deflandre, B., Mucci, A., Gagné, J. P., Guignard, C., & Sundby, B. (2002). Early diagenetic processes in coastal marine sediments disturbed by a catastrophic sedimentation event. *Geochimica et Cosmochimica Acta*, 66, 2547-2558.

Del Amo, Y., Quéguiner, B., Tréguer, P., Breton, H., & Lampert, L. (1997). Impacts of high-nitrate freshwater inputs on macrotidal ecosystems. II. Specific role of the silicic acid pump in the year-round dominance of diatoms in the Bay of Brest (France). *Marine Ecology Progress Series*, *161*, 225–237.

Dell'Anno,A., Bompadre,S., Danovaro,R., 2002. Quantification, base composition, and fate of extracellular DNA in marine sediments. *Limnological Oceanography, 47*, 899–905.

Delmas, R., & Tréguer, P. (1983). Evolution saisonnière des nutriments dans un écosystème eutrophe d’Europe occidentale (la rade de Brest). Interactions marines et terrestres. *Oceanologica Acta, 6*, 345–356.

Dia, a, Guillou, L., Mauger, S., Bigeard, E., Marie, D., Valero, M., & Destombe, C. (2014). Spatiotemporal changes in the genetic diversity of harmful algal blooms caused by the toxic dinoflagellate Alexandrium minutum. *Molecular Ecology*, *23*, 549–60.

Dobell C. (1932). Antony van Leeuwenhoek and his “Little Animals.” Dover, New York.

Domaizon, I., Savichtcheva, O., Debroas, D., Arnaud, F., Villar, C., Pignol, C., … Perga, M. E. (2013). DNA from lake sediments reveals the long-term dynamics and diversity of *Synechococcus* assemblages. *Biogeosciences*, *10*, 3817–3838.

Donders, T. H., Gorissen, P. M., Sangiorgi, F., Cremer, H., Wagner-Cremer, F., & McGee, V. (2008). Three-hundred-year hydrological changes in a subtropical estuary, Rookery Bay (Florida): Human impact versus natural variability. *Geochemistry, Geophysics, Geosystems*, *9*. doi:10.1029/2008GC001980

Donnelly, A., Cooney, T., Jennings, E., Buscardo, E., & Jones, M. (2009). Response of birds to climatic variability ; evidence from the western fringe of Europe. *International Journal of Biometeorology, 53*, 211–220.

Dray, S., Dufour, & A.B. (2007). The ade4 package: implementing the duality diagram for ecologists. *Journal of Statistical Softwar, 22*, 1-20.

Drummond, A. J., Rambaut, A., Shapiro, B., & Pybus, O. G. (2005). Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution*, *22*, 1185–1192.

Durantou, L., Rochon, A., Ledu, D., Massé, G., Schmidt, S., & Babin, M. (2012). Quantitative reconstruction of sea-surface conditions over the last ~150 years in the Beaufort Sea based on dinoflagellate cyst assemblages: the role of large-scale atmospheric circulation patterns. *Biogeosciences*, *9*, 391-5406.

Dyer, K. R., (1986). Coastal and estuarine sediment dynamics: J. Wiley and Sons Publication: 342 .

Dyer, K. R., (1997). Estuaires: A physical introduction: Wiley, London: 140.

E

Edler, L., & Elbrächter, M. (2010). The Utermöhl method for quantitative phytoplankton analysis. *Microscopic and Molecular Methods for Quantitative Phytoplankton Analysis*, *110*.

Edvardsen, B., Eikrem, W., Green, J. C., Andersen, R. A., Moon-van der Staay, S. Y., & Medlin, L. K. (2000). Phylogenetic reconstructions of the Haptophyta inferred from 18S ribosomal DNA sequences and available morphological data. *Phycologia*, *39*, 19–35.

Edwards, K. J. & Macdonald, G. M. (1991). Holocene palynology: Human influence and vegetation change. *Progress in Physical Geography*, *15*, 364-391.

Edwards, M., Beaugrand, G., Helaouët, P., Alheit, J., & Coombs, S. (2013). Marine Ecosystem Response to the Atlantic Multidecadal Oscillation. *PLoS ONE*, *8*, 1–5.

Edwards, M., John, A., Johns, D. & Reid, P. (2001). Case history and persistence of the non-indigenous diatom *Coscinodiscus wailesii* in the north-east Atlantic. *Journal of the Marine Biological Association of the United Kingdom, 81*, 207–211.

Elbrachter, M. (1999). Exotic flagellates of coastal North Sea waters. *Helgoländer Meeresun*, *52*, 235–42.

Ellegaard, M., Clarke, A. L., Reuss, N., Drew, S., Weckström, K., Juggins, S., … Conley, D. J. (2006). Multi-proxy evidence of long-term changes in ecosystem structure in a Danish marine estuary, linked to increased nutrient loading. *Estuarine, Coastal and Shelf Science*, *68*, 567–578.

Ellegaard, M., Figueroa, R. L., & Versteegh, G. J. M. Dinoflagellate life cycles, strategy and diversity: key foci for future research (2013). In: *Biological and Geological Perspectives of Dinoflagellates*. The Micropalaeontological Society, Special Publications, Geological Society, London: 249–262.

Ellegaard, M., Ribeiro, S., Lundholm, N., Andersen, T. J., & Berge, T. (2013). Using the sediment archive of living dinoflagellate cysts and other protist resting stages to study temporal population dynamics, 149–153.

Ellegaard, M., Tuong, N., Nguyen, G., Joest, T., Michelsen, A., Lam, N., … Lund-hansen, L. C. (2014). Estuarine, Coastal and Shelf Science Temporal changes in physical , chemical and biological sediment parameters in a tropical estuary after mangrove deforestation. *Estuarine, Coastal and Shelf Science*, *142*, 32–40.

Elliott, M. & Quintino, V. (2007). The Estuarine Quality Paradox, Environmental Homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed areas. *Marine Pollution Bulletin, 54,* 640-645.

Epp, L. S., Boessenkool, S., Bellemain, E. P., Haile, J., Esposito, A., Riaz, T., … Brochmann, C. (2012). New environmental metabarcodes for analysing soil DNA: potential for studying past and present ecosystems. *Molecular Ecology*, *21*, 1821–33.

Epp, L. S., Stoof, K. R., Trauth, M. H., & Tiedemann, R. (2009). Historical genetics on a sediment core from a Kenyan lake: intraspecific genotype turnover in a tropical rotifer is related to past environmental changes. *Journal of Paleolimnology*, *43*, 939–954.

Erard-Le Denn, E. (1991). Recent occurrence of red-tide dinoflagellate *Alexandrium minutum* Halim for the North Western coasts of France. In: Recent Approaches on Red Tides: 85-98.

Erard-Le Denn, E., Boulay, & V. (1995). Resting cysts of *Alexandrium minutum* in marine sediments: quantification by three methods. In: Harmful Marine Algal Blooms. Lavoisier Publishing, Paris: 257–730.

Erard-Le Denn, E., Desbruyeres, E., & Olu, K. (1993). *Alexandrium minutum*: resting cyst distribution in the sediments collected along the Brittany coast, France. In: Toxic Phytoplankton in the Sea, Elsevier Science Publisher, Amsterdam: 109–114.

Erdner, D. L., Percy, L., Keafer, B., Lewis, J., & Anderson, D. M. (2010). A quantitative real-time PCR assay for the identification and enumeration of Alexandrium cysts in marine sediments. *Deep-Sea Research. Part II, Topical Studies in Oceanography*, *57*, 279–287.

F

Falkowski, P. G. (1994). The role of phytoplankton photosynthesis in global biogeochemical cycles. *Photosynthesis Research*, *39*, 235–258.

Falkowski, P. G., Barber, R. T., & Smetacek, V. (1998). Biogeochemical controls and feedbacks on ocean primary production. *Science*, *281*, 200–206.

Fertouna-Bellakhala, M., Dhib, A., Fathalli, A., Bellakhal, M., Chomérat, N., Masseret, E., Laabir, M., Turkic, S., & Aleyaa, L. (2015). *Alexandrium pacificum* Litaker sp. nov (Group IV): Resting cyst distribution and toxin profile of vegetative cells in Bizerte Lagoon (Tunisia, Southern Mediterranean Sea). *Harmful Algae,* 48, 69-82.

Fertouna-Bellakhala, M., Dhib, Béjaoui, B., Turki, S., & Aleya, L. (2014). Driving factors behind the distribution of dinocyst composition and abundance in surface sediments in a western Mediterranean coastal lagoon: report from a high resolution mapping study. *Marine Pollution Bulletin, 84,* 347-362.

Figueroa, R. I., Garcés, E., & Camp, J. (2010). Reproductive plasticity and local adaptation in the host-parasite system formed by the toxic Alexandrium minutum and the dinoflagellate parasite Parvilucifera sinerae. *Harmful Algae*, *10*, 56–63.

Finkel, Z. V., Beardall, J., Flynn, K. J., Quigg, A., Rees, T. A. V, & Raven, J. A. (2010). Phytoplankton in a changing world: Cell size and elemental stoichiometry. *Journal of Plankton Research*, *32*, 119–137.

Fitzpatrick, E., Caron, D. A., & Schnetzer, A. (2010). Development and environmental application of a genus-specific quantitative PCR approach for *Pseudo-nitzschia* species. *Marine Biology, 157*,1161–69.

Fonseca, V. G., Carvalho, G. R., Sung, W., Johnson, H. F., Power, D. M., Neill, S. P., … Creer, S. (2010). Second-generation environmental sequencing unmasks marine metazoan biodiversity. *Nature Communications*, *1*, 98.

Forster, D., Dunthorn, M., Mahé, F., Dolan, J. R., Audic, S., Bass, D., … Stoeck, T. (2015). Benthic protists: the under-charted majority. *Scientific Reports*, *submitted*, 1–33.

Franco, J. M., Fernandez, P., & Reguera, B. (1994). Toxin profiles of natural populations and cultures of *Alexandrium minutum* Halim from Galician (Spain) coastal waters. *Journal of Applied Phycology*, 6, 275–79.

Fredrick, N. D., Berges, J. A., Twining, B. S., Nuñez-Milland, D., & Hellweger, F. L. (2013). Use of agent-based modeling to explore the mechanisms of intracellular phosphorus heterogeneity in cultured phytoplankton. *Applied and Environmental Microbiology*, *79*, 4359–4368.

Fritz, S., Juggins, S., Battarbee, R., & Engstrom, D. (1991). Reconstruction of past changes in salinity and climate using a diatom-based transfer function. *Nature*.

Fukami, K., Nishijima, T., & Ishida, Y. (1997). Stimulative and inhibitory effects of bacteria on the growth of microalgae. *Hydrobiologia*, *358*, 185–191.

G

Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garce, E., Garcés, E., & Magnani, M. (2004). Development of a Real-Time PCR Assay for Rapid Detection and Quantification of Alexandrium minutum (a Dinoflagellate) Development of a Real-Time PCR Assay for Rapid Detection and Quantification of Alexandrium minutum (a Dinoflagellate). *Applied and Environmental Microbiology*, *70*, 1199–1206.

Garcés, E. (2004). Relationship between vegetative cells and cyst production during *Alexandrium minutum* bloom in Arenys de Mar harbour (NW Mediterranean). *Journal of Plankton Research*, *26*, 637–645.

Garcés, E., & Masó, M. (2001). Phytoplankton potential growth rate versus increase in cell numbers: estimation of cell lysis. *Marine Ecology Progress Series*, *212*, 297–300.

Garneau, M.-È., Schnetzer, A., Countway, P. D., Jones, A. C., Seubert, E. L., & Caron, D. a. (2011). Examination of the seasonal dynamics of the toxic dinoflagellate Alexandrium catenella at Redondo Beach, California, by quantitative PCR. *Applied and Environmental Microbiology*, *77*, 7669–80.

Gattuso, J. P., Frankignoulle, M., & Wollast, R. (1998). Carbon and carbonate metabolism in coastal aquatic ecosystems. *Annual Review of Ecology, Evolution, and Systematics, 29,* 405–433.

Gayoso, A.M. (2001). Observation on *Alexandrium tamarense* (Lebour) Balech and other dinoflagellate population in Golfo Nuevo, Patagonia (Argentina). *Journal of Plankton Research, 23,* 463–468.

Geider, R. J., Delucia, E. H., Falkowski, P. G., Finzi, A. C., Grime, J. P., Grace, J., … Box, P. O. (2001). Primary productivity of planet earth : biological determinants and physical constraints in terrestrial and aquatic habitats. Global Change Biology, 8, 884-882.

Genovesi, B., Laabir, M., Masseret, E., Collos, Y., Vaquer, A., & Grzebyk, D. (2009). Dormancy and germination features in resting cysts of *Alexandrium tamarense* species complex (Dinophyceae) can facilitate bloom formation in a shallow lagoon (Thau, southern France). *Journal of Plankton Research, 31*, 1209–1224.

Genovesi, B., Mouillot, D., Laugier, T., Fiandrino, A., Laabir, M., Vaquer, A., & Grzebyk, D. (2013). Influences of sedimentation and hydrodynamics on the spatial distribution of Alexandrium catenella/tamarense resting cysts in a shellfish farming lagoon impacted by toxic blooms. *Harmful Algae*, *25*, 15–25.

Giguet-Covex, C., Pansu, J., Arnaud, F., Rey, P.-J., Griggo, C., Gielly, L., … Taberlet, P. (2014). Long livestock farming history and human landscape shaping revealed by lake sediment DNA. *Nature Communications*, *5*, 3211.

Gilbert, M. T. P., Bandelt, H.-J., Hofreiter, M., & Barnes, I. (2005). Assessing ancient DNA studies. *Trends in Ecology & Evolution*, *20*, 541–4.

Gimonneau, G., Tchioffo., M. T., Abate, L., Boissière, A., Awono-Ambéné, P. H., Nsango, S. E., Christen, R., & Morlais, I. (2014). Composition of *Anopheles coluzzii* and *Anopheles gambiae* microbiota from larval to adult stages. *Infection, Genetics and Evolution, 28*, 715–724.

Glud, R. N. (2008). Oxygen dynamics of marine sediments. *Marine Biology Research, 4*, 243-289.

Godhe, a, Otta, S. K., Rehnstam-Holm, a S., & Karunasagar, I. (2001). Polymerase chain reaction in detection of Gymnodinium mikimotoi and Alexandrium minutum in field samples from southwest India. *Marine Biotechnology (New York, N.Y.)*, *3*, 152–62.

Godhe, A., Asplund, M. E., Härnström, K., Saravanan, V., Tyagi, A., & Karunasagar, I. (2008). Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. *Applied and Environmental Microbiology*, *74*, 7174–82.

Godhe, A., Rehnstam-Holm, A.-S., Karunasagar, I., & Karunasagar, I. (2002). PCR detection of dinoflagellate cysts in field sediment samples from tropic and temperate environments. *Harmful Algae*, *1*, 361–373.

Gómez, F. (2008). Phytoplankton invasions: comments on the validity of categorizing the non-indigenous dinoflagellates and diatoms in European seas. *Marine Pollution Bulletin*, *56*, 620–8.

Gorgues, T., Planquette, H. (2016). Complexité et efficacité de la pompe biologique de carbone. (https://www.labexmer.eu/fr/recherche/axe2).

Gould, B. A., León, B., Buffen, A. M., & Thompson, L. G. (2010). Evidence of a high-andean, mid-holocene plant community: An ancient DNA analysis of glacially preserved remains. *American Journal of Botany*, *97*, 1579–1584.

Gradstein, F. M., Kristiansen, I. L., Loemo, L., & Kaminski, M. A. (1992). Cenozoic foraminiferal and dinoflagellate cyst biostratigraphy of the central North Sea. *Micropaleontology*, *38*, 101–137.

Gray, M., Wawrik, B., Paul, J., Casper, E., & Gymno-, M. (2003). Molecular Detection and Quantitation of the Red Tide Dinoflagellate Karenia brevis in the Marine Environment. *Applied and Environmental Microbiology*, *69*, 5726–5730.

Green, R. E., Krause, J., Briggs, A. W., Maricic, T., Stenzel, U., Kircher, M., … Pääbo, S. (2010). A draft sequence of the Neandertal genome. *Science (New York, N.Y.)*, *328*, 710–22.

Gregoire, G., Ehrhold, A., Le Roy, P., Jouet, G., & Garlan, T. (2016). Modern morpho-sedimentological patterns in a tide-dominated estuary system: the Bay of Brest (west Brittany, France). *Journal of Maps*. http://doi.org/10.1080/17445647.2016.1139514

Gross J. (1989). Re-occurrence of red tide in Cork Harbor, Ireland. *Red Tide Newsletter*, 2, 4–5.

Gu, H., Sun, J., Kooistra, W. H. C. F., & Zeng, R. (2008). Phylogenetic Position and Morphology of Thecae and Cysts of Scrippsiella (Dinophyceae) Species in the East China Sea. *Journal of Phycology*, *44*, 478–494.

Guardiola, M., Uriz, M. J., Taberlet, P., Coissac, E., Wangensteen, S., & Turon, X. (2015). Deep-Sea , Deep-Sequencing : Metabarcoding Extracellular DNA from Sediments of Marine Canyons. doi:10.5061/dryad.520gq

Guillard, R. R., & Ryther, J. H. (1962). Studies of marine planktonic diatoms : I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve). *Canadian Journal of Microbiology, 8*, 229-239.

Guillaud, J.-F., & Bouriel, L. (2007). Relation concentration-débit et évolution temporelle du nitrate dans 25 rivières de la région Bretagne (France). *Revue Des Sciences de L'eau*, *20*, 213.

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., … Christen, R. (2013). The Protist Ribosomal Reference database (PR2): A catalog of unicellular eukaryote Small Sub-Unit rRNA sequences with curated taxonomy. *Nucleic Acids Research*, *41*, 597–604.

Guillou, L., Viprey, M., Chambouvet, A., Welsh, R. M., Kirkham, A. R., Massana, R., … Worden, A. Z. (2008). Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). *Environmental Microbiology*, *10*, 3349–3365.

H

Hafsaoui, M., Quéguiner, B., & Tréguer, P. (1985). Production primaire et facteurs limitant la croissance du phytoplancton en rade de Brest (1981-1983). *Oceanis*, *11*, 181-195.

Haile, J., Froese, D. G., Macphee, R. D. E., Roberts, R. G., Arnold, L. J., Reyes, A. V, … Willerslev, E. (2009). Ancient DNA reveals late survival of mammoth and horse in interior Alaska. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 22352–22357.

Halim, Y. (1960). *Alexandrium minutum,* dinoflagellé provocant des "eaux rouges", *Vie Milieu*, *11*, 102–105.

Hallegraeff, G. M. (1998). Transport of toxic dinoflagellates via ships' ballast water: bioeconomic risk assessment and effi- cacy of possible ballast water management strategies. *Marine Ecological Progress Series, 168*, 297–309.

Hallegraeff, G. M. (2010). Ocean climate change, phytoplankton community responses, and harmful algal blooms : A formidable predictive challenge. *Journal of Phycology*, *46*, 220–235.

Hallegraeff, G., Steffensen, D.A., & Wetherbee, R. (1988). Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. *Journal of Plankton Research, 10*, 533–41.

Hansen, G., Daugbjerg, N., & Franco, J. . (2003). Morphology, toxin composition and LSU rDNA phylogeny of Alexandrium minutum (Dinophyceae) from Denmark, with some morphological observations on other European strains. *Harmful Algae*, *2*, 317–335.

Härnström, K., Ellegaard, M., Andersen, T. J., & Godhe, A. (2011). Hundred years of genetic structure in a sediment revived diatom population. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 4252–7.

Hattenrath-Lehmann, T.K., Zhen, Y. Wallace, R.B., Tang, Y.Z. & Gobler, C.J. (2016). Mapping the distribution of cysts from the toxic dinoflagellate *Cochlodinium polykrikoides* in bloom-prone estuaries by a novel fluorescence *in situ* hybridization assay. *Applied Environmental Microbiology, 82*, 1114-1125.

Hayes, M.O., (1975). Morphology of sand accumulations in estuaries. Estuarine Research, Academic Press, New York: 3-22.

Hebsgaard, M. B., Gilbert, M. T. P., Arneborg, J., Heyn, P., Allentoft, M. E., Bunce, M., … Willerslev, E. (2009). "The Farm Beneath the Sand"- an archaeological case study on ancient "dirt" DNA. *Antiquity*, *83*, 430–444.

Hernandez Farinas, T., Bacher, C., Soudant, D., Belin, C., & Barille, L. (2015). Assessing phytoplankton realized niches using a French national phytoplankton monitoring network. *Estuarine, Coastal and Shelf Science*, *159*, 15–27.

Hernandez-Farinas, T., Soudant, D., Barille, L., Belin, C., Lefebvre, A., & Bacher, C. (2014). Temporal changes in the phytoplankton community along the French coast of the eastern English Channel and the southern Bight of the North Sea. *ICES J MAR SCI*, *71*, 821–833.

Hily, C. (1991). Is the activity of benthic suspension feeders a factor controlling water quality in the Bay of Brest? *Marine Ecological Progress Series,* **69**, 179–188.

Hily, C., (1989). La megafaune benthique des fonds meubles de la rade de Brest: pré-échantillonage par vidéo sous-marine. *Cahiers de Biologie Marine, 30*, 433-454.

Hofreiter, M. Serre, D., Poinar, H. N., Kuch, M., & Pääbo, S. (2001) Ancient DNA. *Nature Reviews Genetics, 2*, 353–360.

Hopkinson, C. J., & Smith, E. M., (2005). Estuarine respiration: an overview of benthic, pelagic and whole system respiration. In: Respiration in Aquatic Ecosystems, Oxford University Press, New York: 123-147.

Horner, R.A., Greengrove, C.L., Davies-Vollum, K.S., Gawel, J.E., Postel, J.R., & Cox, A.M. (2011). Spatial distribution of benthic cysts of *Alexandrium catenella* in surface sediments of Puget Sound, Washington, USA. *Harmful Algae, 11*, 96–105.

Hou, W., Dong, H., Li, G., Yang, J., Coolen, M. J. L., Liu, X., … Wan, Y. (2014). Identification of photosynthetic plankton communities using sedimentary ancient DNA and their response to late-Holocene climate change on the Tibetan Plateau. *Scientific Reports*, *4*, 6648.

Hu, S. (2016). Protistan diversity and activity inferred from RNA and DNA at a coastal ocean site in the eastern North Pacific. *FEMS Microbiology Ecology*, *92*, 1–39.

Huang, Y., Shuman, B., Wang, Y., & Webb, T. (2004). Hydrogen isotope ratios of individual lipids in lake sediments as novel tracers of climatic and environmental change: A surface sediment test. *Journal of Paleolimnology*, *31*, 363–375.

Hwang, D. F., & Lu, Y. H. (2000). Influence of environmental and nutritional factors on growth, toxicity , and toxin profile of dinoflagellate *Alexandrium minutum*. *Toxicon*, *38*, 1491–1503.

I

Irigoien, X., Harris, R. P., Head, R. N., & Harbour, D. (2000). North Atlantic Oscillation and spring bloom phytoplankton composition in the English Channel. *Journal of Plankton Research*, *22*, 2367–2371.

J

Jacobson, D. M., & Anderson, D. M. (1996). Widespread phagocytosis of ciliates and other protists by marine mixotrophic and heterotrophic thecate dinoflagellates, Journal of Phycology, 32, 279–285.

Jeffrey, S. W., Vesk, M., & Mantoura, R. F. (1997). Phytoplankton pigments: windows into the pastures of the sea. *Nature and Resources*, *33*, 14–29.

John, U., Litaker, R. W., Montresor, M., Murray, S., Brosnahan, M. L., & Anderson, D. M. (2014). Formal revision of the Alexandrium tamarense species complex (Dinophyceae) taxonomy: the introduction of five species with emphasis on molecular-based (rDNA) classification. *Protist*, *165*, 779–804.

Jørgensen, B. B., & Boetius, A. (2007). Feast and famine–microbial life in the deep-sea bed. *Nature Reviews Microbiology, 5*, 770–781.

Jørgensen, T., Haile, J., Möller, P., Andreev, A., Boessenkool, S., Rasmussen, M., … Willerslev, E. (2012). A comparative study of ancient sedimentary DNA, pollen and macrofossils from permafrost sediments of northern Siberia reveals long-term vegetational stability. *Molecular Ecology*, *21*, 1989–2003.

Joyce L. B., Pitcher G. C. (2006). Cysts of *Alexandrium catenella* on the west coast of South Africa: distribution and characteristics of germination. *African Journal of Marine Science, 28,* 295-298.

Joyce, L.B., Pitcher, G.C., du Randt A., & Monteiro, P.M.S. (2005). Dinoflagellate cysts from surface sediments of Saldanha Bay, South Africa: an indication of the potential risk of harmful algal blooms. *Harmful Algae*, 4, 309–318.

K

Kamikawa, R., Nagai, S., Hosoi-Tanabe, S., Itakura, S., Yamaguchi, M., Uchida, Y., … Sako, Y. (2007). Application of real-time PCR assay for detection and quantification of Alexandrium tamarense and Alexandrium catenella cysts from marine sediments. *Harmful Algae*, *6*, 413–420.

Kapkov, V. I., Belenikina, O. A., & Fedorov, V. D. Effect of heavy metals on marine phytoplankton. *Moscow University Biological Sciences Bulletin, 66*,32–36.

Katsanevakis, S., Wallentinus, I., Zenetos, A., Leppäkoski, E., Çinar, M. E., Oztürk, B., … Cardoso, A. C. (2014). Impacts of invasive alien marine species on ecosystem services and biodiversity: a pan-European review. *Aquatic Invasions*, *9*, 391–423.

Keller, M.D., Selvin, R.C, Claus, W., & Guillard R.R.L. (1987). Media for the culture of oceanic ultraphytoplankton. *Journal of Phycology*, *23*, 633–38.

Kermarrec, L., Franc, A., Rimet, F., Chaumeil, P., Humbert, J., & Bouchez, A. (2014). A next-generation sequencing approach to river biomonitoring using benthic diatoms. *Freshwater Science*, *33*, 349–363.

Kim, J. H., Kim, J. H., Wang, P., Park, B. S., & Han, M. S. (2016). An improved quantitative real-time PCR assay for the enumeration of Heterosigma akashiwo (Raphidophyceae) cysts using a DNA debris removal method and a cyst-based standard curve. *PLoS ONE*, *11*, 1–17.

Klais, R., Tamminen, T., Kremp, A., Spilling, K., An, B. W., Hajdu, S. & Olli, K. (2013). Spring phytoplankton communities shaped by interannual weather variability and dispersal limitation: Mechanisms of climate change on key coastal primary producer. *Limnology and Oceanography*, *58*, 753–762.

Klouch, K. Z., Schmidt, S., Andrieux-Loyer, F., Gac, M. Le, Hervio-Heath, D., Qui-Minet, Z. N., … Siano, R. (2016). Historical records from dated sediment cores reveal the multidecadal dynamic of the toxic dinoflagellate Alexandrium minutum in the Bay of Brest (France). *FEMS Microbiology Ecology*, 1–16.

Koinig, K., Schmidt, R., Sommarugaw-Wögrath, S., Tessadri, R., & Psenner, R. (1998). Climate changes as the primary causes for pH shifts in a high alpine lake. *Water Air and Soil Pollution*, *104*, 167–180.

Kooistra, W. H. C. F., Sarno, D., Balzano, S., Gu, H., Andersen, R. A., & Zingone, A. (2008). Global Diversity and Biogeography of Skeletonema Species (Bacillariophyta). *Protist*, *159*, 177–193.

Kremp, A. (2000). Distribution, dynamics and *in situ* seeding potential of *Scrippsiella hangoei* (Dinophyceae) cyst populations from the Baltic Sea. Journal *of Plankton Research, 22*, 2155–2169.

Kremp, A., & Anderson, D. M. (2000). Factors regulating germination of resting cysts of the spring bloom dinoflagellate Scrippsiella hangoei from the northern Baltic Sea, *22*, 1311–1327.

Krüttli, A., Bouwman, A., Akgül, G., Casa, P. Della, Rühli, F., & Warinner, C. (2014). Ancient DNA analysis reveals high frequency of european lactase persistence allele (T-13910) in medieval Central Europe. *PLoS ONE*, *9*. doi:10.1371/journal.pone.0086251

L

Labry, C., Erard-Le Denn, E., Chapelle, A., Fauchot, J., Youenou, A., Crassous, M. P., Lorgeoux, B. (2008). Competition for phosphorus between two dinoflagellates: A toxic *Alexandrium minutum* and a non-toxic *Heterocapsa triquetra*. *Journal of Experimental Marine Biology and Ecology*, *358*, 124–135.

Lagus, A., Suomela, J., Weithoff, G., Heikkilä, K., Helminen, H., & Sipura, J. (2004). Species-specific differences in phytoplankton responses to N and P enrichments and the N:P ratio in the Archipelago Sea, northern Baltic Sea. *Journal of Plankton Research*, *26*, 779–798.

Laird K.R., Fritz S.C., Maasch K.A., & Cumming, B. (1996). Greater drought intensity and frequency before AD 1200 in the Northern Great Plains, USA. *Nature*, *384*, 552–55.

Laird, K. R., Fritz, S. C., Grimm, E. C., & Mueller, P. G. (1996). Century scale paleoclimatic reconstruction from Moon Lake, a closed-basin lake in the northern Great Plains. *Limnology and Oceanography*, *41*, 890–902.

Lakeman, M. B., von Dassow, P., & Cattolico, R. A. (2009). The strain concept in phytoplankton ecology. *Harmful Algae*, *8*, 746–758.

Langer, G., Nehrke, G., Probert, I., Ly, J., & Ziveri, P. (2009). Strain-specific responses of *Emiliania huxleyi* to changing seawater carbonate chemistry. *Biogeosciences Discussions*, *6*, 4361–4383.

Larsonneur, C., (1977). La cartographie des dépôts meubles sur le plateau continental français: methode mise au point et utilisée en Manche. *Journal de Recherche Océanographique, 2,* 3439.

Lazure P., Garnier V., Dumas F., Herry, C., & Chifflet M., (2009). Development of a hydrodynamic model of the Bay of Biscay. Validation of hydrology. *Continental Shelf Research*, *29*, 985-997.

Lazure, P., & Dumas, F. (2008). An external-internal mode coupling for a 3D hydrodynamical model for applications at regional scale (MARS). *Advances in Water Resources, 31*, 233-250.

Le Bescot, N., Mahé, F., Audic, S., Dimier, C., Garet, M.-J., Poulain, J., … Siano, R. (2015). Global patterns of pelagic dinoflagellate diversity across protist size classes unveiled by metabarcoding. *Environmental Microbiology, 18,* 609-626.

Le Pape, O., & Menesguen, A. (1997). Hydrodynamic prevention of eutrophication in the Bay of Brest (France), a modelling approach, in: Ruddick, K. (Ed.) Processes in regions of freshwater influence (PROFILE). *Journal of Marine Systems,12*, 171-186.

Le Pape, O., Del Amo, Y., Menesguen, A., Aminot, A., Quequiner, B., & Treguer, P. (1996). Resistance of a coastal ecosystem to increasing eutrophic conditions: the Bay of Brest (France), a semi-enclosed zone of Western Europe. *Continental Shelf Research*, *16*, 1885–1907.

Leavitt, P. R., & Findlay, D. L. (1994). Comparison of Fossil Pigments with 20 Years of Phytoplankton Data from Eutrophic Lake 227, Experimental Lakes Area, Ontario. *Journal Canadien Des Sciences Halieutiques et Aquatiques*, *51*, 2286–2299.

Leavitt, P. R., Hodgson, D. A. (2001) Sedimentary pigments. In: Developments in paleoenvironmental research, Volume 3. Tracking environmental change using lake sediments: volume 3: terrestrial, algal and siliceous indicators. Kluwer Academic, Dordrecht: 295–325.

Leavitt, P. R., Vinebrooke, R. D., Donald, D. B., Smol, J. P., & Schindler, D. W. (1997). Past ultraviolet radiation environments in lakes derived from fossil pigments. *Nature*, *388*, 457–459.

Lebret, K., Kritzberg, E. S., Figueroa, R., & Rengefors, K. (2012). Genetic diversity within and genetic differentiation between blooms of a microalgal species. *Environmental Microbiology*, *14*, 2395–2404.

Lejzerowicz, F., Esling, P., Majewski, W., Szczucin, W., Decelle, J., Obadia, C., … Pawlowski, J. (2013). Ancient DNA complements microfossil record in deep-sea subsurface sediments. doi:10.5061/dryad.b5m0j

Lejzerowicz, F., Esling, P., Pillet, L., Wilding, T. a, Black, K. D., & Pawlowski, J. (2015). High-throughput sequencing and morphology perform equally well for benthic monitoring of marine ecosystems. *Scientific Reports*, *5*, 13932.

Lett, C., Verley, P., Mullon, C., Parada, C., Brochier, T., Penven, P., & Blanke, B. (2008). A Lagrangian tool for modelling ichthyoplankton dynamics. *Environmental Modelling & Software, 23*, 1210-1214.

Lever, M. A., Torti, A., Eickenbusch, P., Michaud, A. B., Šantl-Temkiv, T., & Jørgensen, B. B. (2015). A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. Frontiers in Microbiology, 6.

Lewis, J., Harris, A. S. D., Jones, K. J., Edmonds, R. L., & Lewis, J. (1999). Long-term survival of marine planktonic diatoms and dinoflagellates in stored sediment samples. *Journal of Plankton Research*, *21*, 343–354.

Lilly, E. L., Halanych, K. M., & Anderson, D. M. (2005). Phylogeny, biogeography, and species boundaries within the Alexandrium minutum group. *Harmful Algae*, *4*, 1004–1020.

Lilly, E. L., Kulis, D. M., Gentien, P. & Anderson, D. M. (2002). Paralytic shell-fish poisoning toxins in France linked to a human-introduced strain of *Alexandrium catenella* from the western Pacific: evidence from DNA and toxin analysis. *Journal of Plankton Research, 24*, 443–452.

Lin, S., Litaker, R. W., & Sunda, W. G. (2016). Phosphorus physiological ecology and molecular mechanisms in marine phytoplankton. *Journal of Phycology*, *52*, 10–36.

Liu, D., Shen, X., Di, B., Shi, Y., Keesing, J., & Wang, Y. (2013). Palaeoecological analysis of phytoplankton regime shifts in response to coastal eutrophication. *Marine Ecology Progress Series*, *475*, 1–14.

Logares, R., Audic, S. S., Santini, S. S., Pernice, M. C., de Vargas, C., & Massana, R. (2012). Diversity patterns and activity of uncultured marine heterotrophic flagellates unveiled with pyrosequencing. *The ISME Journal*, *6*, 1823–1833.

Logares, R., Audic, S., Bass, D., Bittner, L., Boutte, C., Christen, R., … Massana, R. (2014). Patterns of rare and abundant marine microbial eukaryotes. *Current Biology : CB*, *24*, 813–21.

Londeix, L., Herreyre, Y., Turon, J. L., & Fletcher, W. (2009). Last Glacial to Holocene hydrology of the Marmara Sea inferred from a dinoflagellate cyst record. *Review of Palaeobotany and Palynology*, *158*, 52–71.

Lorenzen, C.J., (1967). Determination of chlorophyll and pheopigments: spectrophotometric equations. *Limnological Oceanography, 12*, 343-346.

Loret, P. (2002). No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. *Journal of Plankton Research*, *24*, 735–739.

Lundholm, N., Clarke, a., & Ellegaard, M. (2010). A 100-year record of changing Pseudo-nitzschia species in a sill-fjord in Denmark related to nitrogen loading and temperature. *Harmful Algae*, *9*, 449–457.

Lundholm, N., Ribeiro, S., Andersen, T. J., Koch, T., Godhe, A., Ekelund, F., & Ellegaard, M. (2011). Buried alive – germination of up to a century-old marine protist resting stages. *Phycologia*, *50*, 629–640.

Lydolph, M. C., Jacobsen, J., Arctander, P., Gilbert, M. T. P., Gilichinsky, D. A., Hansen, A. J., … Lange, L. (2005). Beringian paleoecology inferred from permafrost-preserved fungal DNA. *Applied and Environmental Microbiology*, *71*, 1012–1017.

M

Magoč, T., & Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, *27*, 2957–2963.

Mahé, F., Vargas, C. De, Bass, D., Czech, L., Stamatakis, A., Lara, E., Mayor, Jordan Bunge, J., Sernaker, S., Siemensmeyer, T., Trautmann, I., Romac, S., Berney, C., Kozlov, A., Edward, A. D., Seppey, C. V. W., Singer, D., Egge, E.Wirth, R., Trueba, G., & Dunthorn, M. (2016). Soil protists in three neotropical rainforests are hyperdiverse and dominated by parasites, 1-17, doi: http://dx.doi.org/10.1101/050997

Malviya, S., Scalco, E., Audic, S., Vincent, F., Veluchamy, A., & Poulain, J. (2016). Insights into global diatom distribution and diversity in the world's ocean. *PNAS*, 113, E1516–E1525.

Martínez de la Escalera, G., Antoniades, D., Bonilla, S., & Piccini, C. (2014). Application of ancient DNA to the reconstruction of past microbial assemblages and for the detection of toxic cyanobacteria in subtropical freshwater ecosystems. *Molecular Ecology*, *23*, 5791–802.

Massana, R., Castresana, J., Balague, V., Guillou, L., Romari, K., Valentin, K., & Pedro, C. (2004). Phylogenetic and Ecological Analysis of Novel Marine Stramenopiles. *Applied and Environmental Microbiology*, *70*, 3528–3534.

Massana, R., del Campo, J., Sieracki, M. E., Audic, S., & Logares, R. (2014). Exploring the uncultured microeukaryote majority in the oceans: reevaluation of ribogroups within stramenopiles. *The ISME Journal*, *8*, 854–66.

Massana, R., Gobet, A., Audic, S., Bass, D., Bittner, L., Boutte, C., … de Vargas, C. (2015). Marine protist diversity in European coastal waters and sediments as revealed by high-throughput sequencing. *Environmental Microbiology*, *17*, 4035–4049.

Matsuoka, K., Joyce, L.B., Kotani, Y., & Matsuyama, Y. (2003). Modern dinoflagellate cysts in hypertrophic coastal waters of Tokyo Bay, Japan. *Journal of Plankton Research*, 25, 1461–1470.

McCaffrey, M. A., Farrington, J. W., & Repeta, D. J. (1990). The organic geochemistry of Peru margin surface sediments: I. A comparison of the C_{37} alkenone and historical El Niño records. *Geochimica et Cosmochimica Acta*, *54*, 1671–1682.

McCauley, L. a. R., Erdner, D. L., Nagai, S., Richlen, M. L., & Anderson, D. M. (2009). Biogeographic analysis of the globally distributed harmful algal bloom species *Alexandrium minutum* (dinophyceae) based on rRNA gene sequences and microsatellite markers. *Journal of Phycology*, *45*, 454–463.

McCorkle, D. C., Corliss, B. H., & Farnham, C.A. (1997). Vertical distribution and stable isotopic compositions of live (stained) benthic foraminifera from the North Carolina and California continental margins. *Deep-Sea Research I, 44*, 983-1024.

McLusky, D. S. & M. Elliott. 2004. The Estuarine Ecosystem - Ecology, Threats and Management. Oxford University Press, Oxford.

McQuoid, M. R., Godhe, A., & Nordberg, K. (2002). Viability of phytoplankton resting stages in the sediments of a coastal Swedish fjord. *European Journal of Phycology*, *37*, 191–201.

Meehl, G.A., Stocker, T.F., Collins, W.D., Friedlingstein, P., Gaye, A.T., Gregory, J.M., Kitoh, A., Knutti, R., Murphy, J.M., Noda, A., Raper, S.C.B., Watterson, I.G., Weaver, A.J. & Zhao, Z.C. (2007). Global Climate Projections. In: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Meiggs, D., & Taillefert, M. (2011). The effect of riverine discharge on biogeochemical processes in estuarine sediments. *Limnology and Oceanography, 56*, 1797-1810.

Menesguen, A., Cugier, P., & Leblond, I. (2006). A new numerical technique for tracking chemical species in a multisource, coastal ecosystem applied to nitrogen causing *Ulva* blooms in the Bay of Brest (France). *Limnological Oceanography*, *51*, 591-601.

Mertens, K. N., González, C., Delusina, I., & Louwye, S. (2009). 30 000 years of productivity and salinity variations in the late Quaternary Cariaco Basin revealed by dinoflagellate cysts. *Boreas*, *38*, 647–662.

Mertens, K. N., Rengefors, K., Moestrup, Ø., & Ellegaard, M. (2012). A review of recent freshwater dinoflagellate cysts: taxonomy, phylogeny, ecology and palaeocology. *Phycologia*, *51*, 612–619.

Metcalf, J. L., Ursell, L. K., & Knight, R. (2014). Ancient human oral plaque preserves a wealth of biological data. *Nature Genetics*, *46*, 321–323.

Meyer, J., & Riebesell, U. (2015). Reviews and syntheses: Responses of coccolithophores to ocean acidification: A meta-analysis. *Biogeosciences*, *12*, 1671–1682.

Meyer, M., Kircher, M., Gansauge M. T., et al. 2012. A high-coverage genome sequence from an archaic Denisovan individual. Science 338: 222–226.

Middelburg, J. J., Klaver, G., Nieuwenhuize, J., & Vlug, T. (1995). Carbon and nitrogen cycling in intertidal sediments near Doel, Scheldt estuary. *Hydrobiologia*, *311*, 57-69.

Miettinen, A., Koç, N. & Husum, K. (2013). Appearance of the Pacific diatom *Neodenticula seminae* in the northern Nordic Seas– An indication of changes in Arctic sea ice and ocean circulation. *Marine Micropaleontology, 99*, 2–7.

Mitchell, K. J., Llamas, B., Soubrier, J., Rawlence, N. J., Worthy, T. H., Wood, J., … Cooper, A. (2014). Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution. *Science*, *344*, 898–900.

Miyazono, A., Nagai, S., Kudo, I., & Tanizawa, K. (2012). Viability of Alexandrium tamarense cysts in the sediment of Funka Bay, Hokkaido, Japan: Over a hundred year survival times for cysts. *Harmful Algae*, *16*, 81–88

Mizushima, K., & Matsuoka, K. (2004). Vertical distribution and germination ability of Alexandrium spp . cysts (Dinophyceae) in the sediments collected from Kure Bay of the Seto Inland Sea , Japan. *Phycological Research*, 408–413.

Mousing, E.A., Andersen, T.J., & Ellegaard, M. (2013) Changes in the abundance and species composition of phytoplankton in the last 150 years in the southern Black Sea. *Estuaries Coasts*, *36*, 1206–18.

Murray, S. a, Wiese, M., Stüken, A., Brett, S., Kellmann, R., Hallegraeff, G., & Neilan, B. a. (2011). sxtA-based quantitative molecular assay to identify saxitoxin-producing harmful algal blooms in marine waters. *Applied and Environmental Microbiology*, *77*, 7050–7.

N

Nanjappa, D., Audic, S., Romac, S., Kooistra, W. H. C. F., & Zingone, A. (2014). Assessment of species diversity and distribution of an ancient diatom lineage using a DNA metabarcoding approach. *PloS One*, *9*, e103810.

Nehring, S. (1998). Establishment of thermophilic phytoplankton species in the North Sea : biological indicators of climatic changes ? *ICES Journal of Marine Science, 55,* 818–823.

Nixon, S. W. (1995). Coastal marine eutrophication: a definition, social causes, and future concerns. *Ophelia, 41*, 199-219.

Ní Rathaille, A., & Raine, R. (2011). Seasonality in the excystment of Alexandrium minutum and Alexandrium tamarense in Irish Coastal waters. *Harmful Algae, 10*, 629–635.

O

Oksanen, J., Blanchet, G.F., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H, & Wagner, H. (2015). vegan: Community Ecology Package. R package version 2.3-0. http://CRAN.R-project.org/package=vegan.

Orlando, L., Ginolhac, A., Zhang, G., Froese, D., Albrechtsen, A., Stiller, M., … Willerslev, E. (2013). Recalibrating Equus evolution using the genome sequence of an early Middle Pleistocene horse. *Nature*, *499*, 74–8.

Orsi, W., Edgcomb, V., Jeon, S., Leslin, C., Bunge, J., Taylor, G., … Epstein, S. (2011). Protistan microbial observatory in the Cariaco Basin, Caribbean. II. Habitat specialization. *The ISME Journal*, *5*, 1344–56.

P

Pal, S., Gregory-Eaves, I., & Pick, F. R. (2015). Temporal trends in cyanobacteria revealed through DNA and pigment analyses of temperate lake sediment cores. *Journal of Paleolimnology*, *54*, 87–101.

Panieri, G., Lugli, S., Manzi, V., Roveri, M., Schreiber, B. C., & Palinska, K. A. (2010). Ribosomal RNA gene fragments from fossilized cyanobacteria identified in primary gypsum from the late Miocene, Italy. *Geobiology*, *8*, 101–111.

Parducci, L., Matetovici, I., Fontana, S. L., Bennett, K. D., Suyama, Y., Haile, J., … Willerslev, E. (2013). Molecular- and pollen-based vegetation analysis in lake sediments from central Scandinavia. *Molecular Ecology*, *22*, 3511–24.

Park, T.G., Park, Y.T., (2010). Detection of *Cochlodinium polykrikoides* and *Gymnodinium impudicum* (Dinophyceae) in sediment samples from Korea using real-time PCR. *Harmful Algae, 9,* 59-65.

Pasquaud, S., Béguer, M., Hjort Larsen, M., Chaalali, A., Cabral, E. & J. Lobry. (2012). Increase of marine juvenile fish abundances in the middle Gironde estuary related to warmer and more saline waters, due to global changes. *Estuarine, Coastal and Shelf Science, 104*, 46-53.

Pastor, L., Cathalot, C., Deflandre, B., Viollier, E., Soetaert, K., Meysman, F. J. R., Ulses, C., Metzger, E., & Rabouille, C. (2011). Modeling biogeochemical processes in sediments from the Rhône River prodelta area (NW Mediterranean Sea). *Biogeosciences, 8*, 1351–1366.

Pawlowski, J., Christen, R., Lecroq, B., Bachar, D., Shahbazkia, H. R., Amaral-Zettler, L., & Guillou, L. (2011). Eukaryotic richness in the abyss: Insights from pyrotag sequencing. *PLoS ONE*, *6*. doi:10.1371/journal.pone.0018169

Pawlowski, J., Esling, P., Lejzerowicz, F., Cedhagen, T. & Wilding, T. A. (2014). Environmental monitoring through protist next-generation sequencing metabarcoding: assessing the impact of fish farming on benthic foraminifera communities. *Molecular Ecology Resources, 14*, 1129–1140.

Pawlowski, J., Lejzerowicz, F., & Esling, P. (2014). Next-Generation Environmental Diversity Surveys of Foraminifera : Preparing the Future, 93–106.

Pedersen, M. W., Ginolhac, A., Orlando, L., Olsen, J., Andersen, K., Holm, J., … Kjær, K. H. (2013). A comparative study of ancient environmental DNA to pollen and macrofossils from lake sediments reveals taxonomic overlap and additional plant taxa. *Quaternary Science Reviews*, *75*, 161–168.

Pedersen, M. W., Overballe-Petersen, S., Ermini, L., Sarkissian, C. Der, Haile, J., Hellstrom, M., … Willerslev, E. (2015). Ancient and modern environmental DNA. *Philosophical Transactions of the Royal Society of Biology*, *370*.

Penna, A., Battocchi, C., Garcés, E. Anglès, S., Cucchiari, E., Totti, C., Kremp, A., Satta, C., Giacobbe, M. G., Bravo, I., & Bastianini, M. (2010). Detection of microalgal resting cysts in European coastal sediments using a PCR-based assay. *Deep Sea Research Part II, 57*, 288–300.

Penna, A., Battocchi, C., Garcés, E., Anglès, S., Cucchiari, E., Totti, C., … Bastianini, M. (2010). Detection of microalgal resting cysts in European coastal sediments using a PCR-based assay. *Deep Sea Research Part II: Topical Studies in Oceanography*, *57*, 288–300.

Perillo, G. M. E., (1995). Definitions and geomorphologic classifications of estuaries. In: Geomorphology and estuaries Developments in sedimentology. Amsterdam: Elsevier: 17-47.

Perini, F., Casabianca, A., Battocchi, C., Accoroni, S., Totti, C., & Penna, A. (2011). New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate Ostreopsis cf. ovata in marine environment. *PloS One*, *6*, e17699.

Persson, A., Godhe, A., & Karlson, B. (2000). Dinoflagellate cysts in recent sediments from the west coast of Sweden. *Botanica Marina*, 43, 69–79.

Petris, G. (2010). An R package for dynamic linear models. *Journal of Statistical Software,* 36, 1–16.

Pfandl, K., Chatzinotas, A., Dyal, P., & Boenigk, J. (2009). SSU rRNA gene variation resolves population heterogeneity and ecophysiological differentiation within a morphospecies (Stramenopiles, Chrysophyceae). *Limnology and Oceanography*, *54*, 171–181.

Pienitz, R., Smol, J. P., & Macdonaldt, G. M. (1999). Paleolimnological Reconstruction of Holocene Climatic Trends from Two Boreal Treeline Lakes , Northwest Territories, Canada. *Arctic, Antarctic, and Alpine Research*, *31*, 82–93.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M. T., Guerri, G., & Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils*, *45*, 219–235.

Pittera, J., Humily, F., Thorel, M., Grulois, D., Garczarek, L., & Six, C. (2014). Connecting thermal physiology and latitudinal niche partitioning in marine *Synechococcus*. *The ISME journal*, *8*, 1221-1236.

Poinar, H. N., Hofreiter, M., Spaulding, W. G., Martin, P. S., Stankiewicz, B. A., Bland, H., … Wilson, A. C. (1998). Molecular coproscopy: dung and diet of the extinct ground sloth Nothrotheriops shastensis. *Science (New York, N.Y.)*, *281*, 402–6.

Polovina, J. J., Howell, E. A., & Abecassis, M. (2008). Ocean's least productive waters are expanding. *Geophysical Research Letters*, *35*, 2–6.

Pospelova, V., Price, A. M., & Pedersen, T. F. (2015). Palynological evidence for late Quaternary climate and marine primary productivity changes along the California margin. *Paleoceanography*, 1–18.

Pritchard, D., (1955). Estuarine circulation patterns. *Proc. Am. Soc. Civil Eng*, 81, 1-11.

Pritchard, D., (1967). What is an estuary: Physical point of view. In: estuaries,Washington, 3-5.

Probert, I., Siano, R., Poirier, C., Decelle, J., Biard, T., Tuji, A., … Not, F. (2014). Brandtodinium gen. nov. and B. nutricula comb. Nov. (Dinophyceae), a dinoflagellate commonly found in symbiosis with polycystine radiolarians. *Journal of Phycology*, *50*, 388–399.

Psenner, R., & Schmidt, R. (1992). Climate-driven pH control of remote alpine lakes and effects of acid deposition. *Nature*, *360*, 40–46.

Pusceddu, A., Dell'Anno, A., Danovaro, R., Manini, E., Sara, G., & Fabiano, M. (2003). Enzymatically hydrolyzable protein and carbohydrate sedimentary pools as indicators of the trophic state of detritus sink systems: A case study in a Mediterranean coastal lagoon. *Estuaries, 26*, 641-650.

Q

Quéguiner, B., & Tréguer, P. (1984). Studies on the phytoplankton in the Bay of Brest (Western Europe). Seasonal variations in composition, biomass and production in relation to hydrological and chemical features (1981-1982). *Botanica Marina, 27*, 449-459.

Qui-Minet, ZN. (2014). Variabilité interspécifique et intraspécifique des dinoflagellés dans les registres sédimentaires anciens. Rapport de Master 2, Aix-Marseille Université, 37p.

R

R Development Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria : http://www.R-project.org/.

R Development Core Team. (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. https://www.R-project.org/.

R Development Core Team. (2016). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria: https://www.R-project.org/.

Rabalais, N. N., Turner, R. E., Díaz, R. J., & Justić, D. (2009). Global change and eutrophication of coastal waters. *ICES Journal of Marine Science*, *66*, 1528–1537.

Ragueneau, O., Chauvaud, L., Leynaert, A., Thouzeau, G., Paulet, Y. M., Bonnet., Lorrain A, Grall, J., Corvaisier, R., Le Hir, M., Jean, F., & Clavier, J. (2002). Direct evidence of a biologically active coastal silicate pump: Ecological implications. *Limnology and Oceanography,* 47, 1849–1854.

Raimonet, M., Andrieux-Loyer, F., Ragueneau, O., Michaud, E., Kerouel, R., Philippon, X., Nonent, M., & Memery, L. (2013). Strong gradient of benthic biogeochemical processes along a macrotidal temperate estuary: focus on P and Si cycles. *Biogeochemistry, 115*, 399-417.

Rawlence, N. J., Lowe, D. J., Wood, J. R., Young, J. M., Churchman, G. J., Huang, Y.-T., & Cooper, A. (2014). Using palaeoenvironmental DNA to reconstruct past environments: progress and prospects. *Journal of Quaternary Science*, *29*, 610–626.

Raymond, P. A. & Bauer, J. E. (2001). Riverine export of aged terrestrial organic matter to the North Atlantic Ocean, *Nature, 409*, 497– 500.

Redfield, A. (1958). The biological control of chemical factors in the environment. *American Scientist*, *46*, 205–221.

Reid, P. C., Johns, D. G., Edwards, M., Starr, M., Poulin, M. & Snoeijs, P. (2007). A biological consequence of reducing Arctic ice cover : arrival of the Pacific diatom *Neodenticula seminae* in the North Atlantic for the first time in 800 000 years. *Global Change Biology, 13*, 1910–1921.

Reinhard, K. J., Chaves, S. M., Jones, J. G., & Iñiguez, A. M. (2008). Evaluating chloroplast DNA in prehistoric Texas coprolites: medicinal, dietary, or ambient ancient DNA? *Journal of Archaeological Science*, *35*, 1748–1755.

Reuss, N., & Conley, D.J. (2005). Effects of sediment storage conditions on pigment analyses. *Limnology and Oceanography: Methods, 3*, 477-487.

Ribeiro, S., Amorim, A., Andersen, T. J., Abrantes, F., & Ellegaard, M. (2011). Reconstructing the history of an invasion: the toxic phytoplankton species Gymnodinium catenatum in the Northeast Atlantic. *Biological Invasions*, *14*, 969–985.

Ribeiro, S., Berge, T., Lundholm, N., & Ellegaard, M. (2013). Hundred Years of Environmental Change and Phytoplankton Ecophysiological Variability Archived in Coastal Sediments. *PLoS ONE*, *8*, e61184. doi:10.1371/journal.pone.0061184

Ribeiro, S., Berge, T., Lundholm, N., Andersen, T. J., Abrantes, F., & Ellegaard, M. (2011). Phytoplankton growth after a century of dormancy illuminates past resilience to catastrophic darkness. *Nature Communications*, *2*, 311.

Rimet, F., & Bouchez, A. (2012). Life-forms, cell-sizes and ecological guilds of diatoms in European rivers. *Knowledge and Management of Aquatic Ecosystems*, *406,* 1-12.

Rochard, E., G. Castelnaud, & M. Lepage. (1990). Sturgeons (Pisces, Acipenseridae) – Threats and Prospects. *Journal Of Fish Biology, 37*, 123-132.

Rozen, S., & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, USA: 365-86.

Rull, V. (2010). Ecology and Palaeoecology : Two Approaches , One Objective. *The Open Ecology Journal*, *3*, 1–5.

S

Saari, H.K., Schmidt, S., Castaing, P., Blanc, G., Sautour, B., Masson, O., & Cochran, J.K. (2010). The particulate $^{7}Be/^{210}Pb_{xs}$ and $^{234}Th/^{210}Pb_{xs}$ activity ratios as tracers for tidal-to-seasonal particle dynamics in the Gironde estuary (France): Implications for the budget of particle-associated contaminants. *Science of The Total Environment, 408*, 4784–94.

Sarmiento, J. L., Gruber, N., Brzezinski, M. A., & Dunne, J. P., (2004). High-latitude controls of thermocline nutrients and low latitude biological productivity. *Nature, 427*, 56–60.

Satta, C.T., Anglès, S., Lugliè, A., Guillén, J., Sechi, N., Camp, J., & Garcés, E. (2013). Studies on dinoflagellate cyst assemblages in two estuarine Mediterranean bays: a useful tool for the discovery and mapping of harmful algal species. *Harmful Algae, 24*, 65-79.

Schlegel, M., & Hu, N. (2007). Protists – A textbook example for a paraphyletic taxon. *Organisms Diversity & Evolution*, *7*, 166–172.

Schmidt S, Jouanneau J-M, Weber O, Lecroart, P, Radakovitch, O, Gilbert, P, & Jézéquel, D. (2007). Sedimentary processes in the Thau Lagoon (South France): from seasonal to century time scales. *Estuarine Coastal Shelf Science,* 72, 534–42.

Schmidt, S., & De Deckker, P. (2015). Present-day sedimentation rates on the southern and southeastern Australian continental margins. *Australian Journal of Earth Sciences, 62*, 143–50.

Seddon, A. W. R. Paleoecology. (2012). Oxford Bibliographies Online. DOI: 10.1093/OBO/9780199830060-0058

Seity, Y., Brousseau, P., Malardel, S., Hello, G., Bénard, P., Bouttier, F., Lac, C., & Masson, V. (2011). The AROME-France convective scale operational model. *Monthly Weather Review, 139,* 976–991.

Seitzinger, S. P. (1988). Denitrification in freshwater and coastal marine ecosystem: ecological and geochemical significance. *Limnology and Oceanography, 33*, 702-724.

Sekida, S., Horiguchi, T., & Okuda, K. (2001). Development of the cell covering in the dinoflagellate *Scrippsiella hexapraecingula* (Peridiniales, Dinophyceae). *Phycological. Research, 49*, 163–176.

Seppä, P., Helanterä, H., & Chernenko, A. (2009). Population genetics of the black ant Formica lemani (Hymenoptera : Formicidae). *Biological Journal of the Linnean Society*, *97*, 247–258.

Serrano, O., Lavery, P., Masque, P., & Inostroza, K. (2016). Seagrass sediments reveal the long-term deterioration of an estuarine ecosystem, 1523–1531.

Shapiro, B. (2004). Rise and Fall of the Beringian Steppe Bison. *Science*, *306*, 1561–1565.

Shuler, A. J., Paternoster, J., Brim, M., Nowocin, K., Tisdale, T., Neller, K., … & Morton, S. (2012). Spatial and temporal trends of the toxic diatom Pseudo-nitzschia in the Southeastern Atlantic United States. *Harmful Algae*, *17*, 6–13.

Simon, M., López-García, P., Deschamps, P., Moreira, D., Restoux, G., Bertolino, P., & Jardillier, L. (2015). Marked seasonality and high spatial variability of protist communities in shallow freshwater systems. *The ISME Journal*, *9*, 1–13.

Slapeta, J., Moreira, D., & Lopez-Garcia, P. (2005). The extent of protist diversity: insights from molecular ecology of freshwater eukaryotes. *Proceedings of the Royal Society of London*, Series B, *272*, 2073–2081.

Smayda, T. J. (1997). Harmful algal blooms : Their ecophysiology and general relevance to phytoplankton blooms in the sea. *Limnology and Oceanography, 42*, 1137-1153.

Sourisseau, M., Le Guennec, V., Le Gland, G., Plus, M., & Chapelle, A. (2016). Understanding phytoplankton's ecological niche and phenology using traits-based models applied to toxic algae. *Frontiers*, accepted.

Squier, A. H., Hodgson, D. A., & Keely, B. J. (2002). Sedimentary pigments as markers for environmental change in an Antarctic lake. *Organic Geochemistry*, *33*, 1655–1665.

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M. D. M., Breiner, H.-W., & Richards, T. a. (2010). Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. *Molecular Ecology*, *19 Suppl 1*, 21–31.

Stoecker, D. K. (1999). Mixotrophy among Dinoflagellates. *The Journal of Eukaryotic Microbiology*, *46*, 397–401.

Stoof-Leichsenring, K. R., Epp, L. S., Trauth, M. H., & Tiedemann, R. (2012). Hidden diversity in diatoms of Kenyan Lake Naivasha: a genetic approach detects temporal variation. *Molecular Ecology*, *21*, 1918–30.

T

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*, 1789–1793.

Takishita, K., Yubuki, N., Kakizoe, N., Inagaki, Y., & Maruyama, T. (2007). Diversity of microbial eukaryotes in sediment at a deep-sea methane cold seep: Surveys of ribosomal DNA libraries from raw sediment samples and two enrichment cultures. *Extremophiles*, *11*, 563–576.

Tallberg, P., Tréguer, P. Beucher, C., & Corvaisier, R. (2008). Potentially mobile pools of phosphorus and silicon in sediment from the Bay of Brest: Interactions and implications for phosphorus dynamics. *Estuarine, Coastal and Shelf Science, 76,* 85-94.

Taylor, F.J.R., 1987. General group characteristics, special features of interest; and a short history of dinoflagellate study. In: The Biology of Dinoflagellates: Oxford, Blackwell Scientific Publications:1-23.

Taylor, W. A. (2000). Change-Point Analysis: A powerful new tool for detecting changes. http://www.variation.com/cpa/tech/changepoint.html.

Tchioffo, M. T., Boissière, A., Abate, L., Nsango, S. E., Bayibéki, A. N., Awono-Ambéné, P. H., … Morlais, I. (2016). Dynamics of bacterial community composition in the Malaria mosquito's epithelia. *Frontiers in Microbiology*, *6*, 1–9.

Theroux, S., D'Andrea, W.J., Toney, J., Amaral-Zettler, L., & Yongsong, H. (2010).Phylogenetic diversity and evolutionary relatedness of alkenone-producing haptophyte algae in lakes, implications for continental paleotemperature reconstructions. *Earth and Planetary Science Letters, 300*, 311–20.

Tilman, D., Kilham, S. S., & Kilham, P. (1982). Phytoplankton community ecology: the role of limiting nutrients. *Annual Review of Ecology and Systematics*, 13, 349-372.

Torti, A., Lever, M. A., & Jørgensen, B. B. (2015). Origin, dynamics, and implications of extracellular DNA pools in marine sediments. *Marine Genomics*, *24*, 185–196.

Touzet, N., Keady, E., Raine, R., & Maher, M. (2009). Evaluation of taxa-specific real-time PCR, whole-cell FISH and morphotaxonomy analyses for the detection and quantification of the toxic microalgae Alexandrium minutum (Dinophyceae), Global Clade ribotype. *FEMS Microbiology Ecology*, *67*, 329–41.

Touzet, N., Lacaze, J., Maher, M., Turrell, E., & Raine, R. (2011). Summer dynamics of Alexandrium ostenfeldii (Dinophyceae) and spirolide toxins in Cork Harbour, Ireland. *Marine Ecology Progress Series*, *425*, 21–33.

Trikia, H.Z., Daly-Yahiaa, O.K., Maloucheb, D., Komihaa, Y., Deidunc, A., Brahimd, M., & Laabir, M. (2014). Distribution of resting cysts of the potentially toxic dinoflagellate *Alexandrium pseudogonyaulax* in recently-deposited sediment within Bizerte Lagoon (Mediterranean coast, Tunisia). *Marine Pollution Bulletin,* 84, 172-181.

Trommer, G., Leynaert, A., Klein, C., Naegelen, A., & Beker, B. (2013). Phytoplankton phosphorus limitation in a north atlantic coastal ecosystem not predicted by nutrient load. *Journal of Plankton Research, 35*, 1207–1219.

Tyler, M., Coats, D., & Anderson, D. (1982). Encystment in a Dynamic Environment: Deposition of Dinoflagellate Cysts by a Frontal Convergence . *Marine Ecology Progress Series*, *7*, 163–178.

U

Usup, G., Pin, L.C., & Ahmad, A. (2002). *Alexandrium* (Dinophyceae) species in Malaysian waters. *Harmful Algae, 1*, 265–75.

V

Valle-Levinson, A. (2010) Contemporary Issues in Estuarine Physics. Cambridge University Press,, Cambridge.

van Geel, B., Aptroot, A., Baittinger, C., Birks, H. H., Bull, I. D., Cross, H. B., … van Tienderen, P. H. (2008). The ecological implications of a Yakutian mammoth's last meal. *Quaternary Research*, *69*, 361–376.

Vegas-Vilarrubia, T., Rull, V., Montoya, E., & Safont, E. (2011). Quaternary palaeoecology and nature conservation: A general review with examples from the neotropics. *Quaternary Science Reviews*, *30*, 2361–2388.

Verleye, T.G., Mertens, K., Louwye, S., & Arz, H. W. (2009). Holocene salinity changes in the southwestern Black Sea: a reconstruction based on dinoflagellate cysts. *Palynology*, **33**, 77–100.

Verschuren, D., Laird, K. R., & Cumming, B. F. (2000). Rainfall and drought in equatorial east Africa during the past 1,100 years. *Nature*, *403*, 410–414.

Villacorte, L. O., Alizadeh, S. A., Anderson, D. M., Amy, G. L., Schippers, J. C., & Kennedy, M. D. (2015). Seawater reverse osmosis desalination and (harmful) algal blooms. *DES*, *360*, 61–80.

Vilmi, A., Karjalainen, S. M., Landeiro, V. L., & Heino, J. (2015). Freshwater diatoms as environmental indicators: evaluating the effects of eutrophication using species morphology and biological indices. *Environmental Monitoring and Assessment*, *187*.

Vyverman, W., & Sabbe, K. (1995). Diatom-temperature transfer functions based on the altitudinal zonation of diatom assemblages in Papua New Guinea: a possible tool in the reconstruction of regional palaeoclimatic changes. *Journal of Paleolimnology*, *13*, 65–77.

W

Wang, Z., Qi, Y., Lu, S., Wang, Y., & Matsuoka, K. (2004). Seasonal distribution of dinoflagellate resting cyst in surface sediment from Changjang River Estuary. *Phycological Research, 52,* 387–395.

Watson, E. B., Wasson, K., Pasternack, G. B., Woolfolk, A., Dyke, V., Gray, A. B., … Wheatcroft, R. A. (2011). Applications from Paleoecology to Environmental Management and Restoration in a Dynamic Coastal Environment, *19*, 765–775.

Weckström, J., Korhola, A., Erästö, P., & Holmström, L. (2006). Temperature patterns over the past eight centuries in Northern Fennoscandia inferred from sedimentary diatoms. *Quaternary Research*, *66*, 78–86.

West M, Harrison J (1997). Bayesian Forecasting and Dynamic Models. Springer Series in Statistics. New York: 680.

White, A.W., & Lewis, C.M. (1982). Resting cysts of the toxic red tide dinoflagellate *Gonyaulax excavata* in Bay of Fundy sediments. Canadian *Journal of Fisheries and Aquatic Sciences, 39*, 1185–1194.

Willerslev, E., & Cooper, A. (2005). Ancient DNA. *Proceedings of the Royal Society, Series B*, *272*, 3–16.

Willerslev, E., Cappellini, E., Boomsma, W., Nielsen, R., Hebsgaard, M. B., Brand, T. B., … Collins, M. J. (2007). Ancient biomolecules from deep ice cores reveal a forested southern Greenland. *Science (New York, N.Y.)*, *317*, 111–4.

Willerslev, E., Davison, J., Moora, M., Zobel, M., Coissac, E., Edwards, M. E., … Taberlet, P. (2014). Fifty thousand years of Arctic vegetation and megafaunal diet. *Nature*, *506*, 47–51.

Willerslev, E., Hansen, A. J., & Poinar, H. N. (2004). Isolation of nucleic acids and cultures from fossil ice and permafrost. *Trends in Ecology and Evolution*, *19*, 141–147.

Wyatt, T., & Jenkinson, I. R. (1997). Notes on Alexandrium population dynamics. *Journal of Plankton Research*, *19*, 551–575.

Y

Yamaguchi, M., Itakura, S., Nagasaki, K., Imai, I., Yasumoto, T., Oshima, Y., & Fukuyo, Y. (Eds). (1996). Distribution and Abundance of Resting Cysts of the Toxic Dinoflagellates *Alexandrium tamarense* and *A. catenella* in sediments of the eastern Seto Inland Sea, Japan. Intergovernmental Oceanographic Commission of UNESCO, Laboratory of Bioorganic Chemistry, Tohoku University, Japan: 177–180.

Yoon, T., Kang, H., Kang, C., Lee, S. H., Ahn, D., Park, H., & Kim, H. (2016). Development of a cost-effective metabarcoding strategy for analysis of the marine phytoplankton community. *PeerJ* 4:e2115: DOI 10.7717/peerj.2115

Yu, S.-Y., & Berglund, B. E. (2007). A dinoflagellate cyst record of Holocene climate and hydrological changes along the southeastern Swedish Baltic coast. *Quaternary Research*, *67*, 215–224.

Yue, S., Pilon, P., & Cavadias, G. (2002). Power of the Mann-Kendall and Spearman's rho tests for detecting monotonic trends in hydrological series *Journal of Hydrology,259,* 254–71.

Z

Zhai, L., Platt, T., Tang, C., Sathyendranath, S. & Walne, A. (2013). The response of phytoplankton to climate variability associated with the North Atlantic Oscillation. *Deep Sea Research Part II: Topical Studies in Oceanography*, *93*, 159–168.

Zimmermann, J., Gernot, G., Jahn, R., Enke, N., & Gemeinholzer, B. (2015). Metabarcoding vs. morphological identification to assess diatom diversity in environmental studies. Molecular Ecology Ressources, 15, 526–542.

Zimmermann, J., Jahn, R., & Gemeinholzer, B. (2011). Barcoding diatoms: evaluation of the V4 subregion on the 18S rRNA gene, including new primers and protocols. *Organisms Diversity & Evolution*, *11*, 173–192.

Zingone, A., & Enevoldsen, H. O. (2000). The diversity of Harmful algal blooms: a challenge fo science and management. *Ocean & Coastal Management*, 725–748.

Zonneveld, K. A. F., Chen, L., Elshanawany, R., Fischer, H. W., Hoins, M., Ibrahim, M. I., … Versteegh, G. J. M. (2012). The use of dinoflagellate cysts to separate human-induced from natural variability in the trophic state of the Po River discharge plume over the last two centuries. *Marine Pollution Bulletin*, *64*, 114–32.

ANNEXES

ANNEXE 1.

Sediment core sampling in the Bay of Morlaix and the Bay of Vilaine.

This PhD thesis work was originally intended to address the question of *Alexandrium minutum* species invasion and protist community changes at the mesoscale (Brittany regional scale), by analyzing sediment cores collected at different estuaries; the Bay of Brest (Daoulas and Elorn Estuaries), the Bay of Morlaix (Penzé Estuary), and the Bay of Vilaine (Vilaine Estuary). However, different issues identified from sediment analyses of the Bay of Morlaix and Vilaine cores, constrained me to focus my work mainly on the ecosystems of the Bay of Brest (Daoulas and Elorn Estuaries. The reasons of this choice are explained in this Annex.

Sediment core sampling was conducted in October 2013 and April 2014, respectively in the Bay of Morlaix and the Bay of Vilaine. Six sediment cores in each of the sampling sites were collected by scuba divers, using a manual corer. The sediment cores were dedicated to different analyses; two were dedicated for cyst germination, two for genetic analyses and dating and two for chemical analyses (Total Organic Carbone, porosity, granulometry). These last data were not exploited in this thesis. The samples' manipulations and analyses followed the same procedure as described in the Material and methods section of Chapter 1, and in Klouch *et al*. (2016).

- **Bay of Morlaix (Penzé Estuary)**

The sediment core dating was performed by the laboratory EPOC, (Bordeaux, France) and was based on the analysis of different radionoculides' profiles. The sediment analyses revealed a high sedimentation rate in the area, consequently the chronology of the sediment core (34 cm-long) dated only to 1996 ± 5.3, a temporal scale which was not sufficiently long to conduct our ecological analyses. Moreover, these analyses revealed a considerable mixing of the sediment core layers for most of the first layers of the core, preventing our paleocological reconstruction analyses. Although genetic analyses (DNA extraction, real-time PCR amplification, High Throughput Sequencing) has been performed on the core sediment before the acquisition of the dating results, only strains revived by germination experiments from sediment of deep layers (until 17 cm), have been exploited for ecophysiological analyses in the Chapter 4.

Figure A1a. Sampling of the sediment cores in the Bay of Morlaix.

- **Bay of Vilaine (Vilaine Estuary)**

In the Bay of Vilaine, sediment analyses revealed some irregularities in the sediment deposition. The radionuclides (^{234}Th, ^{7}Be) used as indicators of recent deposits have been found in the top 20 cm of the sediment core (40 cm-long) which indicated that the sediment layers were highly mixed. Below this level, an interruption of sediment deposition seems to have occurred. These results indicate that this core could have recorded occasional sedimentary events rather than a regular archive (S. Schimdt pers. comm.). Therefore, it was not suitable for paleoecological reconstructions. The genetic results obtained for this sediment core before the acquisition of sediment dating results, have not been exploited in this PhD thesis.

Figure A1b. Sampling of the sediment cores in the Bay of Vilaine.

ANNEXE 2.

Metabarcoding of the V9 region of 18S RNA gene in Daoulas and Elorn estuaries.

In order to detect short and degraded DNA fragments in the sediments cores, and to complete my metabarcoding approach on a longer fragment (V4 18S rDNA, chapter 1), I used the short-sized barcode V9 region (130 bp) of the 18S rRNA gene on sediments of the DE and EE. From the DNA sediment extracts, the V9 region has been amplified and sequenced using the protocols described in (de Vargas *et al*., 2015; Le Bescot *et al*., 2015). The results showed that over 15% of the total OTUs belonged to prokaryotes (Archaea and Bacteria) (Figure 1). The primers were previously reported for amplifying prokaryotes (de Vargas et al., 2015). Moreover, a large proportion of the OTUs abundance identified after bioinformatics analyses (R. Christen pers. comm.) (72 %) were undetermined (Figure 1). This low taxonomic resolution of the V9 metabarcoding in sediments was attributed to the lack of many reference sequences in the version of the data base (PR2) used for the annotation of the OTUs. Since my approach on the diversity of the protist community needed the taxonomic identifications of the OTUs found in the sediments, I could not use the V9 dataset data for this work.

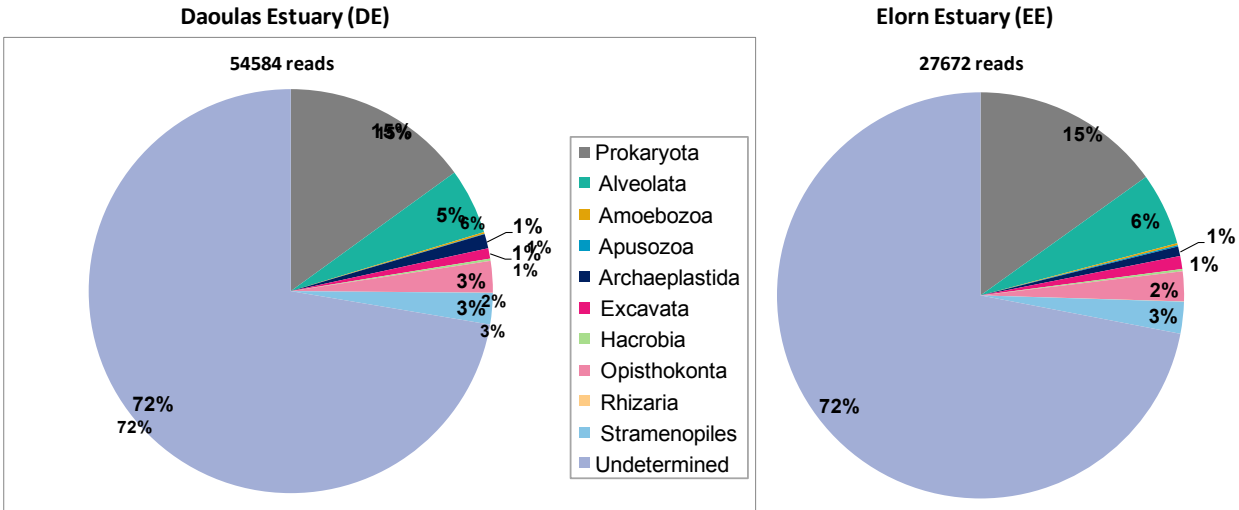


Figure A2. Abundance of taxa (% of OTUs reads) based on the V9 18S rDNA region for the DE (left) and EE (right). All the groups were represented in the pie charts but only those with abundances≥1% are represented.

ANNEXE 3.

Article. Historical records from dated sediment cores reveal the multidecadal dynamic of the toxic dinoflagellate *Alexandrium minutum* in the Bay of Brest (France).

FEMS Microbiology Ecology, 92, 2016, fiw101

doi: 10.1093/femsec/fiw101
Advance Access Publication Date: 8 May 2016
Research Article

RESEARCH ARTICLE

Historical records from dated sediment cores reveal the multidecadal dynamic of the toxic dinoflagellate *Alexandrium minutum* in the Bay of Brest (France)

Khadidja Z. Klouch[1,2], Sabine Schmidt[3], Françoise Andrieux-Loyer[1], Mickaël Le Gac[1], Dominique Hervio-Heath[4], Zujaila N. Qui-Minet[1], Julien Quéré[1], Estelle Bigeard[2], Laure Guillou[2] and Raffaele Siano[1,*]

[1]Ifremer—Centre de Brest, DYNECO PELAGOS, BP 70 ZI Pointe du Diable, F-29280 Plouzané, France, [2]Sorbonne Universités, UPMC Univ. Paris 6, CNRS, Adaptation et Diversité en Milieu Marin (UMR 7144), équipe DIPO, Station Biologique de Roscoff, Place Georges Teissier, CS90074, 29688 Roscoff cedex, France, [3]UMR5805 EPOC, University of Bordeaux, 33605 Pessac, France and [4]Ifremer, RBE, SG2M, Laboratoire Santé, Environnement et Microbiologie, 29280 Plouzané, France

*Corresponding author: Ifremer—Centre de Brest, DYNECO PELAGOS, BP 70 ZI Pointe du Diable, F-29280 Plouzané, France. Tel: +33-02-98-22-42-04; Fax: +33-02-98-22-45-48; E-mail: raffaele.siano@ifremer.fr

One sentence summary: By means of a paleoecological approach (ancient DNA quantification and cyst revivification), a species-specific increasing trend in the concentration of the toxic marine dinoflagellate *Alexandrium minutum* was revealed over a time scale of about 150 years in the Bay of Brest.

Editor: Riks Laanbroek

ABSTRACT

The multiannual dynamic of the cyst-forming and toxic marine dinoflagellate *Alexandrium minutum* was studied over a time scale of about 150 years by a paleoecological approach based on ancient DNA (aDNA) quantification and cyst revivification data obtained from two dated sediment cores of the Bay of Brest (Brittany, France). The first genetic traces of the species presence in the study area dated back to 1873 ± 6. Specific aDNA could be quantified by a newly developed real-time PCR assay in the upper core layers, in which the germination of the species (in up to 17–19-year-old sediments) was also obtained. In both cores studied, our quantitative paleogenetic data showed a statistically significant increasing trend in the abundance of *A. minutum* ITS1 rDNA copies over time, corroborating three decades of local plankton data that have documented an increasing trend in the species cell abundance. By comparison, paleogenetic data of the dinoflagellate *Scrippsiella donghaienis* did not show a coherent trend between the cores studied, supporting the hypothesis of the existence of a species-specific dynamic of *A. minutum* in the study area. This work contributes to the development of paleoecological research, further showing its potential for biogeographical, ecological and evolutionary studies on marine microbes.

Keywords: paleoecology; ancient DNA; real-time PCR; harmful algal blooms (HAB); dinoflagellates; coastal ecology

Received: 18 February 2016; **Accepted:** 4 May 2016

INTRODUCTION

Marine sediments are important resources for paleobiologists as they represent a valuable archive of past environmental communities. The resting stages of plankton, diatom frustules and spores, dinoflagellate cysts, foraminifera shells, and micro- and meso-zooplankton (rotifers, copepods, cladocerans, ciliates), as well as molecules such as sterols and pigments, can be accumulated and preserved in sediments. Their examination combined with a chronological analysis can provide ecological and environmental information about past communities (Ellegaard *et al.* 2013; Liu *et al.* 2013). Benthic resting stages are derived from the rapid response of planktonic stages to unfavorable environmental conditions and are part of the life cycle of species. They are known to be powerful tracers of habitat changes because of their good preservation in sediment (Ellegaard, Figueroa and Versteegh 2013). In particular, phytoplankton resting stages have been widely used to reconstruct past environmental changes in temperature (de Vernal *et al.* 2005; Weckström *et al.* 2006; Durantou *et al.* 2012), salinity (Laird *et al.* 1996; Verleye *et al.* 2009; Mertens *et al.* 2012), productivity (Mertens *et al.* 2009; Pospelova, Price and Pedersen 2015) and eutrophication (Ellegaard *et al.* 2006; Zonneveld *et al.* 2012). Cultures derived from revived phytoplankton resting stages have been used to investigate the impact of environmental changes on the physiological performance of species (e.g. the dinoflagellate *Pentapharsodinium dalei*) in Koljö Fjord, Sweden (Ribeiro *et al.* 2013), or the genetic structure and diversity of populations of diatoms (*Skeletonema marinoi*) revived from sediments of Mariager Fjord, Denmark (Härnström *et al.* 2011).

The analysis of ancient DNA (aDNA) recovered from marine sediments is a complementary approach to the study of other biological remains in sediments that shows great potential. Several recent studies have demonstrated that plankton aDNA can be recovered from lacustrine and marine sediments, from the Holocene to the Pleistocene (Coolen and Overmann 2007; Boere *et al.* 2011) and up to the Miocene (Panieri *et al.* 2010). Most of the DNA stored in marine sediments is believed to be extracellular, as it is protected from nuclease degradation by adsorbing to mineral and organic matrices (Pietramellara *et al.* 2009), but DNA may also originate from resting stages (Godhe *et al.* 2002; Erdner *et al.* 2010). Whatever its origin, this aDNA could be exploitable to study plankton ecological patterns over relatively long-term periods.

The long-term dynamics of marine protist species are usually deduced from multiannual time series, generally obtained from regular spatial-temporal coastal surveys carried out within the framework of monitoring network activities (e.g. Shuler *et al.* 2012; Hernández-Fariñas *et al.* 2014). Long-term series generally focus on potentially toxic species, such as *Alexandrium* spp. (e.g. Touzet *et al.* 2011; Abdenadher *et al.* 2012; Anderson *et al.* 2014; Martin, LeGresley and Hanke 2014; Bazzoni *et al.* 2015). However, most of these historical datasets are relatively short, covering only a few decades (<50 years), and are compromised by the limitations of identifying plankton species in routine optical microscopic analyses. Early information on past communities and species before the implementation of monitoring networks is therefore lacking. Paleoecological data could be an alternative to fill the gaps in the historical information about long-term protist community trends. Previous paleoecological analyses have shown multidecadal shifts and trends for diatom (Lundholm *et al.* 2010) and dinoflagellate species (Ribeiro, Amorim and Andersen 2011), as well as for plankton communities (Mousing, Andersen and Ellegaard 2013) using fossilized remains. aDNA has also been used to describe plankton succession dynamics (e.g. Bissett *et al.* 2005; Epp *et al.* 2010; Theroux *et al.* 2010; Stoof-Leichsenring *et al.* 2012 and Lejzerowicz *et al.* 2013 in old sediment (up to 32 000 years)) and to investigate the response of paleo-planktonic communities to past environmental changes (Coolen *et al.* 2013; Hou *et al.* 2014).

Alexandrium minutum is a cyst-forming dinoflagellate (Alveolata), responsible for several harmful algal blooms (HABs) associated with toxin production causing paralytic shellfish poisoning (PSP). Since its first detection in Alexandria harbor, Egypt (Halim 1960), this species has been detected in several areas: southern Australia (Hallegraeff, Steffensen and Wetherbee 1988), Ireland (Gross 1989), northern France (Belin 1993), the Mediterranean (Honsell 1993), Spain (Franco, Fernandez and Reguera 1994), the North Sea (Nehring 1998; Elbrachter 1999; Hansen, Daugbjerg and Franco 2003), Taiwan (Hwang and Lu 2000), New Zealand (Chang *et al.* 1999), Sweden (Persson, Godhe and Karlson 2000), India (Godhe *et al.* 2001) and Malaysia (Usup, Pin and Ahmad 2002). *Alexandrium minutum* is officially listed as an invasive species in Europe (http://www.europe-aliens.org) and is considered cryptogenic as its geographical origin is unknown (Gómez 2008; Katsanevakis *et al.* 2014). Phylogeographic analyses by Lilly, Halanych and Anderson (2005) and McCauley *et al.* (2009) have shown that *A. minutum* populations can be clearly differentiated into two major clades: the global clade (containing samples from Europe and Australia) and the Pacific clade (containing samples from New Zealand and Taiwan). Yet, it remains quite challenging to determine whether recent *A. minutum* populations were introduced through natural or human-assisted pathways. In the Bay of Brest (Brittany, France), *A. minutum* has been detected since 1990 by the monitoring network REPHY (Reseau de surveillance et d'observation du Phytoplankton et des Phycotoxines) (http://envlit.ifremer.fr/surveillance/phytoplancton_phycotoxines/presentation) but its abundance was rather low (<2000 cells L^{-1}) until recent years when it increased significantly in the area. In July 2012, it reached a record concentration of around 42 million cells L^{-1}. This exceptional bloom induced a cascading effect: toxin accumulation in shellfish, closure of aquaculture activities and economic losses, leading to the Bay of Brest being classified as a new high-risk zone for toxic blooms of *A. minutum* (Chapelle *et al.* 2015).

The objective of this work was to contribute to studies on marine microbial paleoecology and long-term dynamics of HAB dinoflagellate species by collecting historical information on *A. minutum* in the Bay of Brest. More specifically, cyst germination experiments were used to detect the viability of the species in ancient sediments and a newly developed real-time polymerase chain reaction (PCR) assay on aDNA was employed to try to reconstruct the multidecadal dynamic of the species in the area. In order to evaluate the specificity of the *A. minutum* ecological pattern, paleogenetic data obtained for *Scrippsiella donghaienis* were analyzed, as an example of a different, non-toxic, cyst-forming dinoflagellate species of this area. In parallel, plankton-monitoring data collected since 1990 in the Bay of Brest were analyzed in order to verify whether the dynamic of *A. minutum* in the water column was consistent with the results obtained by paleogenetic data during the overlapping period of the two data series.

MATERIALS AND METHODS

Study sites and phytoplankton monitoring

The Elorn and Daoulas estuaries are located in the Bay of Brest (Brittany, North Atlantic, north-west coast of France). They are

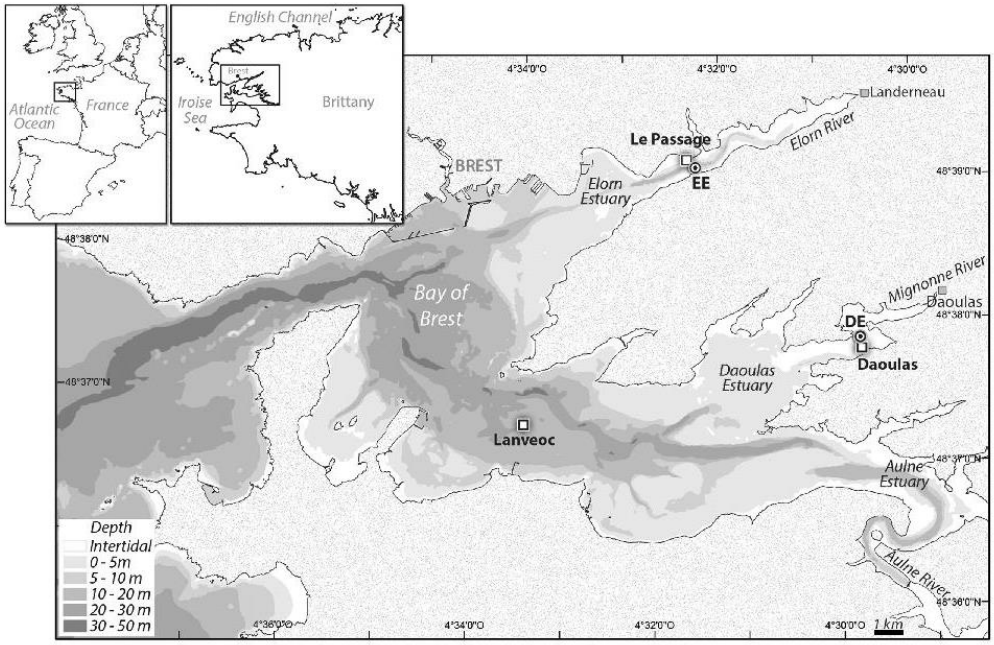


Figure 1. Map of the Bay of Brest with the core sampling sites (Elorn estuary: EE; Daoulas estuary: DE), and the monitored plankton stations (Le Passage, Daoulas, Lanveoc).

both situated near areas of intensive agriculture, domestic waste and industrial discharges, which all contribute to the substantial nutrient supply of these estuaries. Since 1950, coastal ecosystems of the bay have experienced a large increase in their nutrient supply caused by the development of anthropogenic activities. More recently, the ban on washing powders containing orthophosphates has resulted in a decrease in the phosphorous supply leading to a significant imbalance in the N/P ratio (Guillaud and Bouriel 2007). This environmental change has been associated with variations in phytoplankton communities over time, such as an increase in phytoplankton biomass and in the intensity and frequency of HABs (Guillaud and Bouriel 2007). The REPHY monitoring network has been recording occurrences of phytoplankton species in French coastal ecosystems since 1984. In the Bay of Brest, monitoring is carried out at three sampling stations: Le Passage in the Elorn River estuary, from 1990 to 2004 and then from 2012 to date; Lanveoc, from 1993 to date and Daoulas, which became the third spot point in the area after the impressive toxic bloom event of *A. minutum* in July 2012 (42×10^6 cells L^{-1}) (Fig. 1). This last station is monitored during the summer period only (May–August), which corresponds to the highest productivity period when toxic blooms are most likely to occur. Fortnightly, analyses of lugol-fixed samples are carried out using light microscopy. The estimations of *A. minutum* abundance are performed at a specific level, sometimes only during the occurrence of species blooms or high-risk periods. The abundance of non-toxic species is not monitored regularly at all stations and sometimes not at the species level. This is the case for the taxon *Scrippsiella* spp., which, in the case of REPHY monitoring, can include species of other dinoflagellate genera (*Ensiculifera*, *Pentapharsodinium*, *Bysmatrum*) that cannot be distinguished in routine light microscopy analyses by phytoplankton observers. In this study, only samples with non-zero values corresponding to abundances ≥ 100 cells L^{-1}, which is the threshold abundance limit of species detection in the REPHY phytoplankton analysis procedures, were considered and analyzed.

Core sampling and processing

Sediment cores were collected on 11 December 2012 at station EE of the Elorn River estuary (48°23′46.79′N 4°23′2.01′W), and on 21 May 2014 at station DE (48°20′46.6′N 4°17′41.20′W) of the Daoulas estuary (Fig. 1). The sampling sites were selected on the basis of their suitable environmental characteristics for paleoecological analysis (calm hydrodynamics favorable for regular sedimentation (Rawlence *et al.* 2014)) and their proximity to the REPHY stations. Three cores were collected at station EE for genetic (31 cm) germination (25 cm) and dating (29 cm) analyses, respectively. Two cores were collected at DE, one (58 cm) for genetic and dating analyses and the other (59 cm) for cyst germination experiments. Visual inspection revealed no differences in the structure of the cores from the same sampling site. The EE cores were collected by divers at 12 m depth using hand-driven transparent Plexiglas tubes, 9.5 cm in diameter and 60 cm in length. The DE cores were collected at 3 m depth with a corer released from the boat. Immediately after sampling, the sediment cores were delicately extruded from the Plexiglas tubes and then sliced into 1-cm layers. Sterile new equipment was used to slice each layer sample. For DNA and cyst germination analyses, to avoid contamination by smearing between the layers during the core extraction, only the inner part of each slice was sampled, using sterile 6-cm-diameter Petri dishes. The sediment samples for genetic analyses (about 10 g) were preserved in plastic 50-mL cryotubes. The tubes were frozen in liquid

nitrogen and then stored in a −80°C freezer until further analyses. The sediment samples for cyst germination were collected in 50-mL tubes and stored in the dark at 4°C. Finally, sediment aliquots for dating were stored in plastic bags until radionuclide analyses.

^{210}Pb and ^{137}Cs dating

The chronological framework of the sediment cores was determined based on $^{210}Pb_{xs}$ and ^{137}Cs as previously performed in Schmidt *et al.* (2007) and Andresen *et al.* (2014). ^{210}Pb ($T_{1/2} = 22.3$ years) is a naturally occurring radionuclide delivered continuously to the landscape by atmospheric fallouts (Saari *et al.* 2010). This atmospherically derived ^{210}Pb, readily scavenged by sediment, is referred to as ^{210}Pb in excess ($^{210}Pb_{xs}$) of that found within particles due to the decay of its parent isotope, ^{226}Ra. On the contrary, ^{137}Cs ($T_{1/2} = 30$ years) is an artificial radionuclide: its occurrence in the environment is primarily the result of the nuclear weapon test fallout in the early 1960s and the Chernobyl accident in Europe in 1986 of well-known pulse inputs (Avşar *et al.* 2015). In the laboratory, water content was determined by weighing the sediment samples before and after freeze drying, and dry bulk density was subsequently calculated, assuming a mineral density of 2.65 g cm^{-3}. Following this procedure, ^{210}Pb, ^{226}Ra and ^{137}Cs activities were measured using a very low background, high-efficiency, well-shaped γ detector equipped with a Cryo-Cycle (CANBERRA, Meriden, USA) (Schmidt and de Deckker 2015). The γ detector was calibrated using certified reference materials from IAEA (RGU-1; RGTh; SOIL-6). Activities were expressed in mBq g^{-1} and errors were based on 1 SD counting statistics. Excess ^{210}Pb ($^{210}Pb_{xs}$) activities were calculated by subtracting the activity supported by its parent isotope, ^{226}Ra, from the total ^{210}Pb activity in the sediment.

Cyst germination experiments and strain identification

Cyst germination experiments were performed similar to previous paleoecological studies on diatoms (Härnström *et al.* 2011) and dinoflagellates (Miyazono *et al.* 2012). Sediment samples (~5 cm^3) were added to filtered seawater and placed in an ultrasonic bath for 6 min to separate dinoflagellate cysts from inorganic particles. The sediments were sieved with filtered seawater through a 100-μm onto a 20-μm sieve to separate the sediment size fraction containing the majority of dinoflagellate cysts. Different media (F/2, site-specific seawater, K, K/2) and temperature (16°C and 18°C) conditions were tested to optimize cyst germination. Irradiance (60 μmol photons m^{-2} s^{-1}) and photoperiod (12 h:12 h) were kept constant during this optimization. Finally, the combination of 16°C and K medium (Keller *et al.* 1987) was retained. A few drops of the 20–100 μm fraction were distributed onto a 12-well plastic plate containing K medium (2 mL per well). The plates were placed in a culture room at 16°C, under an irradiance of 60 μmol photons m^{-2} s^{-1} and a light:dark cycle of 12 h:12 h. The plates were examined for vegetative cell germination once to three times per week using an inverted microscope (Zeiss Axiovert 135). *Alexandrium minutum* cells were identified by light microscopy, but we failed to establish monoclonal strains in culture. For *Scrippsiella* spp., clonal cultures were established from different sediment layers of both the EE and DE cores. A total of 65 strains of *Scrippsiella* spp. were finally obtained from 12 and 6 sediment layers of the EE and DE cores, respectively. The cultured strains were identified genetically. DNA was extracted from the strain cultures using the DNeasy plant mini kit (QIAGEN, Düsseldorf, Germany) following the manufacturer's instructions. The LSU rDNA region was amplified by PCR using dinoflagellate-specific primers (Ldino6, Ldino1) from Probert *et al.* (2014) and the following PCR program: 35 cycles composed of an initial denaturation at 95°C for 2 min, a denaturation at 95°C for 30 s, a hybridization at 56°C for 30 s, an elongation at 72°C for 1 min and a final elongation at 72°C for 5 min. After purification by the QIAquick PCR purification kit (QIAGEN, Düsseldorf, Germany), the PCR products were sent to GATC Biotech (http://www.gatcbiotech.com/en/products/sanger-services) for Sanger sequencing. All sequences were deposited in GenBank and 32 strain cultures (the remaining 33 strains were lost) are available at the Roscoff Culture Collection (RCC: http://roscoff-culture-collection.org/).

Genetic analyses of sediment samples

DNA extraction and quantification—Genetic analyses were carried out considering the specific precautions that should be taken when working with aDNA in order to avoid cross-contamination between samples and contamination with modern DNA (Gilbert *et al.* 2005). Total DNA was extracted from 5 to 7 g of sediment material from each layer of the EE (31 samples) and the DE (58 samples) cores, using the PowerMax soil isolation kit (Mobio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's instructions. DNA extracts were immediately stored at −80°C. DNA samples were quantified by absorbance measurements using a Take3 trio microplate reader (BioTek, Winooski, VT, USA) on 3 μL of DNA extract, and sterile water was used as a blank. DNA quality was checked by the 260:280 nm ratio to ensure that no contamination by proteins or other components had occurred during DNA extraction. For DNA concentration analyses, in order to determine whether a significant change had occurred in DNA concentrations and, if so, to estimate the change-point in DNA concentration across the sediment core layers, the Change-Point Method (CPM) was applied on the total DNA concentration data along the DE and EE cores. This test is generally used to detect changes in the mean value in a data series (Taylor 2000).

Primer design and specificity—Specific primers for the amplification of *A. minutum* (synonym of *A. lusitanicum*) from genomic rDNA samples are available in the literature (Penna *et al.* 2010) but they generate longer amplicons (>150 bp) than required (50–150 bp) for optimal real-time PCR efficiency. To optimize amplification success in aDNA, the use of short amplicons (ca. 100 bp) is recommended (Coolen *et al.* 2013). Therefore, new primer sets for specific real-time PCR targeting the internal transcribed spacer 1 (ITS1) rDNA region were designed for the two target species in this study, *A. minutum* and *S. donghaienis*. *Scrippsiella donghaienis* was chosen as the second target species in the light of the successful germination analyses in old sediments and given its monophyletic separation in ITS topologies within the genus *Scrippsiella* (Gu *et al.* 2008), which enables a better specific primer design. Its presence in northern European waters (i.e. Sweden) (Gu *et al.* 2008) makes *S. donghaienis* an interesting model to compare with potential invasive species in the North Atlantic. A total of 176 ITS rDNA sequences of *A. minutum* and other *Alexandrium* species and a total of 59 ITS rDNA sequences of *S. donghaienis* and other *Scrippsiella* species were downloaded from GenBank and aligned using Bioedit software (version 7.0.9.0). The primer sets were designed to target a fragment of 100–110 bp (using Primer3 software (http://primer3.wi.mit.edu/, Rozen and Skaletsky 2000)) (Table 1). The primers were analyzed for self/hetero-complementarities and secondary structure using OligoAnalyzer 3.1 software (http://eu.idtdna.com/calc/analyzer).

Table 1. Primer sets designed to target the ITS1 rDNA region of *A. minutum* and *S. donghaienis* by the different PCR amplification methods.

Target species	Genetic region	PCR method	Primer sets	Primer sequences forward (5′-3′) reverse (5′-3′)	Amplicon size (bp)
A. minutum	ITS1	Real-time	Am_48F	TGAGCTGTGGTGGGGTTCC	100
			Am_148 R	GGTCATCAACACAGCAGCA	
A. minutum	ITS1	Semi-nested	Am_55F	TGGTGGGGTTCCTAGGCT	93
			Am_148 R	GGTCATCAACACAGCAGCA	
S. donghaienis	ITS1	Real-time	SD_357F	TATTCTGGCAACACCTTCCAC	110
			SD_468R	AGATGCTTAGCAAGTTGAGCG	

Primer specificity was checked (i) *in silico*, by visual inspection of an alignment of ITS sequences, (ii) *in silico*, using the Primer Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and (iii) in the laboratory, by conventional PCR using DNA from positive (*A. minutum* (three strains) and *S. donghaienis* (14 strains)) and negative (nine dinoflagellate species for a total of 27 strains) controls (Tables S1–S4, Supporting Information). The PCR mixture was identical for the two species except for the concentrations of the reverse and forward primers: 0.3 μM for *A. minutum* and 0.5 μM for *S. donghaienis*, 1.25 u of GoTaq Polymerase, 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 1X of GoTaq Flexi buffer, 2 μL of DNA template and sterile water. The PCR conditions for *A. minutum* were as follows: an initial denaturation step at 95°C for 2 min, 35 cycles at 95°C for 30 s, 62°C for 30 s, 72°C for 30 s and a final elongation step at 72°C for 2 min. For *S. donghaienis*, the cycling program consisted of an initial denaturation step at 95°C for 5 min, 35 cycles at 95°C for 1 s, 61°C for 45 s, 72°C for 1 min and a final elongation step at 72°C for 7 min. The PCR products were loaded onto 1.5% agarose gel to confirm the presence of fragments of the expected length (100 bp for *A. minutum* and 110 bp for *S. donghaienis*) and the absence of non-specific bands.

ITS1 rDNA cloning and standard curve construction—ITS1 rDNA PCR products of *A. minutum* (strain RCC 1490) and *S. donghaienis* (RCC 3047) were cloned separately into plasmids (pCR 4), then chemically transformed into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Several clones were obtained but only one clone of each species was retained for analysis. Plasmid DNA was isolated using the NucleoSpin Plasmid/Plasmid (No-Lid) (MACHERY-NAGEL, Düren, Germany) then linearized with a restriction enzyme 'NotI'. The plasmid was checked on a 1% agarose gel. Standard curves for real-time PCR (serially diluted plasmids containing the ITS1 rDNA fragment of *A. minutum* or *S. donghaienis*) were constructed from the linearized plasmid DNA previously isolated. Plasmid DNA concentration was measured using a Quant-iT PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA, USA) and an FLX 80 fluorescence reader (BioTek, Winooski, VT, USA) according to the manufacturer's instructions. Quantification was performed using lambda DNA standards provided in the kit ranging from 2.5 to 1000 pg μL^{-1}. Plasmid concentration (ng μL^{-1}) was converted into (copy number μL^{-1}) according to the following equation: Number of copies per microliter = (DNA concentration (ng μL^{-1}) / [660 × (plasmid size + insert size) × 10^9 (ng mol^{-1})]) × 6.022 × 10^{23} (molecules). For each target species, a standard curve was constructed with 10-fold serial dilutions of the linearized plasmid containing the ITS1 rDNA sequence of the species. Standard curves ranged from 10^6 to 10 copies μL^{-1} for *A. minutum* and from 10^5 to 10 copies μL^{-1} for *S. donghaienis*.

Real-time PCR—Real-time PCR reactions were performed using the iTaq Universal SYBR Green supermix kit (Bio-Rad, Hercules, California, USA) in a final volume of 20 μL. For *A. minutum* detection, the reaction mixture was composed of 10 μL of SYBR Green supermix (1X) (containing dNTPs, iTaq DNA polymerase, $MgCl_2$ and SYBR Green I), 0.3 μM of the forward primer (Am_48F), 0.2 μM of the reverse primer (Am_148R), sterile water and 2 μL of sample DNA template (variable concentration between 2.42 and 34.30 ng μL^{-1}). The experiments were conducted in 96-well plates containing the standard curve dilutions in duplicate, the target samples and the negative controls composed of water instead of DNA that were both analyzed in triplicate. The plates were loaded onto a Stratagene Mxpro3000P (Agilent Technologies, Santa Clara, CA, USA) thermal cycler with the following cycling conditions: 1 cycle at 95°C for 5 min followed by 40 cycles at 95°C for 5 s and 62°C for 30 s. A melting curve analysis was added at the end of each run to ensure specific *A. minutum* amplification. The optimal annealing temperature of 62°C was initially determined in conventional PCR. To determine the optimal primer concentration, nine different combinations (forward/reverse), ranging from 0.1 to 0.3 μM, were tested on three dilutions of the standard curve (10^6, 10^3, 10 copies μL^{-1}). The primer combination that yielded the lowest threshold cycle value (Ct) and maximum real-time efficiency (Am_48F; 0.3 μM, Am_148R; 0.2 μM) was retained for further analysis. The real-time PCR mixture for *S. donghaienis* detection was identical, except for the primer concentrations; 0.2 μM for the forward and reverse primer (SD_357F /SD_468R). The annealing temperature was determined at 61°C. The cycling conditions were: 1 cycle at 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 61°C for 30 s and 72°C for 30 s, and a final cycle at 95°C for 1 min followed by a melting curve analysis. The reaction efficiency was estimated by the equation $E = 10^{(1/b)} - 1$, where b is the slope of the standard curve. DNA amplifications of target species were considered positive if more than one out of the three replicates tested per sample was positive. The limit of quantification (LOQ) was set at 10 copies per real-time PCR well, which was the smallest quantity of linear plasmid DNA of the standard curves for each real-time PCR assay. Concentrations of *A. minutum* or *S. donghaienis* in each sediment layer were expressed (assuming a 100% DNA extraction efficiency) in terms of copy number per gram of wet sediment, using the following formula: Copy number g^{-1} = copy number μL^{-1} × DNA extraction volume (μL)/sediment wet weight (g).

Sanger sequencing of A. minutum amplicons from sediment layers—In order to confirm the presence of *A. minutum* in sediment layers for which the real-time PCR amplifications were below the LOQ, amplicons resulting from a semi-nested PCR amplification of the DNA extract of the corresponding sediment layers were sequenced. The semi-nested PCR assays were carried out only for *A. minutum* as this was the major target species of our study. After the first amplification using designed primers for real-time PCR (Am_48F/Am_148R) (100 bp), 2 μL of the amplified material was used for the second PCR round performed with a new forward primer (Am_55F) and the same reverse primer

Table 2. *Alexandrium minutum* semi-nested PCR sequences obtained from DE and EE cores with their respective ITS1 rDNA semi-nested sequences.

Species	Core ID	Sediment layers (cm)	Sediment layer dating	Semi-nested sequences (5′-3′)
A. minutum	DE	24	1972 ± 2	ATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	25	1970 ± 2	GGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	31	1955 ± 3	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	33	1950 ± 3	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	41	1931 ± 4	ATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	42	1928 ± 4	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	53	1893 ± 5	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	54	1889 ± 5	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	DE	57	1873 ± 6	GGCATGGCTTGCTTCTGCAAGCGCTTTCATG
A. minutum	EE	11	1991 ± 1	CATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	13	1986 ± 1	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	17	1978 ± 2	GCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	25	1958 ± 2	GGCATGGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT
A. minutum	EE	31	1939 ± 2	GGCTTGCTTCTGCAAGCGCTTTCATGCTGCTGTGTTGAT

(Am_148R) of the first PCR step (Table 1). Both PCR reaction mixtures were carried out in a final volume of 20 μL, using 2 μL of DNA template, 1X of GoTaq Flexi buffer, 2 mM of $MgCl_2$, 0.2 mM of dNTP, 0.5 mM of each primer, 1.25 u of GoTaq polymerase (Promega) and sterile water. The PCR were performed in a PeqStar (OZYME, Montigny-Le-Bretonneux, France) thermocycler with the following conditions: 1 cycle at 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 61°C for 30 s, 72°C for 30 s and a final cycle at 72°C for 2 min. PCR products were purified using the Nucleospin Gel PCR clean-up kit (MACHERY-NAGEL, Düren, Germany) following the manufacturer's instructions, checked on an agarose gel. This semi-nested PCR procedure yielded an amplicon of 93 bp and a sufficient *A. minutum* DNA concentration for Sanger sequencing. Amplification products were sent to GATC Biotech (http://www.gatcbiotech.com/en/products/sanger-services/, Germany) for sequencing. The resulting sequences were identified by basic local alignment search tool (BLAST) and aligned in Bioedit with other *A. minutum* ITS1 rDNA sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2).

Statistical analyses on data series

In order to depict trends in the abundance of target species, the moving averages of real-time PCR data (copy number g^{-1} sediment) were calculated. This method is usually used to calculate long-term trends or cycles from time series data, smoothing out short-term fluctuations in a dataset, and has been used in paleoecological studies (e.g. Yu and Berglund 2007). The moving average of the *A. minutum* and *S. donghaienis* genetic data series (copy number g^{-1} sediment) was calculated with a three-sample interval, meaning that an averaged value of the copy number g^{-1} was calculated for every three consecutive data (e.g. average of layers 1, 2, 3; average of layers 2, 3, 4; etc.). These averaged values were used to produce the curves in Fig. 3 representing the trend of the quantitative genetic data obtained for our studied species in the sediment samples. In addition, the Mann-Kendall trend test was performed on the real-time PCR data (copy number g^{-1} sediment) and the plankton data (cells L^{-1}) across the overlapping periods of the series (from 1989 to 2014) to detect statistically significant trends. The test was applied using the Kendall package in R software version 3.2.3 (R Core Team 2015). The Mann-Kendall trend test is a non-parametric test for monotonic trends, which is typically used to detect whether there is a significant increasing or decreasing trend in a time series (Yue, Pilon and Cavadias 2002). In this test, the initial assumption is the null hypothesis (H0), which assumes that there is no trend in the data series over time. The alternative hypothesis (H_1) is that there is a significant trend (increasing or decreasing) over time. The significance level of the test was set at $\alpha = 0.05$. If the P-value < 0.05, the H0 is rejected, meaning that there is a significant trend in the time series. Kendall's tau (τ) is a value between -1 and 1 that indicates the direction of the trend over time. An increasing trend is indicated by $\tau > 0$ and a decreasing one by $\tau < 0$.

RESULTS

Sediment chronology

In the DE core, ^{210}Pb in excess activity ranged from 45 mBq g^{-1} in the uppermost sediment layers to <1 mBq g^{-1} at 58 cm (Fig. 2). There was a general trend of an exponential decrease in $^{210}Pb_{xs}$ as expected due to the decay of the unsupported ^{210}Pb. The $^{210}Pb_{xs}$ profile in the EE core showed the same trend: due to the shorter length of the sediment core, levels in which $^{210}Pb_{xs}$ became negligible were not reached. In both cores, the $^{210}Pb_{xs}$ decrease presented some irregularities. This could be related to temporary changes in sedimentation intensity, which justified the use of the CSR (constant rate of supply) model for dating (Fig. 2). The 31 cm-long EE core encompassed the last 72 years (from 1939 ± 2 to 2011 ± 1), whereas the DE core covered >100 years (from 1866 ± 7 to 2013 ± 1). The sedimentary profile of ^{137}Cs in the two cores presented the expected shape with two peaks (nuclear weapon test fallout and Chernobyl) in the deepest layers. In the DE core, ^{137}Cs disappeared rapidly to negligible levels below the deepest peak. The EE core was too short to evaluate ^{137}Cs disappearance; however, the expected peak of the second atmospheric fallout was observed. In general, the sedimentary ^{137}Cs peaks in the two cores mimicked the atmospheric fallout rather well, validating the ^{210}Pb chronology (Fig. 2).

Germination experiments

Successful germinations were observed in both EE and DE cores (Table 3). Germinated cells belonged primarily to dinoflagellates (up to 17 cm (1978 ± 2) for EE and 20 cm (1981 ± 2) for DE) and diatoms (up to 11 cm (1991 ± 1) for EE and 13 cm (1995 ± 2) for

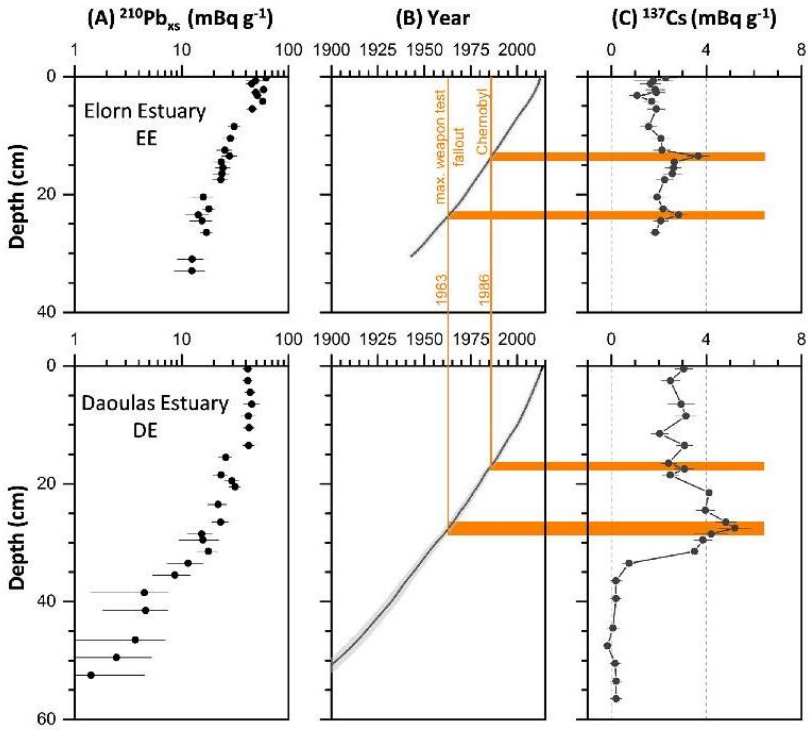


Figure 2. $^{210}Pb_{xs}$ profile (A), ages of sedimentary layers based on CSR model (B) and ^{137}Cs profile (C) with depth along the cores in the Elorn (EE, upper panel) and Daoulas (DE, lower panel) estuaries.

DE). For the EE core, *S. donghaienis* germinated predominantly along the core up to 17 cm (1978 ± 2) while cells of *A. minutum* were observed up to 7 cm (1999 ± 1). For DE, *S. donghaienis* cells were observed up to 20 cm (1981 ± 2) and *A. minutum* was detected up to 13 cm (1995 ± 2) (see dotted line in Fig. 3). Germinated diatom species belonged to *Skeletonema* spp., *Chaetoceros* spp., *Thalassiosira* spp., *Nitzschia* spp., *Lithodesmium* spp., *Paralia* spp. and *Asteromphalus* spp. Other autotrophic (*Peridinium quinquecorne*, *Gymnodinium* spp.) and heterotrophic (*Protoperidinium* spp.) dinoflagellates were recorded along the two cores.

Molecular analyses

Total DNA concentrations extracted from the sediments ranged from 2.42 to 34.30 ng μL^{-1} for EE and from 3.46 to 26.70 ng μL^{-1} for DE. According to the 260:280 nm ratios, the DNA extracts were of sufficient yield and purity to conduct amplification analyses. A decreasing trend in total DNA concentration was observed from the top to the bottom of both EE and DE cores (see Fig. S1, Supporting Information), with higher concentrations found in the first sediment layers of both cores. At 9 cm (10.84 ng μL^{-1}) for EE and 11 cm (16.73 ng μL^{-1}) for DE, the CPM method identified the change in the DNA concentration slope, from a decreasing to a steady trend. For real-time PCR tests, good linear relationships were found between the threshold cycle (Ct) and the initial number of copies ($0.998 \geq R^2 \geq 0.999$). The reaction efficiencies ranged from 95% to 99% for *A. minutum* and from 91.4% to 95.3% for *S. donghaienis*. To ensure specific amplifications, the melting temperature values (Tm) were systematically checked by analyzing the melting curves. For the *A. minutum* assay, the standard curve samples as well as the positive samples showed a specific peak at Tm = 83.05 ± 0.5°C (see Fig. S2, Supporting Information). In the case of *S. donghaienis*, the melting curve peak was observed at Tm = 82.9 ± 0.5°C. Copy numbers < LOQ (=10 copies/well) for both *A. minutum* and *S. donghaienis* were observed in some deep sediment layers. In these cases, the target species were considered present in the core layers, but it was not possible to quantify their ITS rDNA copy numbers. The presence of *A. minutum* in deep layers (indicated with star symbols in Fig. 3) was confirmed by the sequencing of semi-nested PCR products. Nine and five sequences of 31–45 bp were obtained for DE and EE core layers, respectively. The sequences were aligned with other available sequences (see File 1, Supporting Information; Fig. S3, Supporting Information) and identified as *A. minutum* at 100% identity (Table 2).

Multidecadal dynamics of *A. minutum* and *S. donghaienis* detected in sediments

Accurate DNA quantification of *A. minutum* ITS1 rDNA copies g^{-1} sediment was obtained from the 10-cm layer (1993 ± 1) to the 1-cm layer (2011 ± 1) of the EE core and from the 22-cm layer (1976 ± 2) to the 1-cm layer (2013 ± 1) of the DE core (Fig. 3), covering a total period of about 40 years. For both EE and DE, in the layers from which quantitative data were obtained, an increasing trend in the concentration of *A. minutum* ITS1 rDNA copies g^{-1} sediment was observed from the bottom to the top layers. This trend was visualized by the moving average

Table 3. *Scrippsiella* spp. strains obtained from the Elorn estuary (EE) and Daoulas estuary (DE) dated sediment layers with their respective Genbank accession number and Roscoff culture collection (RCC) number.

Species	Strain ID	Core sediment layer (cm)	Sediment layer dating	GenBank accession number	RCC ID
S. donghaienis	IFR-SDO-Sc34	EE2	2010 ± 1	KX009607	4711
S. donghaienis	IFR-SDO-Sc35	EE2	2010 ± 1	KX009606	4712
S. donghaienis	IFR-SDO-Sc38	EE4	2006 ± 1	KX009603	4713
S. donghaienis	IFR-SDO-Sc39	EE4	2006 ± 1	KX009602	4714
S. donghaienis	IFR-SDO-Sc40	EE4	2006 ± 1	KX009601	–
S. donghaienis	IFR-SDO-Sc22	EE9	1995 ± 1	KX009619	–
S. donghaienis	IFR-SDO-Sc23	EE9	1995 ± 1	KX009618	–
S. donghaienis	IFR-SDO-Sc24	EE9	1995 ± 1	KX009617	4715
S. donghaienis	IFR-SDO-Sc25	EE9	1995 ± 1	KX009616	4716
S. donghaienis	IFR-SDO-Sc26	EE9	1995 ± 1	KX009615	4730
S. donghaienis	IFR-SDO-Sc51	EE10	1993 ± 1	KX009592	4717
S. donghaienis	IFR-SDO-Sc53	EE10	1993 ± 1	KX009591	4718
S. donghaienis	IFR-SDO-Sc54	EE10	1993 ± 1	KX009590	–
S. donghaienis	IFR-SDO-Sc1	EE11	1991 ± 1	KX009637	–
S. donghaienis	IFR-SDO-Sc2	EE11	1991 ± 1	KX009636	4733
S. donghaienis	IFR-SDO-Sc3	EE11	1991 ± 1	KX009635	–
S. donghaienis	IFR-SDO-Sc4	EE11	1991 ± 1	KX009634	–
S. donghaienis	IFR-SDO-Sc5	EE11	1991 ± 1	KX009638	–
S. donghaienis	IFR-SDO-Sc6	EE11	1991 ± 1	KX009639	–
S. donghaienis	IFR-SDO-Sc7	EE11	1991 ± 1	KX009640	–
S. donghaienis	IFR-SDO-Sc55	EE12	1989 ± 2	KX009589	–
S. donghaienis	IFR-SDO-Sc57	EE12	1989 ± 2	KX009588	–
S. donghaienis	IFR-SDO-Sc8	EE13	1986 ± 2	KX009633	–
S. donghaienis	IFR-SDO-Sc9	EE13	1986 ± 2	KX009632	–
S. donghaienis	IFR-SDO-Sc10	EE13	1986 ± 2	KX009631	4719
S. donghaienis	IFR-SDO-Sc11	EE13	1986 ± 2	KX009630	–
S. donghaienis	IFR-SDO-Sc12	EE13	1986 ± 2	KX009629	–
S. donghaienis	IFR-SDO-Sc13	EE13	1986 ± 2	KX009628	4720
S. donghaienis	IFR-SDO-Sc14	EE13	1986 ± 2	KX009627	4721
S. donghaienis	IFR-SDO-Sc15	EE13	1986 ± 2	KX009626	4734
S. donghaienis	IFR-SDO-Sc16	EE13	1986 ± 2	KX009625	–
S. donghaienis	IFR-SDO-Sc17	EE13	1986 ± 2	KX009624	–
S. donghaienis	IFR-SDO-Sc18	EE13	1986 ± 2	KX009623	–
S. donghaienis	IFR-SDO-Sc19	EE13	1986 ± 2	KX009622	–
S. donghaienis	IFR-SDO-Sc20	EE13	1986 ± 2	KX009621	4722
S. donghaienis	IFR-SDO-Sc21	EE13	1986 ± 2	KX009620	–
S. donghaienis	IFR-SDO-Sc58	EE14	1984 ± 2	KX009587	–
S. donghaienis	IFR-SDO-Sc59	EE14	1984 ± 2	KX009586	–
S. donghaienis	IFR-SDO-Sc60	EE14	1984 ± 2	KX009585	4731
S. donghaienis	IFR-SDO-Sc61	EE14	1984 ± 2	KX009584	–
S. donghaienis	IFR-SDO-Sc27	EE17	1978 ± 2	KX009614	4723
S. donghaienis	IFR-SDO-Sc28	EE17	1978 ± 2	KX009613	4724
S. donghaienis	IFR-SDO-Sc29	EE17	1978 ± 2	KX009612	4725
S. donghaienis	IFR-SDO-Sc30	EE17	1978 ± 2	KX009611	–
S. donghaienis	IFR-SDO-Sc31	EE17	1978 ± 2	KX009610	4726
S. trochoidea	IFR-STR-Sc36	EE4	2006 ± 1	KX009605	–
S. trochoidea	IFR-STR-Sc37	EE4	2006 ± 1	KX009604	4732
S. trochoidea	IFR-STR-Sc41	EE4	2006 ± 1	KX009600	–
S. trochoidea	IFR-STR-Sc42	EE4	2006 ± 1	KX009599	4727
S. trochoidea	IFR-STR-Sc43	EE4	2006 ± 1	KX009598	–
S. trochoidea	IFR-STR-Sc45	EE4	2006 ± 1	KX009597	4728
S. trochoidea	IFR-STR-Sc32	EE5	2003 ± 1	KX009609	–
S. trochoidea	IFR-STR-Sc33	EE5	2003 ± 1	KX009608	–
S. trochoidea	IFR-STR-Sc46	EE6	2001 ± 1	KX009596	–
S. trochoidea	IFR-STR-Sc47	EE6	2001 ± 1	KX009595	–
S. trochoidea	IFR-STR-Sc48	EE8	1997 ± 1	KX009594	–
S. trochoidea	IFR-STR-Sc49	EE8	1997 ± 1	KX009593	4729
S. donghaienis	IFR-SDO-S5	DE17	1987 ± 2	KX009641	4735
S. donghaienis	IFR-SDO-S8	DE19	1982 ± 2	KX009642	4736
S. donghaienis	IFR-SDO-S7	DE20	1981 ± 2	KX009643	4737
S. trochoidea	IFR-STR-S2	DE10	2001 ± 1	KX009644	4738
S. trochoidea	IFR-STR-S3	DE13	1995 ± 2	KX009645	4739
S. trochoidea	IFR-STR-S9	DE15	1991 ± 2	KX009646	4740
S. trochoidea	IFR-STR-S4	DE17	1987 ± 2	KX009647	4741
S. trochoidea	IFR-STR-S6	DE17	1987 ± 2	KX009648	4742

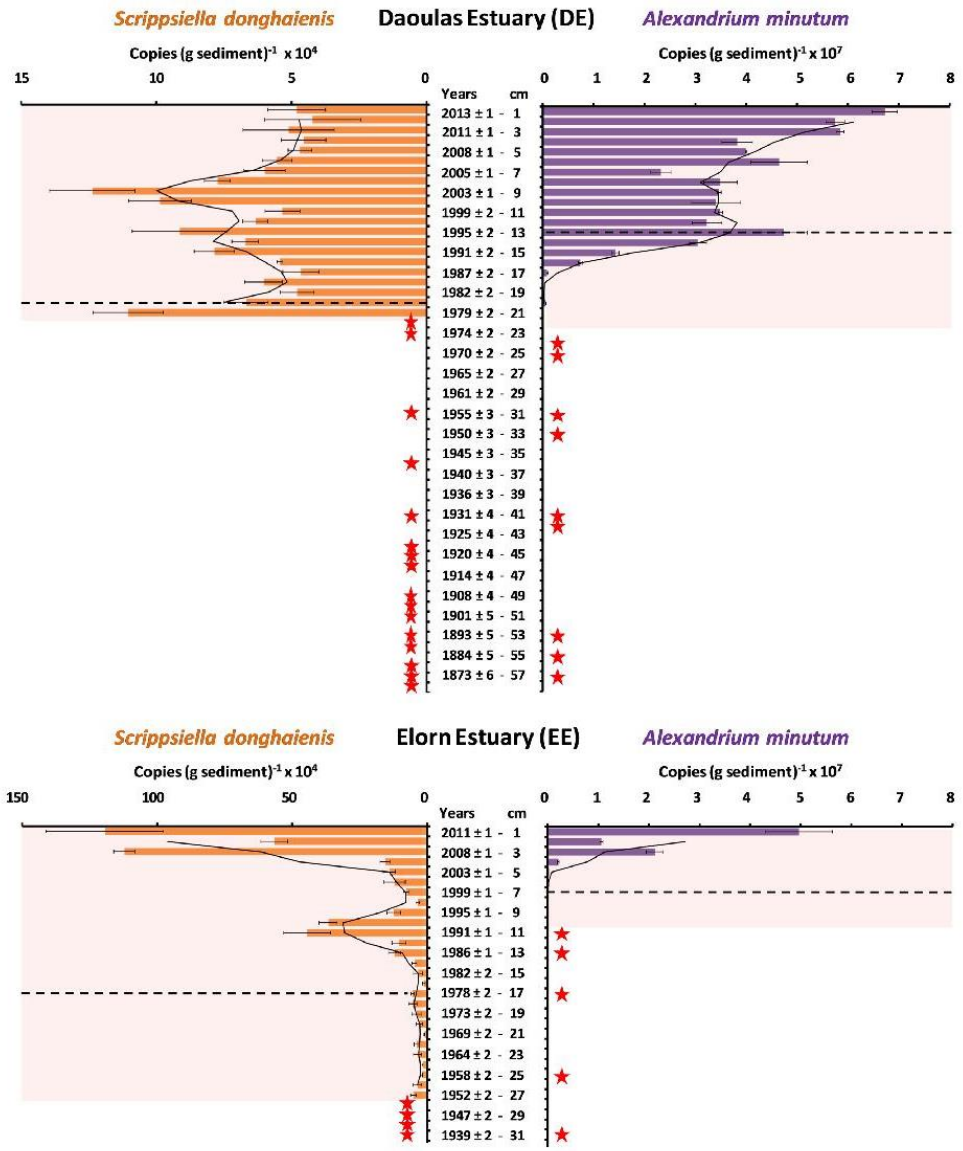


Figure 3. ITS1 rDNA copies g^{-1} sediment for *Scrippsiella donghaienis* (orange) and *Alexandrium minutum* (purple) estimated by real-time PCR in Daoulas (DE) and Elorn (EE) estuaries. Colored shadows indicate the layers for which reliable ITS1 rDNA quantifications were obtained. Stars indicate the layers where the presence of the species was detected but quantitative data were below the LOQ of our real-time PCR assays. The dashed lines indicate the limit layer of respective species germination. The black curves represent calculated moving average on the quantitative data.

calculation (Fig. 3) and statistically confirmed over the period 1989–2014 by the Mann-Kendall test analyses (DE: $\tau = 0.65$, P-value = 0.0005; EE: $\tau = 0.911$, P-value = 0.0003). In particular, at EE, *A. minutum* ITS1 rDNA copies g^{-1} sediment increased from the 10-cm layer (1993 ± 1; 2.72 × 10^4 copies g^{-1} sediment) to reach a peak at the 1-cm layer (2011 ± 1; 4.96 × 10^7 copies g^{-1} sediment). At DE, ITS1 rDNA copy numbers were higher than at EE. They increased from the 22-cm layer (1976 ± 2; 2.49 × 10^4 copies g^{-1} sediment), peaking at the 1-cm layer (2013 ± 1; 6.73 × 10^7 copies g^{-1}). ITS1 rDNA copies g^{-1} were slightly correlated with total DNA concentrations in both cores (EE: $R^2 = 0.51$; DE: $R^2 = 0.55$), which were higher in the top than in the bottom layers (see Fig. S1, Supporting Information). In layers from which non-quantitative data were obtained, the presence of *A. minutum* was detected discontinuously, being present along the cores in 5 out of 21 layers and in 9 out of 35 layers for EE and DE, respectively

(star symbols in Fig. 3). These rDNA traces of *A. minutum* were detected even in the deep sediment layers of both analyzed cores, corresponding to 1939 ± 2 (31 cm) for EE and 1873 ± 6 for DE (57 cm) (Fig. 3).

For *S. donghaienis*, quantification of ITS1 rDNA copies g^{-1} of sediment was obtained from the 27-cm layer (1952 ± 2) to the 1-cm layer (2011 ± 1) for the EE core, and from the 21-cm layer (1979 ± 2) to the 1-cm layer (2013 ± 1) for the DE core (Fig. 3). The pattern of abundance of ITS rDNA copies observed for *S. donghaienis* differed from that of *A. minutum* and varied across the sites studied (Fig. 3). At EE, a bimodal pattern of copy number abundance was evident. From the deepest quantifiable 27-cm layer (1952 ± 2; 5.1 × 10^4 copies g^{-1} sediment), the first peak of abundance was evident at the 11-cm layer (1991 ± 1; 4.4 × 10^5 copies g^{-1} sediment) and a second peak corresponding to the highest copy number observed, was found in the top 3 cm (peak of 1.2 × 10^6 copies g^{-1} sediment at 1 cm (2011 ± 1)). At DE, spikes of abundance were observed at 21 cm (1979 ± 2; 1.1 × 10^5 copies g^{-1} sediment), 13 cm (1995 ± 2; 9.13 × 10^4 copies g^{-1} sediment) and 9 cm (2003 ± 1; 1.24 × 10^5 copies g^{-1} sediment). From the 9-cm layer to the top layer of the DE core, a decrease in copy number abundance was observed (Fig. 3). Over the period 1989–2014, the Mann-Kendall test showed a significant increasing trend in EE ($\tau = 0.601$, *P*-value = 0.0001), but no significant trend was detected in DE ($\tau = -0.295$, *P*-value = 0.0650). The pattern of copies g^{-1} sediment abundance was slightly correlated with total DNA abundance at EE ($R^2 = 0.53$), but not at DE ($R^2 = 0.01$). *Scrippsiella donghaienis* was detected up to the bottom layers of both the EE and DE cores (1939 ± 2 and 1866 ± 7, respectively). In the layers for which non-quantitative data were obtained, the presence of *S. donghaienis* was detected in 4 out of 4 layers for the EE core and in 16 out of 37 layers for the DE core (Fig. 3).

Multidecadal dynamics of *A. minutum* and *S. donghaienis* in the water column (plankton data)

At the Lanveoc station, *A. minutum* concentrations were <7000 cells L^{-1} until 2012, reaching a peak in July 2014 (32 800 cells L^{-1}). At the Le Passage station, regular monitoring activities started when *A. minutum* was first observed in the Bay of Brest in 1990 (<100 cells L^{-1}). From 1992 to 1996, no *A. minutum* cells were detected at the station. From 1997, this species was regularly observed until 2003, but always at low concentrations (<2000 cells L^{-1}). Monitoring in the area stopped in 2003 and was re-established in 2012 when a bloom of 560 000 cells L^{-1} occurred on 11 July 2012. No bloom was observed in the area in 2013 but in 2014, *A. minutum* concentrations reached 199 000 cells L^{-1} on 7 July. The 2012 bloom at Le Passage coincided with a bloom at the Daoulas station (42 × 10^6 cells L^{-1} on 11 July 2012), which represents the highest concentration ever recorded in the bay. Blooms of lower intensity occurred in the following years (293 333 cells L^{-1} in 2013 and 1052 000 cells L^{-1} in 2014) (see Fig. S4, Supporting Information). A statistically significant increasing trend in *A. minutum* concentrations was depicted by our longer plankton time series at Le Passage and Lanveoc in the Bay of Brest by the Mann-Kendall test (Lanveoc: $\tau = 0.247$, *P*-value = 0.0001; Le Passage: $\tau = 0.281$, *P*-value = 0.0050). The Daoulas time series was too short (3 years) to analyze a multiannual trend, thus the Mann-Kendall test was not applied. When qualitatively compared over the overlapping period of analyses, the long-term data series of *A. minutum* obtained from our paleoecological approach (ITS1 rDNA copies g^{-1} sediment) and from the monitoring activity (plankton data Log(cells L^{-1})) of Lanveoc and Le Passage stations nicely showed a coherent increasing trend in species abundance from 1990 until 2014 (Fig. 4). For *Scrippsiella* spp., monitoring was carried out uninterruptedly from 1987 until 2000 at Le Passage, and from 1995 to 2014 at Lanveoc. No monitoring of this taxon has ever been performed at the Daoulas station. Lower concentrations of *Scrippsiella* spp. were recorded at Lanveoc compared to Le Passage. At Lanveoc, *Scrippsiella* spp. abundance remained below 8300 cells L^{-1} until 1998, when it reached 42 000 cells L^{-1}. At Le Passage, *Scrippsiella* spp. abundance reached a maximum of 760 000 cells L^{-1} in 1999. Lower abundances were registered during the following years (see Fig. S4, Supporting Information). At both stations, no trend was detected by the Mann-Kendall coefficient for this taxon (Lanveoc: $\tau = -0.02$, *P*-value = 0.5297; Le Passage: $\tau = -0.0125$, *P*-value = 0.6773).

DISCUSSION

The paleoecological approach used in this study, based on paleogenetic analyses and ancient cyst revivification, provided historical data on two target dinoflagellate species over a time scale of about 150 years. The focus was mainly on the bloom-forming, toxic species *A. minutum* and then paleogenetic data obtained for the non-toxic species *S. donghaienis* were used to compare specific ecological patterns. Both species were detected along dated sediment cores, even in deep sediment layers (dating back to 1873 ± 6 for *A. minutum* and 1866 ± 7 for *S. donghaienis*). Their aDNA concentrations were quantified specifically from 1952 ± 2 or 1976 ± 2 (depending on the species and the cores) using a robust, newly developed real-time PCR assay. Moreover, up to 31–34-year-old resting cysts were successfully revived from core sediments.

Real-time PCR and cyst germination

Real-time PCR is a quantitative technique known to be highly sensitive, enabling species abundances to be estimated accurately in different environments even when the DNA target gene copy number is low. The advantage of real-time PCR is the fast processing of a large number of samples compared to cell or cyst counting microscopic procedures, which are time-consuming and often biased by taxonomic identification problems. Several studies have used real-time PCR to quantify the abundance of a large number of toxic phytoplankton species in seawater samples: *Karenia brevis* (Gray *et al.* 2003); *A. minutum* (Galluzzi *et al.* 2004; Touzet *et al.* 2009); *A. catenella* (Garneau *et al.* 2011); *Ostreopsis* cf. *ovata* (Perini *et al.* 2011); *Pseudo-nitzschia* spp. (Fitzpatrick, Caron and Schnetzer 2010); and different diatoms and dinoflagellates (Godhe *et al.* 2008; Murray *et al.* 2011), as well as in superficial sediment samples (Kamikawa *et al.* 2007; Erdner *et al.* 2010).

Real-time PCR was the most appropriate method for our paleogenetic approach, which aimed to detect ancient genetic traces of the presence and abundance of the target species in the Bay of Brest. Yet, when quantifying genetic material in old sediments, DNA degradation is a major issue to be taken into account. Taphonomic processes that may occur in old sediments and the variable preservation conditions of organic matter occurring from one ecosystem to another may influence DNA conservation and thus its quantitative estimation (Boere *et al.* 2011). DNA degradation mostly occurs in very old sediments, such as those that are of thousand years old, and can cause DNA to be fragmented, which can significantly reduce the efficiency of PCR amplification (d'Abbadie *et al.* 2007). The real-time PCR

were tested and a final optimal combination (temperature, nutrient concentration) was found and systematically applied to all samples, it cannot be excluded that optimal germination conditions vary across sediments of different ages. In fact, *S. donghaienis* and *A. minutum* are able to germinate over a wide range of environmental conditions (Gu *et al.* 2008; Blanco, Lewis and Aldridge 2009).

Interestingly, germination of *A. minutum* and *S. donghaienis* was successful only for layers in which aDNA quantification was possible. It can be questioned whether DNA was amplified and quantified mainly from living stocks, represented by resting cysts, from extracellular genetic material or from both. Most DNA in marine sediments has been shown to be extracellular (Dell'Anno and Danovaro 2005) and well preserved from microbial nuclease degradation (Corinaldesi *et al.* 2011), but DNA inside the resting stages of some plankton species is generally thought to be better preserved than extracellular DNA (Boere *et al.* 2011). Real-time PCR data do not distinguish between extracellular and cyst-preserved genetic material, thus a definitive conclusion regarding the type of DNA quantified in this study is not possible. RNA analysis might have clarified whether active or inactive communities were identified, as explored in Capo *et al.* (2015) where RNA transcripts were found only in the top 2 cm of lacustrine sediments and RNA/DNA analyses showed that genetic material in sediments mainly represented inactive communities. However, RNA analyses and quantitative analyses on cysts were beyond the scope of this study. Our main goal was to obtain historical information about the presence, abundance and viability of *A. minutum* and *S. donghaienis* in order to depict multidecadal ecological patterns.

Historical records of *A. minutum* and *S. donghaienis* and multiannual dynamics

By means of this paleogenetic approach, the first traces of genetic material of *A. minutum* were detected in the Bay of Brest. Starting from two different cores, it could be inferred that the species was present in the bay considerably earlier than its first description in the Mediterranean Sea in 1960 (Halim 1960), as the first detection of the species in the DE sediment core of the Bay of Brest dated back to 1873 ± 6. Similarly, using cyst records in dated sediments, Ribeiro, Amorim and Andersen (2011) established that the dinoflagellate *Gymnodinium catenatum* had been present in the western Iberian Peninsula since 1889 ± 10, before its first description documented in 1939 on planktonic viable material. Our data also suggest that the presence of *S. donghaienis* in the Bay of Brest dated back to 1866 ± 7, but its formal description is relatively recent (Gu *et al.* 2008) and only a few historical and biogeographical data are available, as the species has only been reported in Australia, Sweden and East China (Gu *et al.* 2008). In fact, *A. minutum* and *S. donghaienis* could have been present in the bay even before 1873 ± 6 and 1866 ± 7, respectively. The DE core was not sufficiently long to highlight the absence of the genetic signal along a consistent number of old sediment layers. Regarding the analyzed cores, the presence of both species was detected irregularly across layers from which data below the LOQ were obtained. This could indicate some contamination during the sampling process across the different layers. Yet, our sampling protocol was designed to avoid direct contamination during sampling, both across different layers and with modern DNA (using a clean device for each layer) and along the plastic tube core (collecting only the inner part of each sediment layer). In addition, molecular manipulations in the laboratories were performed following previously developed precautions for paleogenetic analyses to avoid cross-contamination between samples (Gilbert *et al.* 2005; Anderson-Carpenter *et al.* 2011; Boere *et al.* 2011; Domaizon *et al.* 2013). Moreover, similar results were obtained from two different cores, collected and analyzed at two different periods of time. Therefore, we believe that the irregular presence of both species across the two cores is most probably due to the presence of a very small amount of specific genetic material rather than to sample contamination.

In order to validate the multiannual study of species dynamics, the relative species variations in quantitative real-time PCR estimations were compared to those of the plankton time series obtained from monitoring analyses during the overlapping periods of the data series. Previously, real-time PCR has rarely been applied to ancient DNA to compare long-term plankton series and only restrictively to quantify cyanobacteria dynamics in freshwater sediments (Domaizon *et al.* 2013; Martínez de la Escalera *et al.* 2014; Pal, Gregory-Eaves and Pick 2015). To the best of our knowledge, this is the first paleogenetic study carried out in monitored estuarine ecosystems addressing such a comparison between real-time PCR and plankton-monitoring data. Interestingly, the statistically significant increase in concentrations of ITS1 rDNA copy number of *A. minutum* depicted from 25-year-old sediment samples of two different cores corroborates the 24 years of plankton observations carried out at different monitoring stations in the Bay of Brest. When compared qualitatively over the overlapping periods (1989–2014), both the paleogenetic and plankton time data series nicely showed an increasing trend in the concentration of the species *A. minutum* over time (Fig. 4). This trend is particularly evident, and statistically confirmed, from the plankton data of the Lanveoc and Le Passage stations, which have been followed over the long term, and is also proved by the fact that huge blooms of the species have only recently been observed and monitored in the third station of the bay (Daoulas) (Chapelle *et al.* 2015). Conversely, *S. donghaienis* ITS1 rDNA abundances did not show a coherent pattern and trend between the analyzed cores, suggesting a non-homogenous temporal pattern in the Bay of Brest for this species. The increasing concentration trend depicted for *S. donghaienis* from paleogenetic data of the EE core was not confirmed by the DE core data, unlike for *A. minutum* for which both the EE and DE paleogenetic data series showed an increase in species concentration. The comparison with plankton data was not possible since *S. donghaienis* can only be identified genetically (Gu *et al.* 2008) and monitoring data are acquired by optical microscopy analyses, which can only identify genera. Yet, *Scrippsiella* spp. data do not show an increase in the abundance of the taxon and no major bloom of any species of the genus has been registered in the Bay of Brest to date.

Along the Brittany coasts, *A. minutum* has been sporadically observed since 1985, at the very beginning of the REPHY monitoring activities. Today, its ecological success is evident in some areas. For instance, in the Penzé estuary (Bay of Morlaix, north Brittany), this species has produced blooms at more than 10^4 cells L^{-1} every year for the last 27 years (Dia *et al.* 2014). On the north-western Brittany coast (Aber-Wrac'h), blooms are regularly observed, and in the Bay of Brest, high concentrations of the species have been reported since 2012. Our results show that *A. minutum* was present in the Bay of Brest much earlier than its first detection by REPHY monitoring. On the basis of these results, we can put forward the hypothesis that *A. minutum* had a long residence period before blooming in the area. The introduction of the species, if any, may have occurred earlier than our first detection of it (1873 ± 6). Future paleoecological

reconstruction is required to answer this question over a longer time scale.

Our results demonstrate that the species *A. minutum* has increased greatly in the Bay of Brest in recent years. This could be due to the introduction of a new population, potentially genetically different from original populations, or the natural evolution of the local populations, or the adaptation of the local populations to environmental changes (natural or anthropogenic). In fact, local ecosystem-specific constraints have contributed to shaping the population dynamic of the species *A. minutum* in a shorter period of time in relatively close ecosystems (Dia *et al.* 2014). Since our study was performed only at the species level and not at the population level, it is not possible to evaluate to what extent population introduction, adaptation and evolution contributed to the process of *A. minutum* development in the Bay of Brest. Future paleoecological population-based studies could address the issue of species development over a multidecadal time scale at the intraspecific level, contributing to a better understanding of long-term microbial species phenologies in the marine environment.

CONCLUSIONS

This study innovatively associated old-cyst revivification, paleogenetic and plankton-monitoring data to reconstruct the long-term dynamics of two target estuarine dinoflagellate species, *A. minutum* and *S. donghaienis*. Cyst survival time varied between the target species (31–34 years for *S. donghaienis* and 17–19 years for *A. minutum*) and, interestingly, was correlated with their quantifiable genetic material. This finding contributes to the debate about the proportions of intracellular, cyst-protected and extracellular DNA amplifiable from sediments. The paleogenetic data enabled the multidecadal dynamics of the two target species to be studied over a time scale of about 150 years, with quantitative data obtained for about the last 40 years. The most ancient genetic traces found in these two dated core samples suggest that *S. donghaienis* and *A. minutum* have been present in the Bay of Brest since at least 1866 $\pm$ 7 and 1873 $\pm$ 6, respectively. Paleogenetic data clearly showed that *A. minutum* has increased in concentration in recent years, corroborating plankton data over the overlapping period of the two different data series (1989–2014). This study contributes to the development of paleoecological research, showing that this discipline, mostly developed in lacustrine ecosystems to date, can be applied to marine, estuarine ecosystems, providing new perspectives for future research on the biogeography, ecology and evolution of marine microbes.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

The authors wish to thank Xavier Caisey and the other scuba divers from Ifremer for sampling the EE core, and Gwendoline Gregoire and Axel Ehrhold from the laboratory GM/LES of Ifremer for allowing the sampling of the DE in the framework of the project SERABEQ ('Laboratoire d'Excellence' LabexMER (ANR-10-LABX-19)). Pierre Bodenes and Tania Hernández-Fariñas are acknowledged for collaborating in the picture developments and Pascale Malestroit for helping with cyst germination and culture maintenance. Pierre Ramond is acknowledged for helping with statistical tests. Finally, we wish to thank the members of the SG2M/LSEM laboratory of Ifremer for access to their laboratory facilities and for all their technical suggestions. This research falls within the scope of the French GDR (Groupement de Recherche) Phycotox (http://www.phycotox.fr) (2012–18) on harmful microalgae and phycotoxins.

FUNDING

This work was financed by the exploratory projects VEHSPA (2012–2013) and KYNDER (2014–2015), supported by the 'Laboratoire d'Excellence' LabexMER (Agence Nationale de la Recherche (ANR) - 10-LABX-19) and co-funded by the French government under the program 'Investissements d'Avenir', by the projects of the initiative ECosphere Continentale et CÔtière (EC2CO) of the Institut National des Sciences de l'Univers/Centre National de la Recherche Scientifique (INSU/CNRS): PALMITO (2013–2015) and CA'MOMI (2015–2017), and the ANR HAPAR project (2014-2019). This research was carried out within the framework of K. Klouch's PhD and funded by Ifremer and Region Bretagne (Allocation de REcherche Doctorale (ARED) fellowship).

Conflict of interest. None declared.

REFERENCES

Abdenadher M, Hamza A, Fekih W *et al.* Factors determining the dynamics of toxic blooms of *Alexandrium minutum* during a 10-year study along the shallow southwestern Mediterranean coasts. *Estuar Coast Shelf S* 2012;**106**:102–11.

Anderson DM, Keafer BA, Kleindinst JL *et al. Alexandrium fundyense* cysts in the Gulf of Maine: long-term time series of abundance and distribution, and linkages to past and future blooms. *Deep-Sea Res Pt II* 2014;**103**:6–26.

Anderson-Carpenter LL, McLachlan JS, Jackson ST *et al.* Ancient DNA from lake sediments: bridging the gap between paleoecology and genetics. *BMC Evol Biol* 2011;**11**:30.

Andresen CS, Schmidt S, Seidenkrantz M-S *et al.* A 100-year record of changes in water renewal rate in Sermilik Fjord and its influence on calving of Helheim Glacier, Southeast Greenland. *Cont Shelf Res* 2014;**85**:21–9.

Arya M, Shergill IS, Williamson M *et al.* Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn* 2005;**5**:1–11.

Avşar U, Hubert-Ferrari A, De Batist M *et al.* Sedimentary records of past earthquakes in Boraboy Lake during the last ca 600 years (North Anatolian Fault, Turkey). *Palaeogeogr Palaeocl* 2015;**433**:1–9.

Bazzoni AM, Caddeo T, Pulina S *et al.* Spatial distribution and multiannual trends of potentially toxic microalgae in shellfish farms along the Sardinian coast (NW Mediterranean Sea). *Environ Monit Assess* 2015;**187**:86.

Belin C. Distribution of *Dinophysis* spp. and *Alexandrium minutum* along French coasts since 1984 and their DSP and PSP toxicity levels. In: Smayda TJ, Shimizu Y (eds). *Toxic Phytoplankton Blooms in the Sea*. Amsterdam: Elsevier, 1993, 469–74.

Bissett A, Gibson JAE, Jarman SN *et al.* Isolation, amplification, and identification of ancient copepod DNA from lake sediments. *Limnol Oceanogr-Meth* 2005;**3**:533–42.

Blanco EP, Lewis J, Aldridge J. The germination characteristics of *Alexandrium minutum* (Dinophyceae), a toxic dinoflagellate from the Fal estuary (UK). *Harmful Algae* 2009;**8**:518–22.

Boere A, Rijpstra WIC, De Lange GJ *et al.* Preservation potential of ancient plankton DNA in Pleistocene marine sediments. *Geobiology* 2011;**9**:377–93.

Capo E, Debroas D, Arnaud F *et al.* Is planktonic diversity well recorded in sedimentary DNA? Toward the reconstruction of past protistan diversity. *Microb Ecol* 2015;**70**:865–75.

Chang FH, Garthwaite I, Anderson DM *et al.* Immunofluorescent detection of a PSP-producing dinoflagellate, *Alexandrium minutum*, from Bay of Plenty, New Zealand. *New Zeal J Mar Fresh* 1999;**33**:533–43.

Chapelle A, Le Gac M, Labry C *et al.* The Bay of Brest (France), a new risky site for toxic *Alexandrium minutum* blooms and PSP shellfish contamination. *Harmful Algae News* 2015;**51**:4–5.

Coolen MJL, Orsi WD, Balkema C *et al.* Evolution of the plankton paleome in the Black Sea from the Deglacial to Anthropocene. *P Natl Acad Sci USA* 2013;**110**:8609–14.

Coolen MJL, Overmann J. 217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. *Environ Microbiol* 2007;**9**:238–49.

Corinaldesi C, Barucca M, Luna GM *et al.* Preservation, origin and genetic imprint of extracellular DNA in permanently anoxic deep-sea sediments. *Mol Ecol* 2011;**20**:642–54.

d'Abbadie M, Hofreiter M, Vaisman A *et al.* Molecular breeding of polymerases for amplification of ancient DNA. *Nat Biotechnol* 2007;**25**:939–43.

de Vernal A, Eynaud F, Henry M *et al.* Reconstruction of sea-surface conditions at middle to high latitudes of the Northern Hemisphere during the Last Glacial Maximum (LGM) based on dinoflagellate cyst assemblages. *Quaternary Sci Rev* 2005;**24**:897–924.

Dell'Anno A, Danovaro R. Extracellular DNA plays a key role in deep-sea ecosystem functioning. *Science* 2005;**309**:2179.

Dia A, Guillou L, Mauger S *et al.* Spatiotemporal changes in the genetic diversity of harmful algal blooms caused by the toxic dinoflagellate *Alexandrium minutum*. *Mol Ecol* 2014;**23**:549–60.

Domaizon I, Savichtcheva O, Debroas D *et al.* DNA from lake sediments reveals the long-term dynamics and diversity of *Synechococcus* assemblages. *Biogeosciences* 2013;**10**:3817–38.

Durantou L, Rochon A, Ledu D *et al.* Quantitative reconstruction of sea-surface conditions over the last ~150 years in the Beaufort Sea based on dinoflagellate cyst assemblages: the role of large-scale atmospheric circulation patterns. *Biogeosciences* 2012;**9**:5391–406.

Elbrachter M. Exotic flagellates of coastal North Sea waters. *Helgoländer Meeresun* 1999;**52**:235–42.

Ellegaard M, Clarke AL, Reuss N *et al.* Multi-proxy evidence of long-term changes in ecosystem structure in a Danish marine estuary, linked to increased nutrient loading. *Estuar Coast Shelf S* 2006;**68**:567–78.

Ellegaard M, Figueroa RL, Versteegh GJM. Dinoflagellate life cycles, strategy and diversity: key foci for future research. In: Lewis JM, Marret F, Bradley L (eds). *Biological and Geological Perspectives of Dinoflagellates*. London: The Micropalaeontological Society, Special Publications, Geological Society, 2013, 249–62.

Ellegaard M, Ribeiro S, Lundholm N *et al.* Using the sediment archive of living dinoflagellate cysts and other protist resting stages to study temporal population dynamics. In: Lewis JM, Marret F, Bradley L (eds). *Biological and Geological Perspectives of Dinoflagellates*. London: The Micropalaeontological Society, Special Publications, Geological Society, 2013, 149–53.

Epp LS, Stoof KR, Trauth MH *et al.* Historical genetics on a sediment core from a Kenyan lake, intraspecific genotype turnover in a tropical rotifer is related to past environmental changes. *J Paleolimnol* 2010;**43**:939–54.

Erdner DL, Percy L, Keafer B *et al.* A quantitative real-time PCR assay for the identification and enumeration of Alexandrium cysts in marine sediments. *Deep-Sea Res Pt II* 2010;**57**:279–87.

Fitzpatrick E, Caron DA, Schnetzer A. Development and environmental application of a genus-specific quantitative PCR approach for *Pseudo-nitzschia* species. *Mar Biol* 2010;**157**:1161–9.

Franco JM, Fernandez P, Reguera B. Toxin profiles of natural populations and cultures of *Alexandrium minutum* Halim from Galician (Spain) coastal waters. *J Appl Phycol* 1994;**6**:275–9.

Galluzzi L, Penna A, Bertozzini E *et al.* Development of a Real-Time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). *Appl Environ Microb* 2004;**70**:1199–206.

Garneau MÈ, Schnetzer A, Countway PD *et al.* Examination of the seasonal dynamics of the toxic dinoflagellate *Alexandrium catenella* at Redondo Beach, California, by quantitative PCR. *Appl Environ Microb* 2011;**77**:7669–80.

Gilbert MTP, Bandelt H-J, Hofreiter M *et al.* Assessing ancient DNA studies. *Trends Ecol Evol* 2005;**20**:541–4.

Godhe A, Asplund ME, Härnström K *et al.* Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. *Appl Environ Microbiol* 2008;**74**:7174–82.

Godhe A, Otta SK, Rehnstam-Holm AS *et al.* Polymerase chain reaction in detection of *Gymnodinium mikimotoi* and *Alexandrium minutum* in field samples from southwest India. *Mar Biotechnol* 2001;**3**:152–62.

Godhe A, Rehnstam-Holm A-S, Karunasagar I *et al.* PCR detection of dinoflagellate cysts in field sediment samples from tropic and temperate environments. *Harmful Algae* 2002;**1**:361–73.

Gómez F. Phytoplankton invasions: comments on the validity of categorizing the non-indigenous dinoflagellates and diatoms in European seas. *Mar Pollut Bull* 2008;**56**:620–8.

Gray M, Wawrik B, Paul J *et al.* Molecular detection and quantitation of the red tide Dinoflagellate *Karenia brevis* in the marine environment. *Appl Environ Microb* 2003;**69**:5726–30.

Gross J. Re-occurrence of red tide in Cork Harbor, Ireland. *Red Tide Newsl* 1989;**2**:4–5.

Gu H, Sun J, Kooistra WHCF *et al.* Phylogenetic position and morphology of thecae and cysts of *Scrippsiella* (Dinophyceae) species in the East China Sea. *J Phycol* 2008;**44**:478–94.

Guillaud JF, Bouriel L. Relation concentration-débit et évolution temporelle du nitrate dans 25 rivières de la région Bretagne (France). *Rev Sci Eau* 2007;**20**:213–26.

Halim Y. *Alexandrium minutum*, dinoflagellé provocant des "eaux rouges". *Vie Milieu* 1960;**11**:102–5.

Hallegraeff G, Steffensen DA, Wetherbee R. Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. *J Plankton Res* 1988;**10**:533–41.

Hansen G, Daugbjerg N, Franco J. Morphology, toxin composition and LSU rDNA phylogeny of *Alexandrium minutum* (Dinophyceae) from Denmark, with some morphological observations on other European strains. *Harmful Algae* 2003;**2**:317–35.

Härnström K, Ellegaard M, Andersen TJ *et al.* Hundred years of genetic structure in a sediment revived diatom population. *P Natl Acad Sci USA* 2011;**108**:4252–7.

Hernández-Fariñas T, Soudant D, Barillé L *et al.* Temporal changes in the phytoplankton community along the French coast of the eastern English Channel and the southern Bight of the North Sea. *ICES J Mar Sci* 2014;**71**:821–33.

Honsell G. First report of *Alexandrium minutum* in northern Adriatic waters (Mediterranean Sea). In: Smayda TJ, Shimizu Y (eds). *Toxic Phytoplankton Blooms in the Sea*. Amsterdam: Elsevier, 1993, 127–32.

Hou W, Dong H, Li G *et al.* Identification of photosynthetic plankton communities using sedimentary ancient DNA and their response to late-Holocene climate change on the Tibetan plateau. *Sci Rep* 2014;**4**:6648.

Hwang DF, Lu YH. Influence of environmental and nutritional factors on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. *Toxicon* 2000;**38**:1491–503.

John U, Litaker RW, Montresor M *et al.* Formal revision of the *Alexandrium tamarense* species complex (Dinophyceae) taxonomy: the introduction of five species with emphasis on molecular-based (rDNA) classification. *Protist* 2014;**165**:779–804.

Kamikawa R, Nagai S, Hosoi-Tanabe S *et al.* Application of real-time PCR assay for detection and quantification of *Alexandrium tamarense* and *Alexandrium catenella* cysts from marine sediments. *Harmful Algae* 2007;**6**:413–20.

Katsanevakis S, Wallentinus I, Zenetos, A *et al.* Impacts of invasive alien marine species on ecosystem services and biodiversity: a pan-European review. *Aquat Invas* 2014;**9**:391–423.

Keller MD, Selvin RC, Claus W *et al.* Media for the culture of oceanic ultraphytoplankton. *J Phycol* 1987;**23**:633–8.

Kremp A, Anderson DM. Factors regulating germination of resting cysts of the spring bloom dinoflagellate *Scrippsiella hangoei* from the northern Baltic Sea. *J Plankton Res* 2000;**22**:1311–27.

Laird KR, Fritz SC, Maasch KA *et al.* Greater drought intensity and frequency before AD 1200 in the Northern Great Plains, USA. *Nature* 1996;**384**:552–5.

Lejzerowicz F, Esling P, Majewski W *et al.* Ancient DNA complements microfossil record in deep-sea subsurface sediments. *Biol Letters* 2013;**9**:20130283.

Lewis J, Harris ASD, Jones KJ *et al.* Long-term survival of marine planktonic diatoms and dinoflagellates in stored sediment samples. *J Plankton Res* 1999;**21**:343–54.

Lilly EL, Halanych KM, Anderson DM. Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group. *Harmful Algae* 2005;**4**:1004–20.

Liu D, Shen X, Di B *et al.* Palaeoecological analysis of phytoplankton regime shifts in response to coastal eutrophication. *Mar Ecol-Prog Ser* 2013;**475**:1–14.

Lundholm N, Clarke AM, Ellegaard M *et al.* Documenting historical trends in toxic algae: A 100 year-record of changing *Pseudo-nitzschia* species in a sill-fjord related to nutrient loading and temperature. *Harmful Algae* 2010;**9**:449–57.

Lundholm N, Ribeiro S, Andersen TJ *et al.* Buried alive – germination of up to a century-old marine protist resting stages. *Phycologia* 2011;**50**:629–40.

Martin JL, LeGresley MM, Hanke AR. Thirty years – *Alexandrium fundyense* cyst, bloom dynamics and shellfish toxicity in the Bay of Fundy, eastern Canada. *Deep-Sea Res Pt II* 2014;**103**:27–39.

Martínez de la Escalera G, Antoniades D, Bonilla S *et al.* Application of ancient DNA to the reconstruction of past microbial assemblages and for the detection of toxic cyanobacteria in subtropical freshwater ecosystems. *Mol Ecol* 2014;**23**:5791–802.

McCauley LaR, Erdner DL, Nagai S *et al.* Biogeographic analysis of the globally distributed harmful algal bloom species *Alexandrium minutum* (Dinophyceae) based on rRNA gene sequences and microsatellite markers. *J Phycol* 2009;**45**:454–63.

McQuoid MR, Godhe A, Nordberg K. Viability of phytoplankton resting stages in the sediments of a coastal Swedish fjord. *Eur J Phycol* 2002;**37**:191–201.

Mertens KN, Bradley LR, Takano Y *et al.* Quantitative estimation of Holocene surface salinity variation in the Black Sea using dinoflagellate cyst process length. *Quaternary Sci Rev* 2012;**39**:45–59.

Mertens KN, González C, Delusina I *et al.* 30 000 years of productivity and salinity variations in the late Quaternary Cariaco Basin revealed by dinoflagellate cysts. *Boreas* 2009;**38**:647–62.

Miyazono A, Nagai S, Kudo I *et al.* Viability of *Alexandrium tamarense* cysts in the sediment of Funka Bay, Hokkaido, Japan: over a hundred year survival times for cysts. *Harmful Algae* 2012;**16**:81–8.

Mizushima K, Matsuoka K. Vertical distribution and germination ability of *Alexandrium* spp. cysts (Dinophyceae) in the sediments collected from Kure Bay of the Seto Inland Sea, Japan. *Phycol Res* 2004;**52**:408–13.

Mousing EA, Andersen TJ, Ellegaard M. Changes in the abundance and species composition of phytoplankton in the last 150 years in the southern Black Sea. *Estuaries Coasts* 2013;**36**:1206–18.

Murray S, Wiese M, Stüken A *et al.* sxtA-based quantitative molecular assay to identify saxitoxin-producing harmful algal blooms in marine waters. *Appl Environ Microb* 2011;**77**:7050–7.

Nehring S. Non-indigenous phytoplankton species in the North Sea: supposed region of origin and possible transport vector. *Arch Fish Mar Res* 1998;**46**:181–94.

Pal S, Gregory-Eaves I, Pick FR. Temporal trends in cyanobacteria revealed through DNA and pigment analyses of temperate lake sediment cores. *J Paleolimnol* 2015;**54**:87–101.

Panieri G, Lugli S, Manzi V *et al.* Ribosomal RNA gene fragments from fossilized cyanobacteria identified in primary gypsum from the late Miocene, Italy. *Geobiology* 2010;**8**:101–11.

Penna A, Battocchi C, Garcés E *et al.* Detection of microalgal resting cysts in European coastal sediments using a PCR-based assay. *Deep-Sea Res Pt II* 2010;**57**:288–300.

Perini F, Casabianca A, Battocchi C *et al.* New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. *PloS One* 2011:**6**:1–9.

Persson A, Godhe A, Karlson B. Dinoflagellate cysts in recent sediments from the west coast of Sweden. *Bot Mar* 2000;**43**:69–79.

Pietramellara G, Ascher J, Borgogni F *et al.* Extracellular DNA in soil and sediment: fate and ecological relevance. *Biol Fert Soils* 2009;**45**:219–35.

Pospelova V, Price AM, Pedersen TF. Palynological evidence for late Quaternary climate and marine primary productivity changes along the California margin. *Paleoceanography* 2015;**30**:1–18.

Probert I, Siano R, Poirier C *et al.* *Brandtodinium* gen. nov. and *B. nutricula* comb. Nov. (Dinophyceae), a dinoflagellate commonly found in symbiosis with polycystine radiolarians. *J Phycol* 2014;**50**:388–99.

R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2015. https://www.R-project.org/ (12 May 2016, date last accessed).

Rawlence NJ, Lowe DJ, Wood JR *et al.* Using palaeoenvironmental DNA to reconstruct past environments: progress and prospects. *J Quaternary Sci* 2014;**29**:610–26.

Ribeiro S, Amorim A, Andersen TJ. Reconstructing the history of an invasion: the toxic phytoplankton species *Gymnodinium catenatum* in the Northeast Atlantic. *Biol Invas* 2011;**14**:969–85.

Ribeiro S, Terje B, Lundholm N *et al.* Hundred years of environmental change and phytoplankton ecophysiological variability archived in coastal sediments. *PloS One* 2013;**8**:1–8.

Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds). *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Totowa, NJ, USA: Humana Press, 2000, 365–86.

Saari HK, Schmidt S, Castaing P *et al.* The particulate $^{7}Be/^{210}Pb_{xs}$ and $^{234}Th/^{210}Pb_{xs}$ activity ratios as tracers for tidal-to-seasonal particle dynamics in the Gironde estuary (France): Implications for the budget of particle-associated contaminants. *Sci Total Environ* 2010;**408**:4784–94.

Schmidt S, De Deckker P. Present-day sedimentation rates on the southern and southeastern Australian continental margins. *Aust J Earth Sci* 2015;**62**:143–50.

Schmidt S, Jouanneau J-M, Weber O *et al.* Sedimentary processes in the Thau Lagoon (South France): from seasonal to century time scales. *Estuar Coast Shelf S* 2007;**72**:534–42.

Shuler AJ, Paternoster J, Brim M *et al.* Spatial and temporal trends of the toxic diatom *Pseudo-nitzschia* in the Southeastern Atlantic United States. *Harmful Algae* 2012;**17**:6–13.

Stoof-Leichsenring KR, Epp LS, Trauth MH *et al.* Hidden diversity in diatoms of Kenyan Lake Naivasha: a genetic approach detects temporal variation. *Mol Ecol* 2012;**21**:1918–30.

Taylor WA. *Change-Point Analysis: A Powerful New Tool for Detecting Changes*. 2000. http://www.variation.com/cpa/tech/changepoint.html (12 May 2016, date last accessed).

Theroux S, D'Andrea WJ, Toney J *et al.* Phylogenetic diversity and evolutionary relatedness of alkenone-producing haptophyte algae in lakes, implications for continental paleotemperature reconstructions. *Earth Planet Sc Lett* 2010;**300**:311–20.

Touzet N, Keady E, Raine R *et al.* Evaluation of taxa-specific real-time PCR, whole-cell FISH and morphotaxonomy analyses for the detection and quantification of the toxic microalgae *Alexandrium minutum* (Dinophyceae), Global Clade ribotype. *FEMS Microbiol Ecol* 2009;**67**:329–41.

Touzet N, Lacaze J, Maher M *et al.* Summer dynamics of *Alexandrium ostenfeldii* (Dinophyceae) and spirolide toxins in Cork Harbour, Ireland. *Mar Ecol-Prog Ser* 2011;**425**:21–33.

Usup G, Pin LC, Ahmad A. *Alexandrium* (Dinophyceae) species in Malaysian waters. *Harmful Algae* 2002;**1**:265–75.

Verleye TG, Mertens K, Louwye S *et al.* Holocene salinity changes in the southwestern Black Sea: a reconstruction based on dinoflagellate cysts. *Palynology* 2009;**33**:77–100.

Weckström J, Korhola A, Erästö P *et al.* Temperature patterns over the past eight centuries in Northern Fennoscandia inferred from sedimentary diatoms. *Quaternary Res* 2006;**66**:78–86.

Yu S-Y, Berglund BE. A dinoflagellate cyst record of Holocene climate and hydrological changes along the southeastern Swedish Baltic coast. *Quaternary Res* 2007;**67**:215–24.

Yue S, Pilon P, Cavadias G. Power of the Mann-Kendall and Spearman's rho tests for detecting monotonic trends in hydrological series. *J Hydrol* 2002;**259**:254–71.

Zonneveld KAF, Chen L, Elshanawany R *et al.* The use of dinoflagellate cysts to separate human-induced from natural variability in the trophic state of the Po River discharge plume over the last two centuries. *Mar Pollut Bull* 2012;**64**:114–32.

ANNEXE 4.

Intra-interspecific variability in S*crippsiella* species dynamics.

Given the germination success of the *Scrippsiella* spp. species from the EE core, the temporal dynamics of *Scrippsiella trochoidea* clade STR1*, Scrippsiella trochoidea* clade STR2, *and Scrippsiella donghaienis* were analyzed by real-time PCR from ancient DNA extracts of the Elorn sediment layers in the frame of the Master 2 internship of Z.N. Qui-Minet that I supervised with R. Siano in 2014 (Qui-Minet 2014). Beyond the study of the temporal dynamic of the selected taxa, this study was carried out to compare the *A. minutum* multiannual dynamics to other dinoflagellate species, in order to evaluate its specificity. Interestingly, *S. trochoidea* STR1 showed a peak in DNA copies at 1995 ± 1, while *S. trochoidea* STR2 and *S. donghaienis* were not abundant, revealing patterns of intra- and interspecific variability in the dynamics of the three species (Figure A4). These results were only partially exploited in the frame of my PhD thesis. Only the dynamics of *S. donghaienis* was exploited for comparison with the *A. minutum* dynamics in the chapter 2 and in Klouch *et al*., (2016), given the high specifity of the real-time PCR primers used and the higher efficiency of the real time PCR assays.

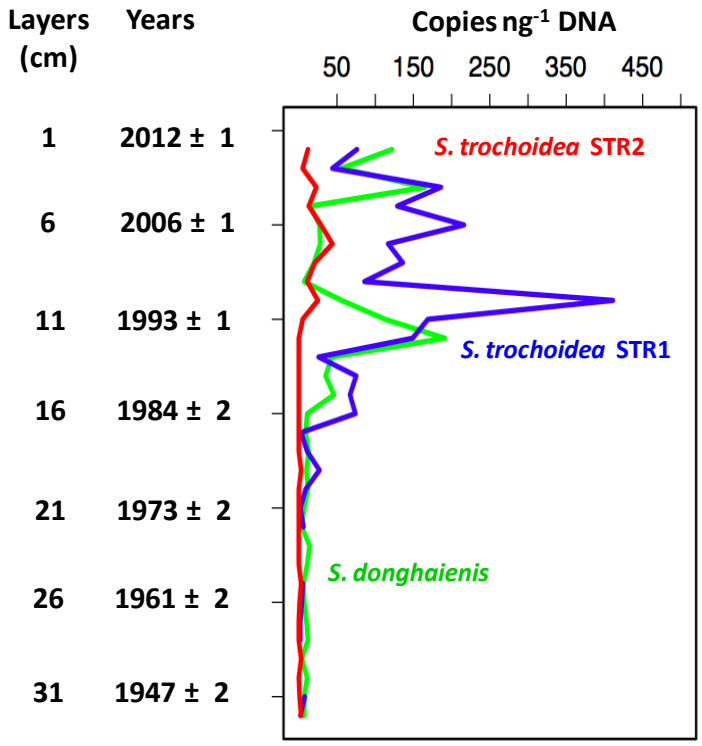


Figure A4. Dynamics of *Scrippsiella* species in the Elorn Estuary (EE) obtained by real-time PCR.

ANNEXE 5.

Poster- Cross approaches of molecular and micropaleontological signals to infer the anthropogenic and climatic impacts in the Bay of Brest.

In the frame of my PhD thesis I had the chance to collaborate with palynologists (Aurélie Penaud (researcher) and Clément Lambert (PhD)) from the IUEM institute (Brest). This collaboration was in the frame of the EC2CO project CA'MOMI (2014-2015) and was intended to compare our paleogenetic data with other paleoecological indicators, such as pollens and dinoflagellate cysts. The aim was to cross paleogenetic and micropaleontological data to infer the anthropogenic and climatic impacts in the coastal environment of the Bay of Brest.

This work has been performed on the Daoulas Estuary sediment core (DE). Different biological fossils (pollen, dinoflagellate cysts, benthic foraminifera shells, maërl algae) have been analyzed to infer terrestrial landscapes, and hydrological conditions of the sea (temperature, salinity, nutrient concentration) and compared to our DNA-based data (*A. minutum* multidecadal dynamic inferred by real-time PCR). Palynological results revealed an increase in the abundances of the Aulne tree pollens (*Aulnus*) from the 70'. Moreover, increasing abundances of the pollen of some tree taxa were correlated with the AMO (Atlantic Multidecadal Oscillation) index, indicating a strong climatic imprint on the trees' pollinisation. This increase is similar with the change in protist community (chapter 1) and in the *A. minutum* dynamics (chapter 2) revealed by my paleogenetic analyses. This multiproxy approaches suggest a potential link between different biological variables (tree pollens and marine dinoflagellates) and climate change which will be deepened in the next future and will be the object of new research article. The first results of the CA'MOMI project have been presented in the poster presented at the EC2CO congress held in Paris on the 1st Juin 2016 (Figure A5).

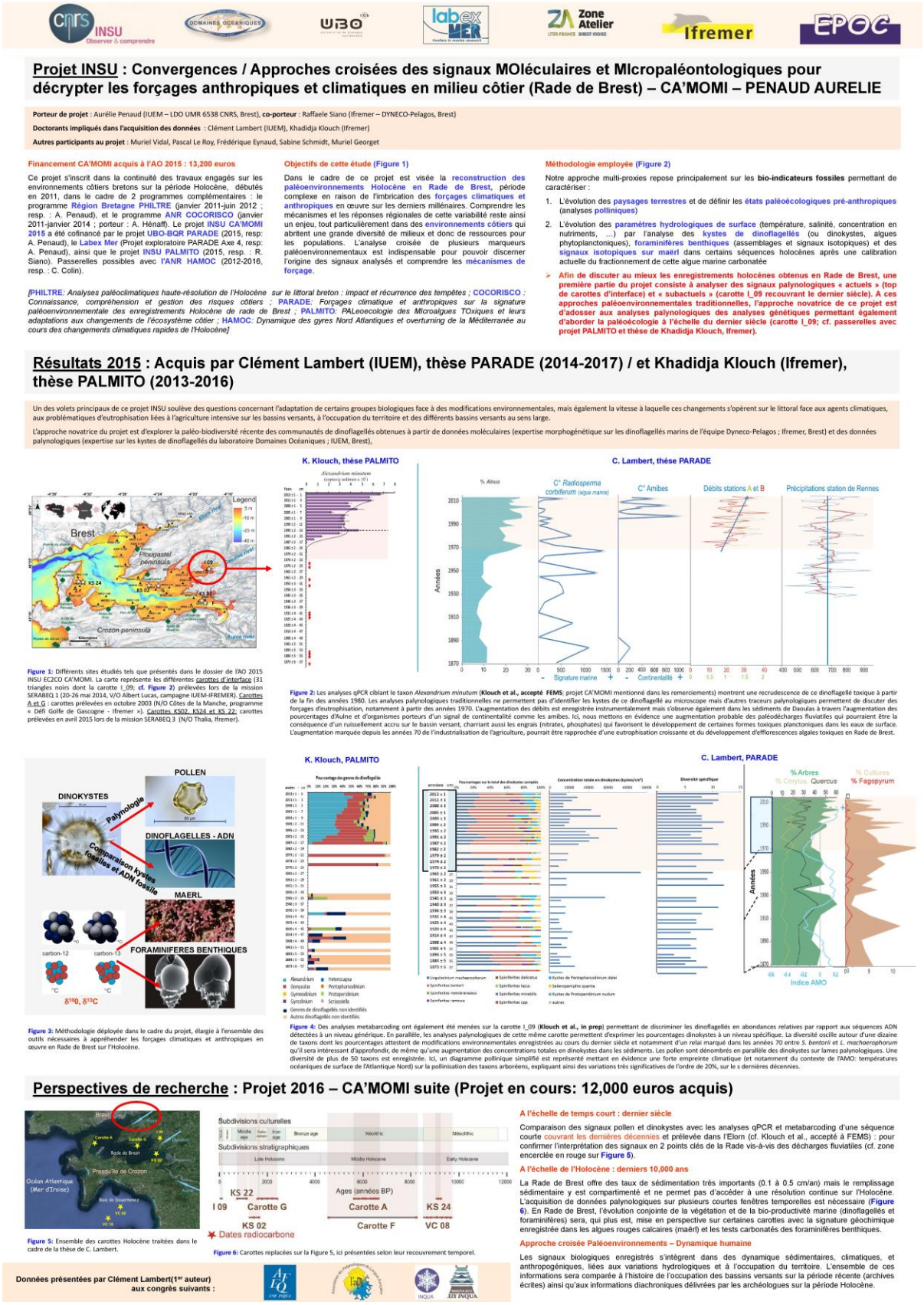


Figure A5. Poster- Cross approaches of molecular and micropaleontological signals to infer the anthropogenic and climatic impacts in the Bay of Brest.

Protist paleoecology from biological archives of the marine coastal ecosystems

Abstract

Protists are important contributors to the biogeochemical cycles of the marine environment. The composition of their communities and their temporal dynamics are traditionally studied by analyzing data sets acquired in the frame of monitoring/observation networks of plankton, whose implementation is however relatively recent (≤40 years). In this study, we analyzed the biological traces (resting stages and ancient DNA) preserved in sediments covering a time scale of 150 years in order to study changes in the composition and the temporal dynamics of marine protists, focusing mainly on two estuarine ecosystems of the Bay of Brest (Brittany, France). These changes were studied at three levels of biological complexity: communities, species and populations. The diversity of protists and their preservation potential in sediments over the time were evaluated using a metabarcoding approach (barcode: V4 region of the 18S rDNA) on the DNA extracted from ancient sediments. The results showed that only a minor part (16-18%) of the protists richness (#OTUs) of superficial sediments is retrieved in deep sediments, and that most of the protists found in ancient sediments are known to produce resting stages. Two main paleocommunities were differentiated (before/after 1950), suggesting the existence of a distinct and specific biodiversity for the identified periods. The relative abundances of dinoflagellates showed a decreasing trend since the 70s' and *Alexandrium* and *Gonyaulax* genera showed an opposite dynamic in terms of relative abundance over the time. These changes in the community composition are probably the result of the hydrological changes occurred in the Bay of Brest during the last century. The importance of the genus *Alexandrium* unveiled by the metabarcoding analysis, encourgaged the study of the multidecadal dynamics of the species *Alexandrium minutum*. This species is classified as invasive in Europe and gained attention in the Bay of Brest after the toxic bloom events occurred since 2012. Paleogenetic data (real-time PCR) suggest that *A. minutum* is present in the Bay of Brest since at least 1873 ± 7 and that, across a time scale of about 150 years, the species has proliferated only recently in the estuaries of the bay. This recent proliferation of *A. minutum* in the estuaries questioned about the current distribution of the species in the whole Bay of Brest. The analysis of *A. minutum* DNA traces and the cysts germination experiments performed from superficial sediments samples in thirty stations sampled during two consecutive winters (December 2013 and January 2015) revealed that the species currently occupies the whole area of the Bay of Brest and is potentially able to germinate in all the studied sites. Real-time PCR data suggest that the south-eastern part of the bay, where muddy sediment are more abundant, is potentially more favorable for the accumulation of the species cysts. This heterogeneity of the spatial distribution of the species in the sediments was also found in water. The Lagrangian model simulations of particle trajectories showed a disconnection between the estuaries of the Bay of Brest and suggest the presence of potential barriers for *A. minutum* dispersal in the water. In order to explain the reasons of the recent proliferation of *A. minutum* in the Brittany estuaries, we compared the physiological performances of *A. minutum* and *Scrippsiella donghaienis* (non-toxic dinoflagellate) strains. These strains were obtained from the germination of their cysts accumulated in sediments at different periods of time. Mimicking the historical changes of Nitrogen/Phosphorus ratio (N/P) occured in the Brittany estuaries during the last century, the growth rate, phosphorus assimilation rate, and the maximal biomass attained by the different strains were compared in culture media with different N/P ratios (25 and 100). The results showed a strong phenotypic intraspecific variability for both species and for both analyzed media. The results of this thesis work, discussed in the context of the changes of protist communities over the time, contribute to the research in sedimentary paleoecology, showing the advantages and limits of this approach to reveal still underexplored biological patterns.

Keywords: Paleoecology; Coastal ecosystems; Protists; Dinoflagellates; Harmful Algal Blooms (HABs); Resting stages; Ancient DNA; Plankton; Molecular ecology; Metabarcoding; Real-time PCR.

Paléoécologie des protistes à partir d'archives biologiques provenant d'écosystèmes marins côtiers

Résumé

Les protistes sont d'importants contributeurs aux cycles biogéochimiques de l'environnement marin. La composition de leurs communautés et leur dynamique temporelle sont traditionnellement étudiées en analysant des séries de données acquises dans le cadre de réseaux de surveillance/observation du plancton dont la mise en place est relativement récente (≤40 ans). Dans cette étude, nous avons analysé les traces biologiques (formes de résistance et ADN ancien) préservés dans des sédiments couvrant une échelle temporelle d'environ 150 ans afin d'étudier les changements de la composition et la dynamique temporelle des protistes marins, principalement dans deux écosystèmes estuariens de la rade de Brest (Bretagne, France). Ces changements ont été étudiés à trois niveaux de complexité biologique: communautés, espèces et populations. La diversité des protistes et leur potentiel de préservation dans les sédiments au fil du temps ont été évalués à travers l'utilisation d'une approche de *metabarcoding* (*barcode*: région V4 du 18S ADNr) sur l'ADN extrait à partir de sédiments anciens. Les résultats montrent que seulement une partie minoritaire (16-18%) de la richesse des protistes (#OTUs) des sédiments superficiels est retrouvée dans les sédiments profonds et que la plupart des protistes présents dans les sédiments anciens sont connus pour être capables de produire des formes de résistance. La composition des communautés de protistes étaient différenciées en deux principales paléocommunautés (avant/après 1950), suggérant une biodiversité spécifique à chaque période. Les abondances relatives des dinoflagellés ont montré une tendance à la baisse depuis les années 70' et les genres *Alexandrium* et *Gonyaulax* ont montré une dynamique opposée en termes d'abondance relative à travers le temps. Ces changements de communautés sont probablement la conséquence des variations hydrologiques documentées en rade de Brest au cours du dernier siècle. La prise de conscience de l'importance du genre *Alexandrium*, dévoilée par l'étude de *metabarcoding*, a conduit à l'étude de la dynamique multidécennale de l'espèce *Alexandrium minutum*. Cette dernière est classifiée comme invasive en Europe et a attiré l'attention en rade de Brest suite aux évènements d'efflorescences toxiques apparus depuis 2012. Les données paléogénétiques (PCR en temps réel) suggèrent qu'*A. minutum* est présente dans la rade depuis au moins 1873 ± 7 et qu'au cours des derniers 150 ans, l'espèce est devenue envahissante, proliférant dans les estuaires de la rade seulement ces dernières années. La mise en évidence de la prolifération récente d'*A. minutum* dans les estuaires de la rade de Brest nous a amené à nous interroger sur la distribution actuelle de l'espèce dans l'ensemble de cet écosystème. L'analyse des traces d'ADN d'*A. minutum* et des expériences de germination des kystes effectuées sur des sédiments superficiels de trente sites échantillonnés pendant deux hivers consécutifs (décembre 2013 et janvier 2015) a révélé que l'espèce occupe et qu'elle est potentiellement capable de germer dans tous les sites étudiés. Les données de PCR en temps réel suggèrent que la partie sud-est de la rade, où des sédiments à typologie vaseuse sont plus abondants, est potentiellement plus favorable à l'accumulation des kystes de l'espèce. Cette hétérogénéité de la distribution spatiale dans le sédiment a également été retrouvée dans l'eau. La simulation par modèle lagrangien des trajectoires de particules physiques montre une déconnexion entre les estuaires de la rade et suggère la présence de barrière de dispersions des cellules d'*A. minutum* dans l'eau. Pour essayer d'expliquer les raisons des proliférations récentes d'*A. minutum* dans les écosystèmes bretons, nous avons comparé les performances physiologiques de souches d'*A. minutum* et *Scrippsiella donghaienis* (dinoflagellé non toxique). Ces souches ont été obtenues à partir de la germination des kystes accumulés dans les sédiments d'époques différentes. En mimant les changements historiques des rapports Azote/Phosphore (N/P) qui ont eu lieu dans les écosystèmes bretons au cours du dernier siècle, les taux de croissance et de consommation de phosphore, et la biomasse maximale atteinte par les différentes souches ont été mesurés dans des milieux de cultures ayant un rapport N/P différent (25 et 100). Les résultats montrent une forte variabilité phénotypique intraspécifique au sein des deux espèces et dans les deux milieux étudiés. Les résultats de ces travaux de thèse, discutés dans un contexte de changements des communautés de protistes à travers le temps, contribuent au domaine de la recherche en paléoécologie sédimentaire, montrant les avantages et les limites de cette approche pour révéler des patrons biologiques encore peu explorés.

Mots-clés : Paléoécologie ; Ecosystèmes côtiers ; Protistes ; Dinoflagellés ; Efflorescences d'algues toxiques (HABs) ; formes de résistance ; ADN ancien ; Plancton ; Ecologie moléculaire ; Metabarcoding ; PCR en temps réel.