VILLE FRANCHE SUR MER

*REPUBLIQUE FRANCAISE*
*DEPARTEMENT DES ALPES-MARITIMES* *ARRONDISSEMENT DE NICE*

Extrait du registre des délibérations du Conseil Municipal
**REUNION DU CONSEIL MUNICIPAL DU 23 JUILLET 2014 À 17 HEURES**

L'an deux mille quatorze, le vingt-trois juillet le Conseil Municipal de cette Commune, régulièrement convoqué le dix-sept juillet, s'est réuni au nombre prescrit par la loi, sous la présidence de Monsieur Christophe TROJANI, Maire

- **Étaient Présents :** Monsieur André BEZZINA, Madame Catherine BARRAJA, Madame Joëlle BRAVETTI, Monsieur Jean-Paul GEAY, Madame Pasquale HATTEMBERG, Monsieur Jean-Louis ZAMBERNARDI, Madame Juliana CHICHMANIAN, Monsieur Jean-Louis BAUCHET, Madame Anne RAINAUD, Monsieur André BIANCHERI, Madame Monique LAUGIER, Monsieur Joseph COSENTINO, Madame Christiane FROUTE, Madame Marie ADAMO-BRONSONE, Monsieur Régis BELLI, Madame Claudine KHOKHLOV, Monsieur Jean-François GIAUME, Madame Isabelle PALAZZOLI, Monsieur Florian VIALLA, Madame Gisèle AMEDEO , Monsieur Bernard REBUFFEL, Monsieur Jean-Pierre MANGIAPAN, Madame Christine PETRUCCELLI, Madame Patricia DEGUS, Monsieur Richard CONTE, Madame Marie-Paule ZANOTTI

**Absents avec procurations :**

- Monsieur Robert BOJANOVICH donne procuration à Monsieur le Maire
- Monsieur Cédric CIRASA donne procuration à Monsieur Richard CONTE

Monsieur Florian VIALLA est élu secrétaire de séance

**6/ OBJET : SURVEILLANCE DE LA QUALITÉ DES EAUX DE BAIGNADE-RECHERCHE ET SUIVI DU DÉVELOPPEMENT DES DINOFLAGELLES TOXIQUES DU GENRE OSTRÉOPSIS AU NIVEAU DU LITTORAL DE LA COMMUNE DE VILLEFRANCHE-SUR-MER – POURSUITE DES ÉTUDES – DEMANDE DE SUBVENTION AU CONSEIL GÉNÉRAL DES ALPES-MARITIMES**

**Madame Anne RAINAUD, Conseillère Municipale, expose à ses collègues :**

Mes Chers Collègues,

Lors de sa séance du 27 juin 2013, le Conseil Municipal avait accepté le principe d'un suivi du développement des dinoflagellés toxiques du genre ostréopsis, au niveau des plages de Villefranche-sur-Mer.

Il avait décidé de confier cette étude au laboratoire océanologique de Villefranche-sur-Mer (LOV, CNRS, UPMC / UMR 7093).

Le financement du Conseil Général des Alpes-Maritimes a été obtenu à hauteur de 50% du montant des études.

Un rapport final a été établi par Monsieur Rodolphe LEMÉE, du Laboratoire océanologique de Villefranche.

Les conclusions de ce rapport préconisent la poursuite des analyses durant l'été 2014.

Elle leur propose :

- de bien vouloir accepter le principe d'un suivi du développement des dinoflagellés toxiques du genre ostréopsis au niveau des plages de Villefranche-sur-Mer durant l'été 2014 ;
- De solliciter une subvention du Conseil Général des Alpes-Maritimes à hauteur de 50% du montant prévisionnel, qui s'élève à 12.000€ TTC
- de confier cette étude au laboratoire océanologique de Villefranche-sur-Mer (LOV/CNRS/UPMC/IMR 7093)
- d'autoriser Monsieur le Maire à signer la convention à intervenir avec l'UMPC / LOV et le Conseil Général des Alpes-Maritimes, dont le projet était joint en de l'ordre du jour
- d'approuver le plan de financement suivant :
    - Montant de la dépense : 12.000 euros
    - Participation du Conseil Général des Alpes-Maritimes : 6.000 euros
    - Participation communale : 6.000 euros

Le montant de la dépense sera inscrit au Budget 2014 au compte 6042-414

Le rapport 2013 du L.O.V était également joint en annexe de l'ordre du jour.

**LE CONSEIL MUNICIPAL**
**Après en avoir délibéré à l'unanimité**
**ADOPTE**

Le Maire,

Christophe TROJANI

La présente délibération est susceptible d'être contestée dans un délai de deux mois à compter de sa date d'exécution :
- soit en exerçant un recours administratif (gracieux ou hiérarchique)
- soit en exerçant un recours contentieux devant les juridictions administratives

# Surveillance de la qualité des eaux de baignade : recherche et suivi du développement des dinoflagellés toxiques du genre *Ostreopsis* au niveau du littoral de Villefranche-sur-Mer. Eté 2013

**Rodolphe Lemée**

Laboratoire d'Océanographie de Villefranche, CNRS UMR 7093,
Observatoire Océanologique de Villefranche-sur-Mer
Université Pierre et Marie Curie
Contact : lemee@obs-vlfr.fr

Un grand merci aux étudiantes du LOV de l'été 2013 : Kraïma Narjis, Marne Segond et Adeline Williams (Licence 3, Université de Nice Sophia Antipolis).

Sommaire

**Titre** : Surveillance de la qualité des eaux de baignade : recherche et suivi du développement des dinoflagellés toxiques du genre *Ostreopsis* au niveau du littoral de Villefranche-sur-Mer. Eté 2013

**Etat de l'art** : Le changement climatique à l'échelle planétaire et ses répercussions aux échelles locales n'ont pas seulement des effets sur la température moyenne de l'air et de l'eau, ou sur la fréquence et l'intensité des évènements météorologiques remarquables. Tous ces paramètres peuvent influencer les aires de répartition de nombreuses espèces animales et végétales. Il est possible, par exemple, de voir proliférer sous des latitudes tempérées des organismes parasites ou toxiques qui étaient en limite de leurs aires de répartition. C'est peut-être le cas de certaines espèces du genre *Ostreopsis* (dinoflagellés benthiques), souvent associées aux espèces toxiques du genre *Gambierdiscus*, dont le développement est généralement limité aux zones tropicales. Ces espèces vivent généralement entre 35° N et 35° S de latitude, à l'exception de *Ostreopsis siamensis* et *Ostreopsis ovata* qui ont été inventoriées relativement récemment en Méditerranée. Des espèces du genre *Ostreopsis* se développent donc en Méditerranée depuis plusieurs dizaines d'années, mais leurs proliférations, impliquant des effets néfastes aux niveaux écologiques, sanitaires et socio-économiques, sont beaucoup plus récentes et limitées pour l'instant au bassin occidental et à l'Adriatique.

Le genre *Ostreopsis* est connu sous les tropiques pour être à l'origine d'intoxications alimentaires (souvent mortelles) suite à l'accumulation de la palytoxine et de ses dérivés dans des crabes, des oursins ou des poissons. La palytoxine est une macromolécule (2680 Daltons) hydrosoluble complexe polyhydroxylée dont la structure chimique a été caractérisée dans les années 1980. Quelle que soit son origine, il existe très peu de différences entre les structures chimiques de la palytoxine et ses analogues. La palytoxicose est caractérisée par les symptômes d'intoxication suivants : hypersalivation, crampes abdominales, nausée, forte diarrhée, paresthésie des extrémités, spasmes musculaires et difficultés respiratoires suivi de décès dans les cas les plus graves. La forte toxicité de la palytoxine chez les mammifères en fait une des substances d'origine marine les plus toxiques connues. A titre d'exemple, la dose létale 50 (DL50) de la palytoxine chez la souris par voie intrapéritonéale est de 0,75 µg/kg. Au niveau cellulaire, la palytoxine provoque un large éventail d'effets pharmacologiques qui incluent : un efflux de potassium et un influx de sodium ; une dépolarisation des membranes excitables et l'activation secondaire des canaux calcium ; une augmentation et une mobilisation du calcium intracellulaire ; l'activation de l'échangeur sodium-calcium ; une contraction des muscles striés, lisses et cardiaques et une augmentation de la libération de neuromédiateurs au niveau des terminaisons nerveuses.

En Méditerranée, la concentration de toxines dans la chaîne alimentaire suite à des proliférations d'*Ostreopsis* spp. a été suggérée dans des poissons et des mollusques (bivalves et gastéropodes) et confirmée par des études récentes. Une intoxication par contact direct est également possible. Les espèces du genre *Ostreopsis* se développent préférentiellement à très faible profondeur, sur d'autres végétaux (macroalgues ou phanérogames) ou directement sur le substrat. Lorsque les conditions sont favorables (période estivale), ces microalgues peuvent proliférer et se retrouver en suspension dans l'eau, formant parfois des agrégats relativement

importante. Suite à la lyse des cellules ou à une exsudation, les toxines se retrouvent dans l'eau de mer, dans les agrégats et même dans les aérosols dispersés par les vents. Les conséquences peuvent alors être néfastes, aussi bien pour la santé humaine que pour l'écosystème. Les conditions deviennent hypoxiques, voir anoxiques, avec une importante mortalité des invertébrés (principalement des oursins et des coquillages). Au niveau de la santé humaine, les réactions sont diverses : irritations cutanées, affections respiratoires, conjonctivites et fièvre. La toxine ou les cellules étant transportées par les aérosols marins, même les personnes n'étant pas en contact direct avec l'eau de mer peuvent être atteintes (en particulier les vacanciers sur les plages). A Gênes, durant l'été 2005, plusieurs dizaines de personnes ont ressenti ces différents symptômes et plusieurs dizaines d'entre elles ont été hospitalisées durant plusieurs jours. Les plages atteintes ont été interdites au public pendant plus d'une semaine, en plein été, avec des conséquences économiques certaines. En Espagne, plusieurs habitants d'un immeuble situé en bordure de plage ont été intoxiqués. En Algérie, plusieurs centaines de personnes ont été légèrement intoxiquées.

**Objectifs :** A la demande de la commune de Villefranche-sur-Mer et du Conseil Général des Alpes-Maritimes, un suivi du développement d'*Ostreopsis* cf. *ovata* a été prévu durant l'été 2013 dans la baie de Villefranche. Ce suivi, qui a fait l'objet d'une convention, présente un avantage très important : une analyse rapide (moins de 48h) permettant d'émettre un « Bulletin de surveillance » hebdomadaire envoyé par mail. Seule cette rapidité dans les analyses permet de faire un travail préventif efficace.

## Matériel et Méthodes

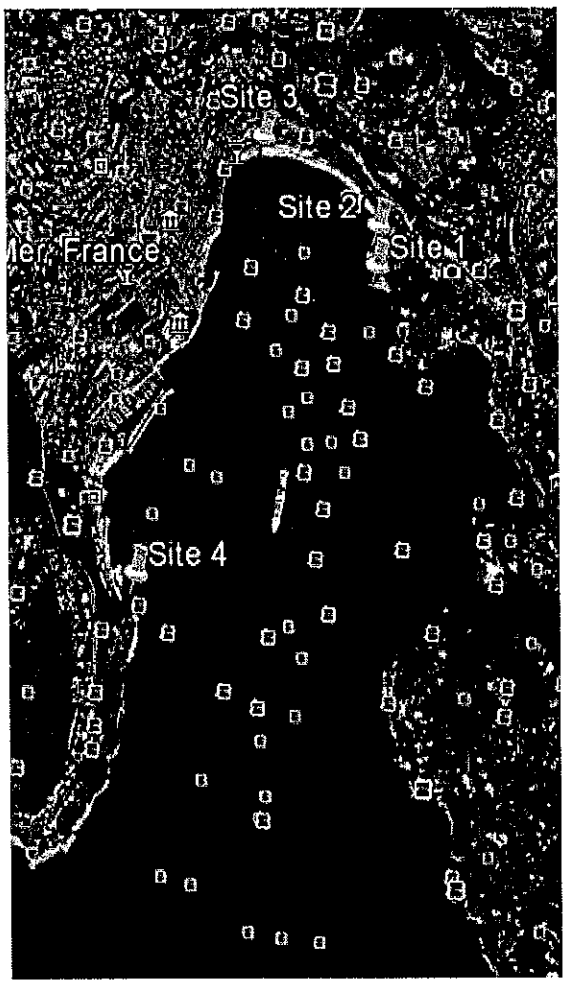
Stratégie d'échantillonnage : Nous avons réalisé des prélèvements une fois par semaine, entre **le 19 juin et 26 août 2013,** au niveau de la baie de Villefranche-sur-Mer. Quatre zones (sites 1, 2, 3 et 4) de prélèvements ont été définies (cf. Carte 1) pour ce suivi hebdomadaire. Au niveau du site 4, 3 sous-sites ont été analysés (4a, 4b et 4c), afin de mesurer la variabilité à petite échelle de l'abondance d'*Ostreopsis* cf. *ovata* (analyse du 19 juin au 21 août).

**Localisation des sites**
**Site 1** : plage des jeunes, côté nord de la digue rocheuse artificielle sud
**Site 2** : Marinières, côté ouest de la digue rocheuse artificielle à l'est du poste de secours
**Site 3** : Marinières, côté est des rochers au niveau des barrières d'entrée du parking
**Site 4** : Rochambeau, zone interne de la crique, juste en face de l'immeuble sud-est
**Sites 4a, 4b et 4c** : Au niveau du site 4, du sud (4a) au nord (4c). Distance entre les sites : environ 10 m.

Méthode de récolte : Au niveau de tous les sites, des prélèvements d'eau ont été effectués à 30 cm de profondeur et à 20 cm du substrat. Ils ont été utilisés pour évaluer le nombre de microalgues dans l'eau de mer (prélèvement dans des flacons en polypropylène de 250 ml). Un autre prélèvement a permis de récolter des macroalgues (à 50 cm de profondeur) et l'eau environnante afin de déterminer la quantité de microalgues épiphytes (flacons de 250 ml).

Comptage des microalgues dans l'eau de mer : Environ une heure après les prélèvements d'eau, nous avons ajouté 2 % de lugol acide pour fixer et préserver les échantillons. Ils ont ensuite été conservés à 4°C dans l'obscurité jusqu'à l'analyse. Pour évaluer le nombre de cellules de microalgues dans l'eau, nous avons utilisé la méthode d'Utermöhl en faisant sédimenter 50 ml de prélèvement. Les microalgues ont ensuite été déterminées et comptées à l'aide d'un microscope inversé (Axiovert 35).

Comptage des microalgues sur les macroalgues : Environ 30 minutes après les prélèvements des macroalgues avec l'eau environnante, nous avons ajouté 2 % de lugol acide afin de préserver les échantillons. De retour au laboratoire, nous avons vigoureusement agité les échantillons pour détacher les microalgues des macroalgues, puis nous avons tamisé ces échantillons sur un filtre de 500 µm de vide de maille en acier inoxydable. Les macroalgues ont ensuite été rincées 2 fois avec 100 ml d'eau de mer filtrée sur 0.2 µm (avec une agitation vigoureuse à chaque rinçage) afin de décrocher la totalité des microalgues. Les macroalgues (sur le tamis) ont alors été identifiées puis pesées (Poids Frais = PF). Elles ont ensuite été séchées 48 h à 60 °C dans une étuve afin de déterminer le Poids Sec (PS). Le filtrat (sous le filtre de 500 µm) est recueilli. Une fraction de ce dernier est observée au microscope (Alphaphot 2-YS2, Nikon) à l'aide de lames calibrées (1 ml ou 100 µl, en fonction de la concentration en cellules).




**Carte 1** : Sites de prélèvement d'*Ostreopsis* cf. *ovata* dans la baie de Villefranche-sur-Mer au cours de la saison estivale 2013. Carte Google Earth.

**Résultats et Discussion** : Un bilan des analyses hebdomadaires a été envoyé par mail (1) à la Mairie de Villefranche-sur-Mer et (2) au Conseil Général des Alpes-Maritimes, sous forme de « Bulletins de Surveillance ».

Evolution du nombre de dinoflagellés toxiques sur substrats rocheux et au niveau de la plage : Au niveau de tous les sites, nous avons prélevé les macroalgues majoritaires de chacun des sites (soit *Halopteris scoparia* (= *Stypocaulon scoparium*), soit *Padina pavonica* , soit des Dictyotales) pour suivre l'évolution d'*Ostreopsis* spp. sur la macroflore. Les figures 1 et 2 présentent l'évolution pendant l'été 2013 du nombre d'*Ostreopsis* spp. sur les macroalgues et dans l'eau pour les sites 1 à 4. Au niveau du site 4, c'est toujours le sous-site 4b qui est présenté dans ces premiers graphes.

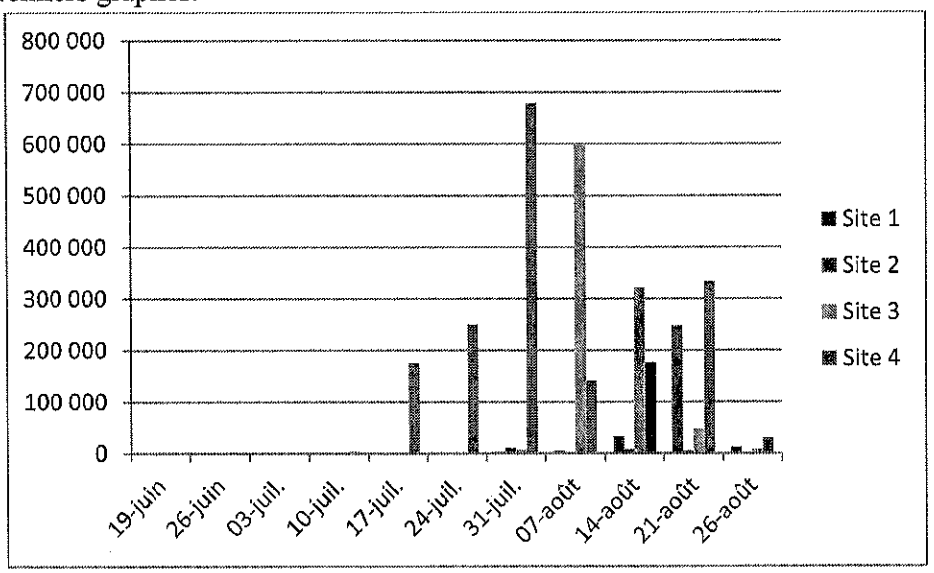



**Figure 1** : Evolution de la quantité d'*Ostreopsis* cf. *ovata* par gramme de Poids Frais de macroalgues au niveau des sites 1, 2, 3 et 4b dans la baie de Villefranche au cours de la saison estivale 2013.

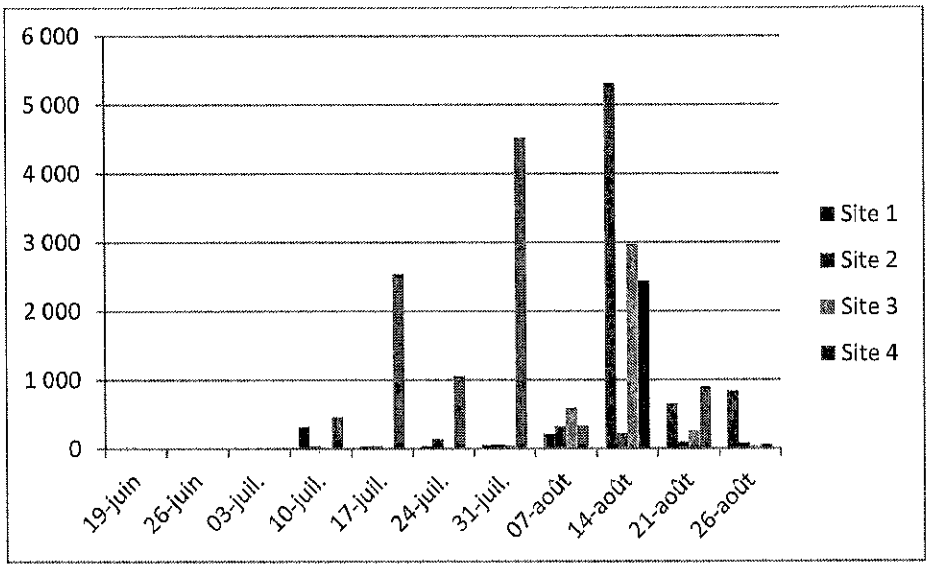



**Figure 2** : Evolution de la quantité d'*Ostreopsis* cf. *ovata* par litre d'eau de mer au niveau des sites 1, 2, 3 et 4b dans la baie de Villefranche au cours de la saison estivale 2013.

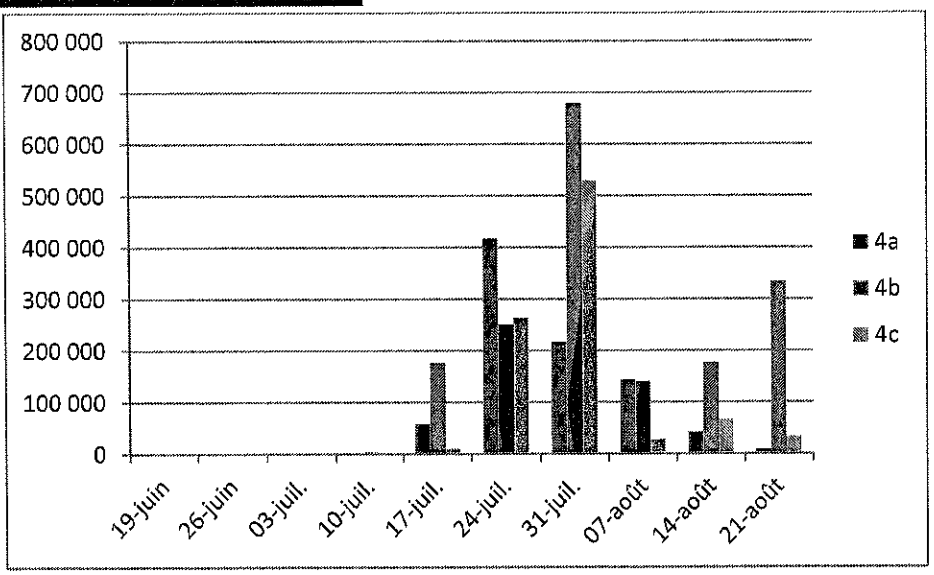



**Figure 3** : Quantité d'*Ostreopsis* cf. *ovata* par gramme de Poids Frais de macroalgues au niveau des sites 4a, 4b et 4c dans la baie de Villefranche durant l'été 2013.

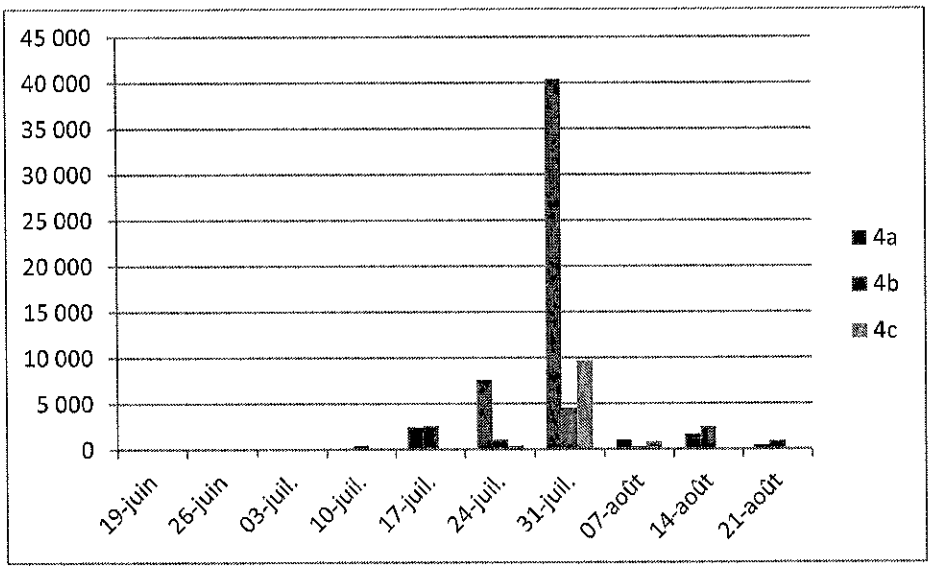



**Figure 4** : Quantité d'*Ostreopsis* cf. *ovata* par litre d'eau de mer au niveau des sites 4a, 4b et 4c dans la baie de Villefranche durant l'été 2013.

Comme durant l'été 2012, c'est le site 4 (Rochambeau) qui présente les plus fortes concentrations en *Ostreopsis* cf. *ovata*, dépassant légèrement les valeurs benthiques observées au site 3 (début de la plage des Marinières). De nombreuses fleurs d'eau ont été observées au site 4 début août (figure 5).

Contrairement à 2012, on commence à retrouver en 2013 des quantités non négligeables d'*Ostreopsis* à la Plage des Jeunes (site 1). Ce site était connu pour avoir de très fortes abondances de cette microalgue en 2008 et 2009, puis presque aucun développement de l'algue en 2012, lors des travaux de réaménagement (pas de suivi en 2010 et 2011).

Concernant la période du développement d'*Ostreopsis* cf. *ovata*, elle présente un léger décalage comparer à celle de 2012 (environ 1 semaine de retard), probablement en relation avec le printemps relativement froid dans notre région en 2013.

**Figure 5** : Fleurs d'eau à *Ostreopsis* cf. *ovata* observées au site 4 durant l'été 2013. On remarque également la présence de méduses (*Pelagia noctiluca*).

**Conclusion et recommandations pour les années futures :**

En France, durant l'été 2008, le seuil de pré-alerte était de 4 000 cellules d'*Ostreopsis* cf. *ovata* par litre d'eau de mer et le seuil d'alerte était de 30 000 cellules/litre (sources : ARS PACA). Depuis l'été 2009, les seuils officiels sont plus élevés : «pré-alerte» à 30 000 cellules/litre et «alerte» à 100 000 cellules/litre. Ces seuils n'ont pas évolué depuis 2009.

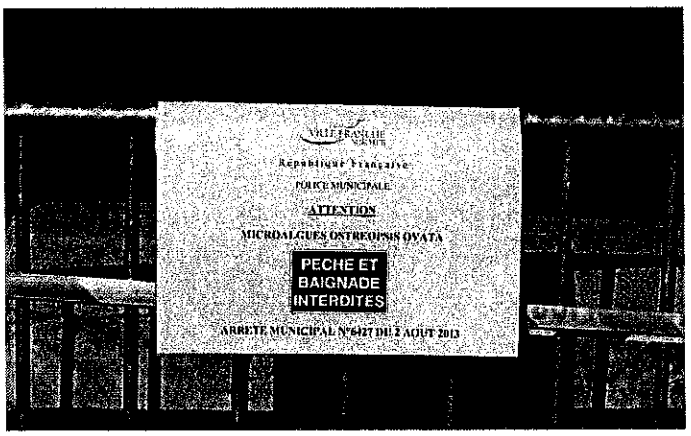



**Figure 6** : Affiche présentant l'interdiction de pêche et de baignade au site 4 durant l'été 2013.

un fort développement d'*Ostreopsis* cf. *ovata* dans la baie de Villefranche, aussi bien sur les macroalgues que dans l'eau. Les quantités de microalgues planctoniques ont dépassé le seuil d'alerte de 2008 et le seuil de pré-alerte de 2009 (ARS). Un arrêté municipal a été pris afin d'interdire la baignade et la pêche pendant une semaine, à partir du 2 août (figure 6) au niveau du site 4 (Rochambeau).

Les sites 3 et 4 sont toujours des sites à risque. Le site 1 (plage de jeunes) semble également redevenir une zone propice au développement de la microalgue toxique.

Enfin l'étude des 3 sous-sites (4a, 4b et 4c) montre bien la nécessité d'effectuer plus d'un prélèvement afin d'avoir une bonne estimation de la quantité d'*Ostreopsis* cf. *ovata* dans une zone.

Il est toujours très difficile de prévoir l'intensité et la période de prolifération d'*Ostreopsis* cf. *ovata*, en liaison avec le climat (à différentes échelles de temps). Par contre, se sont souvent les mêmes sites qui sont touchés d'une année sur l'autre. Nous conseillons de focaliser les efforts de surveillance sur ces zones sensibles, surtout lorsqu'elles sont très fréquentées et/ou bordés d'immeubles.

**Publications du responsable du projet, en relation avec le sujet.**
**Ces publications sont disponibles sur simple demande.**

Mangialajo L, Bertolotto R, Cattaneo Vietti R, Chiantore M, Grillo C, **Lemée R**, Melchiorre N, Moretto P, Povero P, Ruggieri N. (2008). The toxic benthic dinoflagellate *Ostreopsis ovata*: Quantification of proliferation along Genoa coastline (Italy, NW Mediterranean Sea) in the summer of 2006. Marine Pollution Bulletin, 56 (6): 1209-1214.

Tichadou L, Glaizal M, Armengaud A, Grossel H, **Lemée R**, Kantin R, Lasalle JL, Drouet G, Rambaud L, Malfait P, de Haro L. (2010). Health impact of unicellular algae of the *Ostreopsis* genus blooms in the Mediterranean Sea: experience of the French Mediterranean Coast Surveillance Network from 2006 to 2009. Clinical Toxicology, 48: 839-844.

Mangialajo L, Ganzin N, Accoroni S, Asnaghi V, Blanfuné A, Cabrini M, Cattaneo-Vietti R, Chavanon F, Chiantore M, Cohu S, Costa E, Fornasaro D, Grossel H, Marco-Mirailles F, Maso M, Rene A, Rossi AM, Sala MM, Thibaut T, Totti C, Vila M, **Lemee R.** (2011). Trends in *Ostreopsis* proliferation along the Northern Mediterranean coasts. Toxicon, 57(3): 408-420.

Cohu S, Thibaut T, Mangialajo L, Labat JP, Passafiume O, Blanfuné A, Simon N, Cottalorda JM & **Lemée R**. (2011). Occurence of the toxic dinoflagellate *Ostreopsis* cf. *ovata* in relation with environmental factors in Monaco (NW Mediterrean). Mar. Poll. Bull. 62 (12): 2681-2691.

Amzil Z, Sibat M, Chomerat N, Grossel H, Mirrales F, **Lemee R**, Nezan E & Sechet V. (2012). Ovatoxin-a and Palytoxin accumulation in seafood in relation with *Ostreopsis* cf. *ovata* blooms on the French Mediterranean coast. Mar. Drugs 10: 477-496.

Cohu S & **Lemée R.** (2012). Vertical distribution of the toxic epibenthic dinoflagellates *Ostreopsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis* in the NW Mediterranean Sea. Cahier de Biologie Marine 53: 373-380.

**Lemée R**, Mangialajo L, Cohu S, Amzil Z, Blanfuné A, Chomerat N, Ganzin N, Gasparini S, Grossel H, Guidi-Guilvard L, Hoareau L, le Duff F, Marro S, Simon N, Nezan E, Pedrotti ML, Sechet V, Soliveres O, Thibaut T (2012). Interactions between scientists, managers and policy makers in the framework of the French MediOS project on *Ostreopsis* (2008-2010). Cryptogamie Algologie, 33 (2): 137-142.

Guidi-Guilvard L, Gasparini S & **Lemée R.** (2012). The negative impact of *Ostreopsis* cf. *ovata* on phytal meiofauna from the coastal NW Mediterranean. Cryptogamie Algologie, 33 (2): 121-128.

Blanfuné A, Cohu S, Mangialajo L, **Lemée R** & Thibaut T (2012). Preliminary assessents of the impact of *Ostreopsis* cf. *ovata* (Dinophyceae) development on macroinvertebrates in the North Western Mediterranean Sea. Cryptogamie Algologie, 33 (2): 129-136.

Sechet V, Sibat M, Chomérat N, Nézan E, Grossel H, Lehebel-Peron JB, Jauffrais T, Ganzin N, Marci-Miralles F, **Lemée R** & Amzil Z (2012). *Ostreopsis* cf. *ovata* in the French Mediterranean coast: molecular characterization and toxin profile. Cryptogamie Algologie, 33 (2) : 89-98. 3)

L. (2012). Proceedings of the International Congress on *Ostreopsis* Development (ICOD, April 2011, France). Cryptogamie Algologie, 33 (2): 79-80.

ICOD (2012). Round Table 1 of the International. Conference of *Ostreopsis* development: Secondary metabolites and toxicity of *Ostreopsis*, Cryptogamie Algologie 33(2): 81-84.

ICOD (2012). Round Table 2 of the International Conference on *Ostreopsis* Development: Environmental, Health and Economic management, state of the art and perspectives, Cryptogamie Algologie, 33(2): 85-87.

Cohu S, Mangialajo L, Thibaut T, Blanfuné A, Marro S & **Lemée R.** (2013). Proliferation of the toxic dinoflagellates *Ostreopsis* cf. *ovata* in relation with depth, biotic substrate and environmental factors in NW Mediterranean Sea. Harmful Algae, 24: 32-44.

Biré R, Trotereau S, **Lemée R**, Delpont C, Chabot B, Aumond Y, Krys S. (2013). Occurrence of palytoxines in marine organisms from different trophic levels harvested on the French Mediterrean coast in 2009. Harmful Algae, 28: 10-22.

Les 2 dernières publications sont annexées à ce rapport.

Toutes les publications issues des communications orales ou affichées lors du congrès international ICOD de 2011 à Villefranche sont disponibles sur le site internet de l'Accord International RAMOGE (en bas de la page : http://www.ramoge.org/fr/ostreopsis_ovata.aspx).

Pour les gestionnaires du littoral, je recommande les publications suivantes :

ICOD (2012). Round Table 2 of the International Conference on *Ostreopsis* Development: Environmental, Health and Economic management, state of the art and perspectives, Cryptogamie Algologie, 33(2): 85-87.

et

Lemée R, Mangialajo L, Cohu S, Amzil Z, Blanfuné A, Chomerat N, Ganzin N, Gasparini S, Grossel H, Guidi-Guilvard L, Hoareau L, le Duff F, Marro S, Simon N, Nezan E, Pedrotti ML, Sechet V, Soliveres O, Thibaut T (2012). Interactions between scientists, managers and policy makers in the framework of the French MediOS project on *Ostreopsis* (2008-2010). Cryptogamie Algologie, 33 (2): 137-1

armful Algae 24 (2013) 32–44

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

# Proliferation of the toxic dinoflagellate *Ostreopsis* cf. *ovata* in relation to depth, biotic substrate and environmental factors in the North West Mediterranean Sea

S. Cohu [a,b,c], L. Mangialajo [c], T. Thibaut [c], A. Blanfuné [a,b,c], S. Marro [a,b], R. Lemée [a,b,*]

[a] Université Pierre et Marie Curie-Paris 6, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer cedex, France
[b] CNRS, Marine Microbial Ecology and Biogeochemistry, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer cedex, France
[c] Université Nice-Sophia Antipolis, Laboratoire Ecosystèmes Côtiers Marins et Réponse aux Stress, EA 4228, Parc Valrose, 06108 Nice Cedex2, France

ARTICLE INFO

*Article history:*
Received 10 December 2011
Received in revised form 11 January 2013
Accepted 11 January 2013

*Keywords:*
Dinoflagellate
HAB
Mediterranean Sea
*Ostreopsis*

ABSTRACT

In recent decades, the North West Mediterranean Sea has been seriously affected by the development of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata*, which is associated with harmful effects on human health and the environment. The present work aims to provide a large overview of the multiple environmental factors assumed to regulate or influence the growth of *Ostreopsis*. An intensive sampling campaign over a full annual cycle was performed along the French and Italian coasts (in six sites from Cassis to Genoa), to determine patterns of temporal and spatial distributions of both *O.* cf. *ovata* epiphytic and planktonic cells. Results highlighted substantial seasonal variations in the abundance of *Ostreopsis*. These variations correlated to seawater temperature, with an optimum growth temperature ranging from 23 °C to 27.5 °C. Phosphate concentration, rather than nitrogen or silicate, was also positively associated with *Ostreopsis*. Decreases in oxygen and increases in chlorophyll *a* concentrations were recorded during the summer blooming period. The maximal *Ostreopsis* epiphytic abundance was generally higher on *Dictyota* spp. than on the other two sampled macroalgae (up to $8.54 \times 10^6$ cells $g^{-1}$ FW), even though statistical analysis did not support a clear substrate preference. Epiphytic abundances were significantly higher at a very shallow depth (0.5 m), than at 1 and/or 3 m depths. High anthropogenic pressure (related to population density) seems to have promoted the occurrence of blooms in urbanized areas, which could partly explain the strong demarcation in *Ostreopsis* development between Western and Eastern sampling sites. The ecological niche of *Ostreopsis* cf. *ovata* needs precise definition, which will require further *in situ* and *in vitro* experimental studies, to determine the relative importance of distinct environmental parameters.



## 1. Introduction

Blooms of the dinoflagellate *Ostreopsis* J. Schmidt represent an increasing and worrying issue throughout the world. These harmful benthic microalgae grow in tropical and subtropical areas (Ballantine et al., 1988; Morton et al., 1992; Grzebyk et al., 1994; Faust, 1995; Faust et al., 1996; Parsons and Preskitt, 2007; Mattos Nascimento et al., 2008). However, in the last few decades the frequency of *Ostreopsis* proliferations has increased in temperate areas. Coastal zones of the Mediterranean Sea are particularly affected, with the highest abundance recorded in the North Western part (Mangialajo et al., 2008b, 2011; Totti et al., 2010; Cohu et al., 2011; Parsons et al., 2012). Blooms have also been reported in temperate areas of New Zealand (Chang et al., 2000; Rhodes et al., 2000; Shears and Ross, 2009, 2010), Tasmania and South Australia (Pearce et al., 2001; Rhodes et al., 2010), the islands of Kyushu, Shikoku and Okinawa in Japan (Taniyama et al., 2003; Rhodes, 2011) and Peter Great Bay in Eastern Russia (Selina and Orlova, 2010; Selina and Levchenko, 2011).

The proliferation of *Ostreopsis* is a complex phenomenon linked to many interacting factors. As is the case for other harmful algae, human activities might be involved. Indeed, various factors have possibly contributed to the expansion of HABs. These include: transport of resting cysts in ballast waters or on floating debris (Maso et al., 2003; Vila and Maso, 2005); coastal water eutrophication (Maso and Garcés, 2006); building harbors which increase water residence-time (Vila et al., 2001a; Vila and Maso, 2005); global climate change (Maso and Garcés, 2006; Miraglia et al., 2009).

*Ostreopsis* bloom events may have important environmental and health consequences. *Ostreopsis* species produce potent palytoxin and its derivatives (Onuma et al., 1999; Taniyama et al., 2003; Lenoir et al., 2004; Riobo et al., 2006), which are

* Corresponding author at: Université Pierre et Marie Curie-Paris 6, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer cedex, France.
*E-mail address:* lemee@obs-vlfr.fr (R. Lemée).

1568-9883/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.hal.2013.01.002

thought to be responsible for tropical Neurotoxic Shellfish Poisonings via the consumption of contaminated seafood (Ito et al., 1998; Yasumoto, 1998; Deeds and Schwartz, 2010). Around the Mediterranean Sea, blooms of *Ostreopsis* cf. *ovata* Fukuyo frequently cause cases of skin and eye irritations and less frequently respiratory distress (Simoni et al., 2003; Brescianini et al., 2006; Ciminiello et al., 2006; Barroso Garcia et al., 2008; Tichadou et al., 2010). *Ostreopsis* proliferation can also cause significant ecological damage: mass mortalities of invertebrates occurred during blooms in New Zealand (Shears and Ross, 2009, 2010), Brazil (Mattos Nascimento et al., 2008) and the Mediterranean Sea (Simoni et al., 2003; Totti et al., 2010).

The abundance of *Ostreopsis* shows high seasonal variation, mostly in temperate areas (Vila et al., 2001a,b; Simoni et al., 2003; Turki, 2005; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011) and an important spatial heterogeneity at different scales, *i.e.* from hundreds of meters (Okolodkov et al., 2007; Shears and Ross, 2009; Cohu et al., 2011) to several kilometers (Grzebyk et al., 1994; Chang et al., 2000; Aligizaki and Nikolaidis, 2006; Parsons and Preskitt, 2007). In the Mediterranean Sea, the seasonal trend of *Ostreopsis* spp. clearly follows the wide range in variations of temperature (Vila et al., 2001a,b; Aligizaki and Nikolaidis, 2006; Ungaro et al., 2010). However, the impact of temperature during the summer period seems less evident due to strong interactions with other factors. The marked heterogeneity in the distribution of *Ostreopsis* spp. among several substrates (*i.e.* macroalgae, sea grasses, dead biotic substrates, rock and sand) could partially explain the spatial variability of epibenthic cells (Grzebyk et al., 1994; Faust et al., 1996; Turki, 2005; Aligizaki and Nikolaidis, 2006; Parsons and Preskitt, 2007; Mangialajo et al., 2008b; Totti et al., 2010). Moreover, the 3-dimensional structure of macroalgae hosts and/or the production of secondary metabolites may affect *Ostreopsis* colonization (Parsons and Preskitt, 2007; Totti et al., 2010). Depth (as related to light intensity) could also strongly restrict *Ostreopsis* development, although most observations have been limited to a depth between 0.2 and 5 m (*e.g.* Aligizaki and Nikolaidis, 2006; Monti et al., 2007; Battocchi et al., 2010; Cohu et al., 2011). In addition, the distribution of *Ostreopsis* cells in deeper waters is unknown. The influence of nutrient availability has been poorly studied and contrasting results have been reported for tropical and temperate areas (Vila et al., 2001b; Parsons and Preskitt, 2007; Ungaro et al., 2010). Likewise, the relationships between the development of *Ostreopsis* and other parameters such as oxygen saturation and concentrations of algal pigments (*e.g.* chlorophyll *a* and pheophytin) are poorly understood. While several *in vitro* experiments have highlighted bacterial species associated with the toxicity of *Ostreopsis* spp. (Tosteson et al., 1989; Carballeira et al., 1998; Ashton et al., 2003; Pérez-Guzmán et al., 2008), the impact of *Ostreopsis* blooms on bacterial abundance in the natural environment has not been examined.

The present study aims to provide a base for our understanding of *Ostreopsis* ecology in the Mediterranean Sea. An intensive, wide-ranging sampling campaign documenting the development of *O.* cf. *ovata* in relation to a suite of environmental factors (substrate, depth, temperature, nutrients, oxygen, chlorophyll, pheophytin and bacterial abundance) was conducted over one year along the South-Eastern French and Ligurian coasts, from Cassis (France) to Genoa (Italy).

## 2. Materials and methods

### 2.1. Ostreopsis *cf.* ovata

From February 2008 to February 2009, sampling was conducted by snorkeling and diving in six sites along the Gulf of Lions and Ligurian coasts: Cassis, Ramatuelle, Saint-Raphaël, Nice, Villefranche-sur-Mer and Genoa (Fig. 1). Each site was sampled once per season in Winter, Spring and Fall, and four times in Summer (Table 1). For each site, the hierarchical sampling design described below was conducted at 0.5, 1 and 3 m depths in three randomly chosen sub-sites, nearly 10 m apart, in order to constitute triplicates.

Concentrations of planktonic *Ostreopsis* were determined from seawater samples collected in a 250 ml plastic flask, at about 20 cm above the rocky substrate. To determine epiphytic cell abundance, three macroalgae species were collected on rocky substrate: the bush-like Phaeophyceae *Halopteris scoparia* (Linnaeus) Sauvageau, the flat-ribbon Phaeophyceae *Dictyota* spp. (mostly *Dictyota dichotoma*) and the articulated corallinales (Rhodophyceae) *Corallina elongata* (Ellis & Solander) or rarely *Jania rubens* (Linnaeus). Each macroalga was carefully sampled in a 250 ml plastic flask with the surrounding seawater, avoiding any loss of

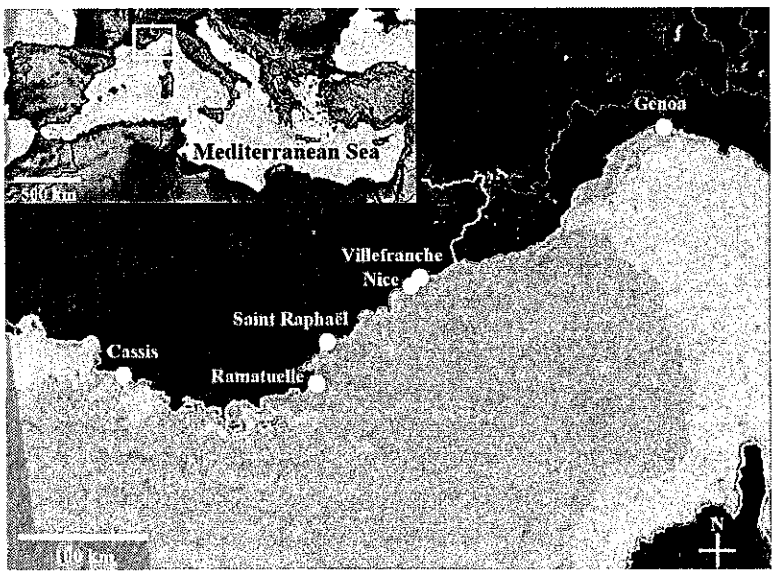



**Fig. 1.** The Mediterranean Sea showing the positions of the six sampled sites, Cassis, Ramatuelle, Saint Raphaël, Nice, Villefranche-sur-Mer and Genoa along the Gulf of Lions and Ligurian coasts.

**Table 1**
Characteristics of the sampled sites: dates of sampling, geographical coordinates, nature of substrate and hydrodynamic mode (+ = sheltered, ++ = moderate, +++ = exposed). The maximal epiphytic abundances and concentrations of *Ostreopsis* cf. *ovata* are indicated for each site with the depth, the substrate and the season at which they were measured.

| Time (code) | Cassis | Ramatuelle | Saint Raphaël | Nice | Villefranche-sur-Mer | Genoa |
|---|---|---|---|---|---|---|
| Winter (W) | 13th March 2008 | 4th March 2008 | 20th February 2008 | 2nd February 2008 | 14th February 2008 | 20th March |
| Spring (Sp) | 7th May 2008 | 25th April 2008 | 23th April 2008 | 18th April 2008 | 15th April 2008 | 14th May 2 |
| Summer1 (Su1) | 2nd July 2008 | 6th June 2008 | 9th June 2008 | 2nd June 2008 | 2nd June 2008 | 26th June 2 |
| Summer2 (Su2) | 24th July 2008 | 4th July 2008 | 16th July 2008 | 18th July 2008 | 7th July 2008 | 11th July 2 |
| Summer3 (Su3) | 6th August 2008 | 29th July 2008 | 29th July 2008 | 30th July 2008 | 30th July 2008 | 31st July 20 |
| Summer4 (Su4) | 22nd August 2008 | 25th August 2008 | 25th August 2008 | 27th August 2008 | 27th August 2008 | 19th Augus |
| Fall/Winter (F/W) | 5th December 2008 | 17th November 2008 | 3rd February 2009 | 16th January 2009 | 14th November 2008 | 17th Febru |
| GPS coordinates | 43°11.26′N | 43°11.26′N | 43°25.20′N | 43°41.45′N | 43°42.16′N | 44°23.29′N |
| | 5°32.11′E | 6°38.73′E | 6°51.55′E | 7°17.59′E | 7°19.19′E | 8°59.61′E |
| Nature of substrate | Artificial | Natural | Natural | Natural | Artificial | Natural |
| Hydrodynamic mode | +++ | + | ++ | +++ | + | ++ |
| Population density (pers/km$^2$) | 290 | 65 | 380 | 4795 | 1279 | 2496 |
| Max. epiphytic abundance (cells g$^{-1}$ FW) | $0.06 \times 10^6$ on *Dictyota* spp. | $0.01 \times 10^6$ on *Dictyota* spp. | $0.02 \times 10^6$ on *Corallina elongata* | $1.98 \times 10^6$ on *Dictyota* spp. | $8.54 \times 10^6$ on *Dictyota* spp. | $2.81 \times 10^6$ o *Dictyota* spp |
| Max. planktonic concentration (cells l$^{-1}$) | $1 \times 10^3$ | $3 \times 10^3$ | $0.4 \times 10^3$ | $12 \times 10^3$ | $7 \times 10^3$ | $68 \times 10^3$ |

microalgae as much as possible. Depending on their growth cycle and the environmental disturbance, some macroalgae species were missing in some sites/depths, particularly during winter, and therefore could not be collected. *Ostreopsis* cells were fixed by adding acidic Lugol at 1% (vol./vol.) in water samples and 2% (vol./vol.) in macroalgae samples. Concentrations of planktonic cells in the water samples were evaluated with an inverted microscope using the Utermöhl method (Utermöhl, 1958) and settling 50 ml of sea water. *Ostreopsis* concentrations were recorded as the number of cells per liter (cells l$^{-1}$). Macroalgae samples were vigorously shaken and passed through a 500 μm meshed filter to separate macroalgae and water containing microalgae. Macroalgae were rinsed twice with 100 ml of 0.2 μm filtered seawater to recover a maximum of microalgae, then dried on absorbent paper and weighed (±0.01 g). Epiphytic cell abundances in the rinsed water were determined in 1 ml volume Sedgwick Rafter cells using a standard light microscope and recorded as the number of cells per gram of fresh weight of macroalgae (cells g$^{-1}$ FW).

### 2.2. Measurement of environmental factors

#### 2.2.1. Temperature and oxygen

Seawater temperature (±0.1 °C) and oxygen saturation (±0.1%) were measured in each site at both 0.5 and 3 m depths during sampling sessions, using an oxymetric/temperature sensor (Oxy1971, WTX). The oxygen saturation is the ratio, expressed in percentage, between the measured oxygen concentration and the theoretical solubility calculated from the seawater temperature.

#### 2.2.2. Nutrient and pigments

In order to quantify nutrients and concentrations of chlorophyll *a* (Chl *a*) and pheophytin, samples of seawater were collected at 0.5 and 3 m depths in triplicate, following the same method used for planktonic cells. Samples intended for nutrient analysis were fixed with 0.06% of $HgCl_2$ solution and kept in the dark at 4 °C. Concentrations of nitrite, nitrate, phosphate and silicate were measured using an automatic analysis chain (EV2-Alliance Instrument) according to the methodology of Tréguer and Corre (1975). Samples for Chl *a* and pheophytin quantification were filtered (1 l per sample) on a Whatman GF/F. Filters were kept frozen (−20 °C) before extraction in a 90% acetone solution. Chl *a* and pheophytin concentrations were measured using fluorometry, according to the Lorenzen method (Lorenzen and Newton Downs, 1986).

#### 2.2.3. Bacteria

Bacterial abundance was determined from water samples collected at 0.5 and 3 m depths, in triplicate. Samples (1.5 ml) were fixed in glutaraldehyde (0.65% final concentration), kept in the dark for 15 min at ambient temperature, flash frozen in liquid nitrogen and stored at −80 °C. Concentrations of heterotrophic bacteria were determined by flow cytometry (Becton Dickinson, FACSCalibur) after staining with SYBRGreen I (Molecular Probes) as described by Gasol and Del Giorgio (2000). Fluorescent 1 μm latex beads ($10^5$ beads ml$^{-1}$) were systematically added to the bacterial samples as an internal quality standard (Polyscience Inc., Europe).

### 2.3. Statistical analyses

As data on the abundance of *Ostreopsis* did not show a normal distribution, the effects of factors such as site, time, substrate and depth on both epiphytic and planktonic abundances were tested by separate PERMANOVAs (Anderson, 2005), following the orthogonal design: site (3 levels, fixed), time (4 levels, fixed, crossed), substrate (3 levels, fixed, crossed), depth (3 levels, fixed, crossed), with $n = 3$ replicates per combination of factors. This statistical

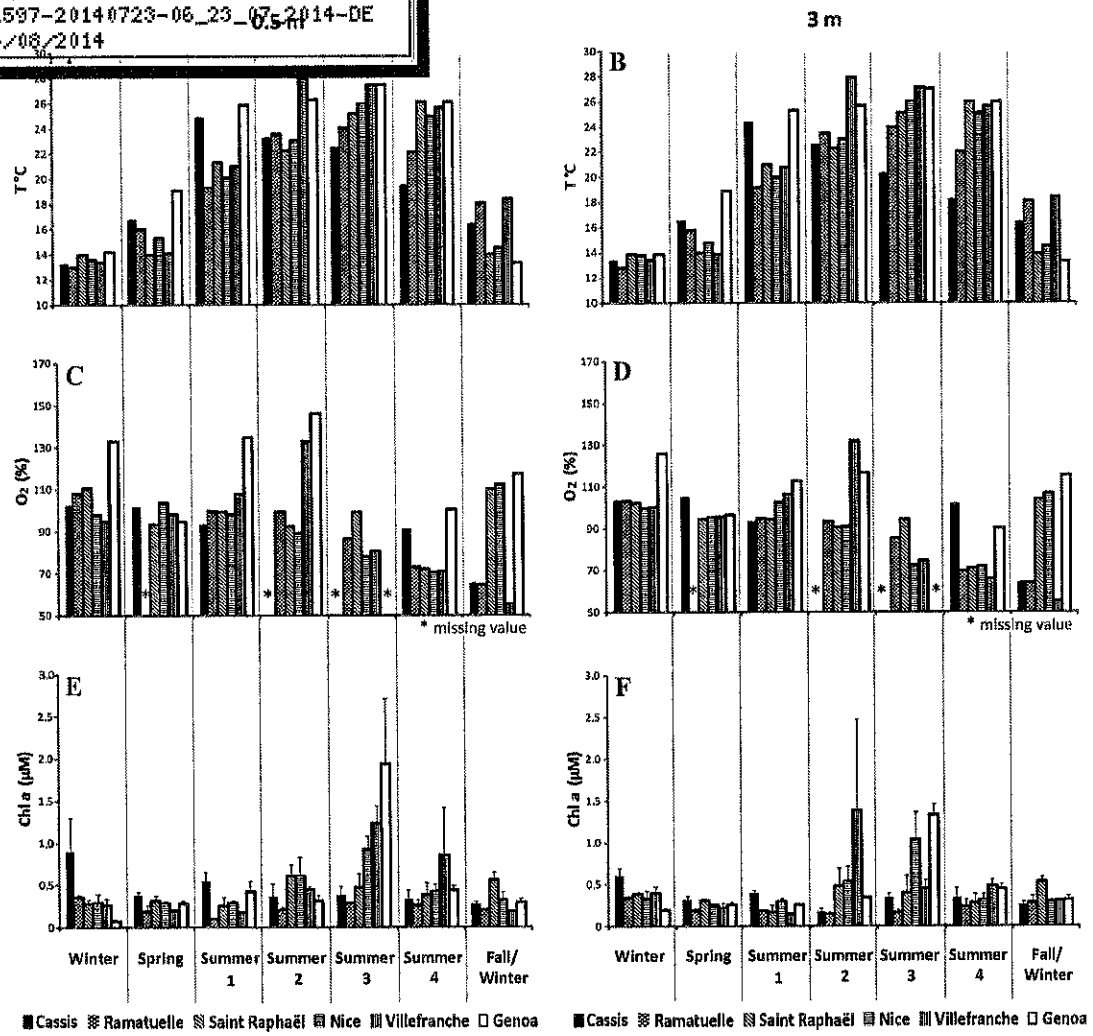



**Fig. 2.** Temperature (a and b), oxygen saturation (c and d) and mean chlorophyll a concentration ± SE (e and f) measured at 0.5 and 3 m depths in the six studied sites during the seven sampling periods.

design was restricted to the data collected in the eastern sites of Nice, Villefranche-sur-Mer and Genoa during the bloom summer period ("Summer1" to "Summer4") in order to avoid a bias in results induced by the frequent null abundances recorded during the other seasons and in the three western sites. PERMANOVAs were performed with 9999 random permutations, using the PERMANOVA+ program of PRIMER 6 (PRIMER-E Ltd.). Analyses were based on Euclidean distance-transformed data. Significant terms were examined individually using pair-wise tests.

The non-parametric Spearman correlation test (STATISTICA 7.0, Stat Soft, Inc.) was applied on the complete data set in order to highlight eventual relationships between *Ostreopsis* abundance and environmental parameters, as well as between the parameters themselves.

## 3. Results

### 3.1. Environmental characteristics of sites

All the sampled sites were natural rocky areas, except Cassis and Villefranche-sur-Mer which were composed of artificial rocky substrate (Table 1). Cassis and Nice sites were highly exposed to hydrodynamic conditions. The towns of Nice, Genoa and Villefranche-sur-Mer have the highest population densities respectively (data in Table 1 from the last 2008 French population census: http://www.recensement.insee.fr and from the Genoa town hall: http://www.comune-italia.it/comune-genova.html).

Trends of environmental parameters measured in the six sites at 0.5 and 3 m depths are reported in Figs. 2 and 3. Each environmental parameter showed approximately the same range of values at both sampled depths, except for nitrate and silicate concentrations which were consistently more abundant at the depth of 0.5 m than at 3 m, in Cassis.

The total temperature range encountered varied from 12.8 to 28 °C, in Ramatuelle and Villefranche-sur-Mer respectively (Fig. 2a and b). Oxygen saturation ranged from 55.2% in Villefranche-sur-Mer to 146.2% in Genoa (Fig. 2c and d). Chlorophyll *a* concentrations varied from 0.03 µM in Saint-Raphaël to 3.19 µM in Genoa (Fig. 2e and f). Concerning nutrients, Nitrite followed a typical summer depletion (Fig. 3a and b), varying from 0.01 µM in Ramatuelle to 0.35 µM in Genoa. Nitrate concentrations were often non-detectable in Genoa and Ramatuelle, and reached 20.55 µM in Cassis (Fig. 3c and d). Phosphate concentrations ranged between 0.01 µM in Villefranche-sur-Mer and 0.56 µM in Saint-Raphaël (Fig. 3e and f). Silicate varied between 0.59 and 25.08 µM, both values measured in Cassis (Fig. 3g and h). As a general rule, a longitudinal gradient of nutrient concentrations was not observed between the Eastern and Western sites. The total

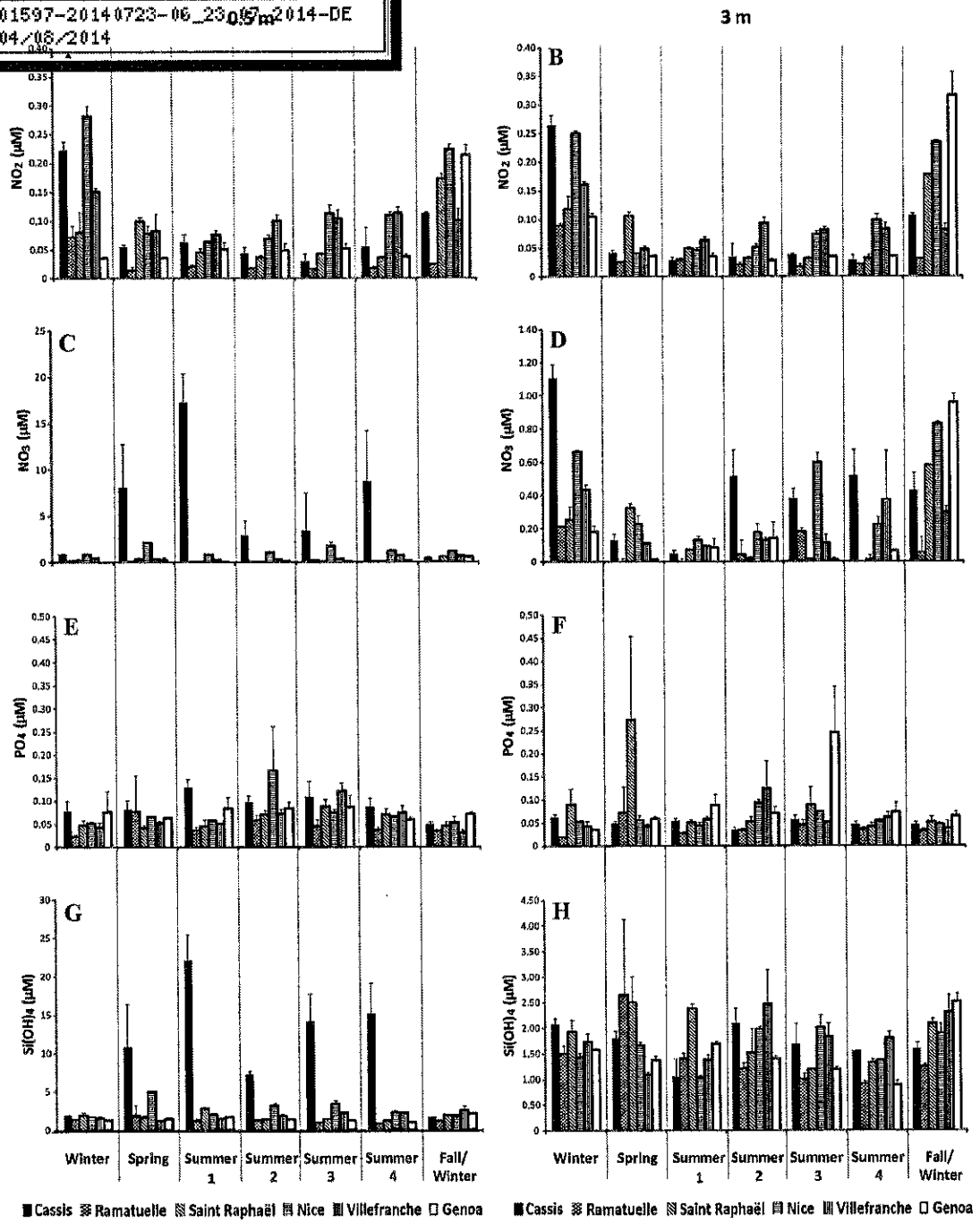



**Fig. 3.** Mean nutrient concentrations ± SE measured at 0.5 and 3 m depths in the six studied sites during the seven sampling periods: nitrites (a and b), nitrates (c and d), phosphates (e and f) and silicates (g and h).

bacterial abundance varied from $0.02 \times 10^6$ cells $l^{-1}$ in Nice to $2.33 \times 10^6$ cells $l^{-1}$ in Saint-Raphaël.

### 3.2. Temporal and spatial variation in Ostreopsis abundance

The presence of epiphytic *Ostreopsis* cf. *ovata* was observed for the first time on March 4th 2008 (Winter) at 0.5 m in Ramatuelle. Planktonic cells were detected later, on April 18th (Spring), at 1 m in Nice. The first epiphytic bloom was measured mainly on *Dictyota* spp. on July 11th in Genoa (Fig. 4, during Summer2, up to $1.32 \times 10^6$ cells $g^{-1}$ FW at 0.5 m), simultaneously with the first registered peak in planktonic concentrations (up to $8 \times 10^3$ cells $l^{-1}$ at 0.5 m). Subsequently, high population abundances of *Ostreopsis* were observed during the last two weeks of July, in the three eastern sites (Nice, Villefranche-sur-Mer and Genoa). The maximal epiphytic abundance ($8.54 \times 10^6$ cells $g^{-1}$ FW) was recorded at a depth of 1 m on *Dictyota* spp., on July 30th (Summer3) in Villefranche-sur-Mer. The maximum planktonic concentration ($68 \times 10^3$ cells $l^{-1}$) was recorded on July 31st, in Genoa at 0.5 m. From mid-August, epiphytic and planktonic abundances remained low or below the limit of detection. The presence of *Ostreopsis* cells in samples was detected up to December 5th (Fall/Winter) in Cassis. The abundance

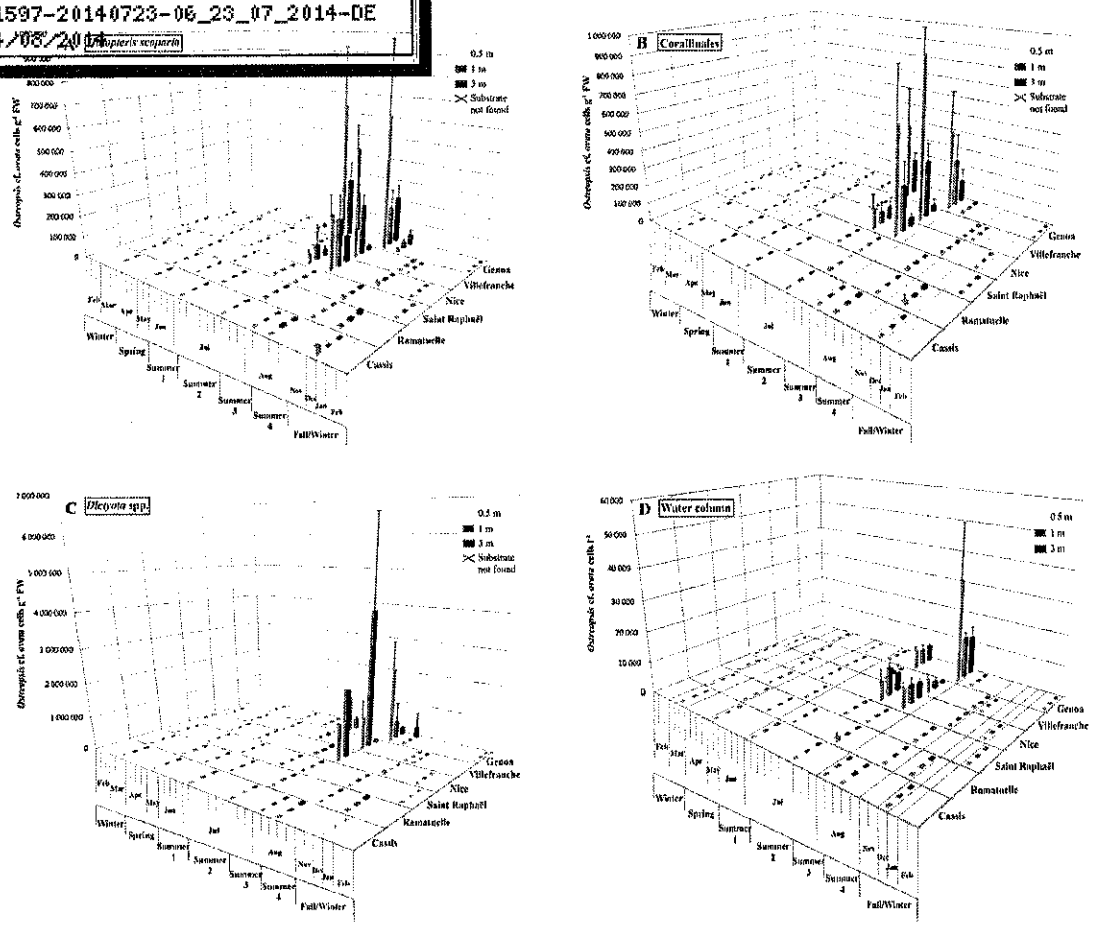



**Fig. 4.** Mean *Ostreopsis* epiphytic abundances ± SE measured on (a) *Halopteris scoparia* ($N = 360$), (b) *Corallinales* ($N = 366$), (c) *Dictyota* spp. ($N = 270$) and (d) mean *Ostreopsis* planktonic concentrations ± SE ($N = 378$) at 0.5, 1 and 3 m depths during the seven sampling periods. For clarity, the annual time scale is variable, each division on the *x*-axis corresponding to one sampling date.

of *Ostreopsis* showed a standard seasonal development, with the highest values found from June to August.

In addition to the temporal variation, a high spatial heterogeneity was clearly evident. Indeed, epiphytic and planktonic *Ostreopsis* abundances exhibited high spatial variation at a small scale within each site, as shown by the important standard errors (SE) related to the triplicates (for each substrate and depth, Fig. 4). In addition, a high heterogeneity was observed at a larger spatial scale between the sampled sites, mostly during summer. Over the whole bloom season (from Summer1 to Summer4), the mean epiphytic abundances (all substrates and depths included) reached $0.24 \times 10^6$ ($\pm$ SE of $0.09 \times 10^6$) cells $g^{-1}$ FW in Genoa, $0.21 \times 10^6$ ($\pm 0.02 \times 10^6$) cells $g^{-1}$ FW in Villefranche-sur-Mer and $0.11 \times 10^6$ ($\pm 0.06 \times 10^6$) cells $g^{-1}$ FW in Nice. In the western sites, the measured mean abundances were lower: $0.03 \times 10^6$ ($\pm 0.02 \times 10^6$) in Cassis, $0.03 \times 10^6$ ($\pm 0.01 \times 10^6$) in Saint Raphaël, and $0.01 \times 10^6$ ($\pm 0.01 \times 10^6$) in Ramatuelle. Planktonic concentrations followed the same trend with higher mean values (all depths included) recorded in Genoa ($6 \times 10^3 \pm 4 \times 10^3$ cells $l^{-1}$), Nice ($3 \times 10^3 \pm 1 \times 10^3$ cells $l^{-1}$) and Villefranche-sur-Mer ($0.8 \times 10^3 \pm 0.5 \times 10^3$ cells $l^{-1}$). Very low mean concentrations ($\leq 0.1 \times 10^3$ cells $l^{-1}$) were observed in Cassis, Saint Raphaël and Ramatuelle. For both epiphytic and planktonic cells, blooms were only recorded in Genoa, Villefranche-sur-Mer and Nice, which indicates a geographical distinction between eastern and western sites.

The amplitude of epiphytic blooms strongly differed between the substrates, although the abundance of *Ostreopsis* on the three collected macroalgae and in the water column were positively correlated (Spearman test, Table 2).

Results of the four-way PERMANOVAs performed on *Ostreopsis* cf. *ovata* epiphytic and planktonic abundances during summer in the three eastern sites (periods/sites of blooms occurrence) are shown in Table 3a. Since the main effects of the factors tested individually are not considered meaningful in the presence of significant interactions (Anderson, 2005), only significant interactions using pair-wise tests are detailed (results in Table 3b).

Concerning epiphytic data, results of PERMANOVAs indicated a significant effect of the interaction time × substrate ($p = 0.03$, Table 3a). It showed that the distribution of *Ostreopsis* between the three collected substrates varied according to the sampling period. However, significant differences in the abundance of *Ostreopsis* between macroalgae were only observed during Summer1, with a higher abundance on Corallinales than *Halopteris scoparia* (pair-wise test, Table 3b). Inversely, the temporal evolution of epiphytic *Ostreopsis* cells depended on the substrate: abundances on Corallinales and *H. scoparia* were very variable during summer with significantly higher values recorded in Summer3 (late July–early August). Abundances on *Dictyota* spp. exhibited less temporal variation, without any significant differences between Summer3, Summer2 and Summer4 (higher values), as well as between Summer1 and Summer4.

Table 2
Results of the Spearman test performed on the complete data set (coefficients $r_S$ of Spearman, bold when significant with $p < 0.05$, bold and underlined when significant with $p < 0.01$).

| | O g$^{-1}$ Hal. | O g$^{-1}$ Cor. | O g$^{-1}$ Dic. | O l$^{-1}$ | $NO_3$ | $NO_2$ | $PO_4$ | $Si(OH)_4$ | Ntotal/P | $PO_4$/$Si(OH)_4$ | Chl a | Pheo. | $T$ (°C) | $O_2$ | Bacteria |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| O g$^{-1}$ Hal. | 1.000 | **0.901** | **0.893** | **0.890** | −0.061 | **−0.160** | **0.349** | −0.093 | **−0.191** | **0.362** | **0.412** | **0.193** | **0.738** | **−0.392** | 0.067 |
| O g$^{-1}$ Cor. | | 1.000 | **0.907** | **0.894** | **−0.149** | **−0.179** | **0.277** | **−0.181** | **−0.258** | **0.403** | **0.392** | **0.153** | **0.737** | **−0.362** | 0.119 |
| O g$^{-1}$ Dic. | | | 1.000 | **0.849** | −0.095 | **−0.151** | **0.322** | −0.093 | **−0.215** | **0.385** | **0.367** | 0.119 | **0.722** | **−0.295** | 0.015 |
| O l$^{-1}$ | | | | 1.000 | **−0.157** | **−0.207** | **0.362** | **−0.150** | **−0.290** | **0.450** | **0.394** | **0.197** | **0.749** | **−0.332** | 0.070 |
| $NO_3$ | | | | | 1.000 | **0.673** | **0.211** | **0.632** | **0.927** | **−0.372** | **0.236** | **0.243** | **−0.307** | 0.049 | **−0.264** |
| $NO_2$ | | | | | | 1.000 | 0.115 | **0.478** | **0.656** | **−0.176** | **0.288** | **0.404** | **−0.413** | **0.216** | **−0.231** |
| $PO_4$ | | | | | | | 1.000 | **0.396** | −0.106 | **0.459** | **0.495** | **0.407** | **0.358** | 0.100 | 0.026 |
| $Si(OH)_4$ | | | | | | | | 1.000 | **0.525** | **−0.516** | **0.284** | **0.266** | **−0.138** | 0.087 | **−0.125** |
| Ntotal/P | | | | | | | | | 1.000 | **−0.583** | 0.104 | **0.149** | **−0.440** | 0.063 | **−0.245** |
| $PO_4$/$Si(OH)_4$ | | | | | | | | | | 1.000 | **0.190** | **0.151** | **0.409** | 0.012 | 0.061 |
| Chl a | | | | | | | | | | | 1.000 | **0.711** | **0.256** | −0.018 | 0.103 |
| Pheo. | | | | | | | | | | | | 1.000 | 0.067 | **0.172** | −0.116 |
| $T$ (°C) | | | | | | | | | | | | | 1.000 | **−0.296** | 0.091 |
| $O_2$ | | | | | | | | | | | | | | 1.000 | 0.091 |
| Bacteria | | | | | | | | | | | | | | | 1.000 |

*Abbreviations*: O g$^{-1}$ Hal = *Ostreopsis* cf. *ovata* cells g$^{-1}$ FW of *Halopteris scoparia*; O g$^{-1}$ Cor = *Ostreopsis* cf. *ovata* cells g$^{-1}$ FW of Corallinales; O g$^{-1}$ Dic = *Ostreopsis* cf. *ovata* cells g$^{-1}$ FW of *Dictyota* spp.; O l$^{-1}$ = *Ostreopsis* cf. *ovata* cells l$^{-1}$; Chl a = chlorophyll a; Pheo = pheophytine.

PERMANOVAs also highlighted a significant effect of the interaction time × depth on both epiphytic ($p = 0.02$) and planktonic ($p = 0.03$) abundances (Table 3a). Epiphytic abundances were higher overall at 0.5 m compared to 3 m, with intermediate abundances at 1 m, except during Summer4 when no significant differences appeared between the three depths (Table 3b). In contrast, planktonic concentrations were not significantly related to depth, except during Summer1 with higher values recorded at 0.5 and 1 m than at 3 m, following the epiphytic distribution at the same period. Conversely, the temporal changes in both epiphytic and planktonic *Ostreopsis* cells varied with depth. The temporal variation in epiphytic abundances seemed to decrease with depth, with all sampling periods significantly different at 0.5 m, three significantly different periods at 1 m, and two significantly different periods at 3 m (Table 3b). Planktonic concentrations did not follow this marked trend and the highest temporal variability appeared at 1 m depth. Overall, regardless of depth, both epiphytic and planktonic abundances were higher in Summer3 and lower in Summer1, with or without significant differences with Summer4 or Summer2.

The interaction sites × time was only significant with regard to the planktonic concentrations ($p < 0.001$, Table 3a). In the three sites, higher values were recorded in Summer3 – late July/early August and in Summer2 – early July in Nice (Table 3b). Conversely, it was not possible to highlight any clear pattern of the site ranking, due to the high temporal variability, although Genoa consistently showed higher planktonic concentrations than Nice and/or Villefranche, except in Summer4.

### 3.3. Ostreopsis *development related to environmental factors*

#### 3.3.1. *Oxygen, pigments and bacteria concentrations*

A decrease in the oxygen saturation at 0.5 and 3 m depths was observed during summer, particularly between the sampling periods Summer2 (pre-bloom period) and Summer3/Summer4 (mean bloom and post-bloom periods respectively) in the eastern sites of Nice, Genoa and mostly Villefranche-sur-Mer (Fig. 2a). The Spearman test showed significant negative relationships between oxygen saturation and *Ostreopsis* epiphytic and planktonic abundances (Table 2).

Marked increases in chlorophyll *a* concentrations occurred during the period Summer3 in Genoa, Villefranche-sur-Mer and Nice, mostly at 0.5 m (Fig. 2a). Chlorophyll *a* positively correlated with all epiphytic and planktonic *Ostreopsis* abundances. Similar results were obtained for pheophytin, except an absence of a correlation with cell abundance on *Dictyota* spp. Chlorophyll *a* and pheophytin concentrations were strongly correlated. In contrast to pheophytin, chlorophyll *a* was positively related to the temperature.

Finally, bacteria concentration did not correlate with *Ostreopsis* abundances, regardless of substrate.

#### 3.3.2. *Temperature*

Over the entire study period and all sites included, the Spearman test showed high positive correlations between temperature and *Ostreopsis* cf. *ovata* epiphytic and planktonic abundances (Table 2). These correlations were not linear (Fig. 5) and we assumed the presence of minimal and maximal temperature thresholds allowing *Ostreopsis* growth. The first epiphytic cells were detected at the temperature of 13.0 °C and high abundances (>10,000 cells g$^{-1}$ FW) began to occur at 18 °C, which could represent a first minimal threshold for a substantial development of *Ostreopsis*. Planktonic cells were recorded in the water column from 18 °C and were high in number (>5000 cells l$^{-1}$) from 23 °C. Therefore, temperatures ranging from 23 °C to 27.5 °C seemed to favor the occurrence of epiphytic and planktonic blooms. Although maximal *Ostreopsis* peaks were observed at 27.5 °C, cell abundances were relatively low at the maximal measured temperature of 28.0 °C.

#### 3.3.3. *Nutrient concentrations*

The Spearman test highlighted significant negative correlations between nitrite and both epiphytic and planktonic *Ostreopsis* abundances, although low Spearman coefficients indicated weak relations (Table 2). Nitrate and silicate concentrations were weakly and negatively correlated with *Ostreopsis* abundances, only on Corallinales and in the water column. In contrary to the other nutrients, phosphate concentrations were positively correlated with *Ostreopsis* epiphytic cells on all substrates and with planktonic cells. Phosphate also positively correlated with nitrate and silicate.

The ratio N:P (with N = sum of nitrate and nitrite) was significantly and negatively related to *Ostreopsis* abundances on all substrates, contrary to the ratio P:S.

Finally, all nutrients were negatively correlated with temperature, except for phosphate.

## 4. Discussion

### 4.1. Ostreopsis *cf.* ovata *nomenclature*

Despite the morphological concordance between the Mediterranean strains of *Ostreopsis ovata* and the first description of the species by Fukuyo (1981), the high diversity in the size and shape of cells, and the lack of genetic analysis on cells from the

**Table 4**
Results of PERMANOVA (a) and conclusions of pair-wise tests (b) performed on epiphytic and planktonic data collected during summer sampling periods (Summer1 to Summer4). Bold *p* values are significant with $p < 0.05$. In the pair-wise tests conclusions, significantly similar factors ($p < 0.05$) are joined by crossed-bar.

a).

| Source of variation | df | Epiphytic data (N = 268) MS | F | p | Planktonic data (N = 108) MS | F | p |
|---|---|---|---|---|---|---|---|
| Sites (Si) | 2 | $1.81 \times 10^{11}$ | 0.81 | 0.4182 | $2.87 \times 10^{8}$ | 10.82 | **0.0001** |
| Time (Ti) | 3 | $3.93 \times 10^{12}$ | 17.60 | **0.0004** | $5.20 \times 10^{8}$ | 19.60 | **0.0001** |
| Substrate (Su) | 2 | $1.27 \times 10^{12}$ | 5.67 | **0.0227** | | | |
| Depth (De) | 2 | $1.04 \times 10^{12}$ | 4.64 | **0.0346** | $5.23 \times 10^{7}$ | 1.97 | 0.1309 |
| SixTi | 6 | $3.94 \times 10^{11}$ | 1.76 | 0.1301 | $1.98 \times 10^{8}$ | 7.45 | **0.0001** |
| SixSu | 4 | $7.60 \times 10^{10}$ | 0.34 | 0.8289 | | | |
| SixDe | 4 | $3.42 \times 10^{11}$ | 1.53 | 0.1692 | $3.87 \times 10^{7}$ | 1.46 | 0.2044 |
| TixSu | 6 | $8.77 \times 10^{11}$ | 3.93 | **0.0313** | | | |
| TixDe | 6 | $8.49 \times 10^{11}$ | 3.80 | **0.0230** | $5.23 \times 10^{7}$ | 1.97 | **0.0331** |
| SuxDe | 4 | $2.94 \times 10^{11}$ | 1.32 | 0.2376 | | | |
| SixTixSu | 12 | $9.83 \times 10^{10}$ | 0.44 | 0.9107 | | | |
| SixTixDe | 12 | $3.59 \times 10^{11}$ | 1.61 | 0.1166 | $2.90 \times 10^{7}$ | 1.09 | 0.3589 |
| SixSuxDe | 8 | $4.52 \times 10^{11}$ | 2.02 | 0.0689 | | | |
| TixSuxDe | 12 | $4.13 \times 10^{11}$ | 1.85 | 0.0818 | | | |
| SixTixSuxDe | 19 | $3.79 \times 10^{11}$ | 1.70 | 0.0858 | | | |
| Residues | 165/72 | $2.23 \times 10^{11}$ | | | $2.65 \times 10^{7}$ | | |

b).

**Pair-wise tests on interactions for Epiphytic data:**

**Time x Substrate**

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| HAL DIC COR | COR = HAL = DIC | HAL = COR = DIC | COR = HAL = DIC |

| Corallinales | *Halopteris scoparia* | *Dictyota* sp. |
|---|---|---|
| Su1 < Su4 < Su2 < Su3 | Su1 < Su4 < Su2 < Su3 | Su1 Su4 Su2 Su3 |

**Time x Depth**

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| 3 m < 1 m 0.5 m | 3 m < 1 m < 0.5 m | 3 m 1 m 0.5 m | 1 m = 0.5 m = 3 m |

| 0.5 m | 1 m | 3 m |
|---|---|---|
| Su1 < Su4 < Su2 < Su3 | Su1 < Su2 Su4 Su3 | Su1 < Su2 Su4 Su3 |

**Pair-wise tests on interactions for Planktonic data:**

**Sites x Time**

| Nice | Villefranche | Genoa |
|---|---|---|
| Su1 < Su4 < Su2 Su3 | Su1 < Su4 Su2 < Su3 | Su1 Su4 < Su2 < Su3 |

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| NIC VLF < GEN | VLF < GEN NIC | VLF < NIC < GEN | GEN = NIC = VLF |

**Time x Depth**

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| 3 m < 1 m 0.5 m | 3 m = 1 m = 0.5 m | 3 m = 1 m = 0.5 m | 3 m = 1 m = 0.5 m |

| 0.5 m | 1 m | 3 m |
|---|---|---|
| Su1 Su4 < Su2 Su3 | Su1 < Su4 < Su2 < Su3 | Su1 < Su4 < Su2 Su3 |

*Abbreviations:* NIC = Nice; VLF = Villefranche; GEN = Genoa; Su1 = Summer1; Su2 = Summer2; Su3 = Summer3; Su4 = Summer4; HAL = *Halopteris scoparia*; DIC = *Dictyota* spp., COR = Corallinales.

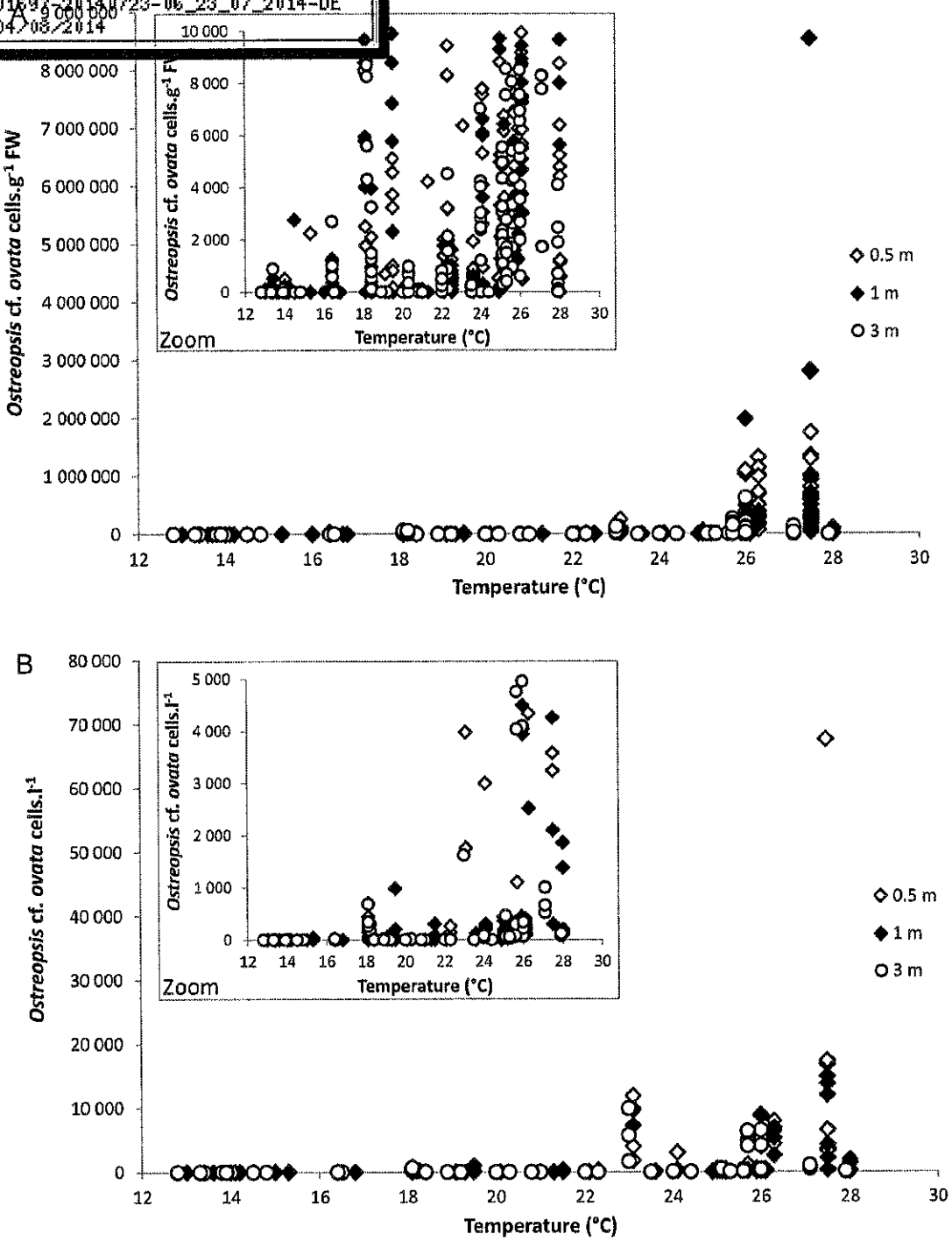



**Fig. 5.** (a) Epiphytic ($N = 988$) and (b) planktonic ($N = 363$) *Ostreopsis* abundances depending on the temperature at 0.5, 1 and 3 m depths.

type locality, makes the term "*Ostreopsis* cf. *ovata*" more appropriate for the specimen from the Mediterranean Sea (Penna et al., 2005, 2010). Moreover, genetic analysis carried out on Mediterranean strains by Penna et al. (2005, 2010) showed phylogenetic differences with the first genetic sequences of *O. ovata* registered on GenBank, which belong to a Malaysian strain (Pin et al., 2001).

### 4.2. Temporal and spatial variation in Ostreopsis abundance

*Ostreopsis* spp. proliferation followed a marked seasonal trend, as previously shown in other temperate areas (*e.g.* in the Mediterranean Sea and in New Zealand; Chang et al., 2000; Rhodes et al., 2000; Vila et al., 2001a,b; Simoni et al., 2003, 2004; Turki, 2005; Aligizaki and Nikolaidis, 2006; Shears and Ross, 2009; Ungaro et al., 2010; Mangialajo et al., 2011) and in tropical environments (Ballantine et al., 1988; Morton et al., 1992; Briggs and Leff, 2007; Okolodkov et al., 2007; Parsons and Preskitt, 2007). The present study also highlighted significant variations in *Ostreopsis* cf. *ovata* abundances in the summer period.

We previously reported a high heterogeneity of *Ostreopsis* cf. *ovata* abundances at a small spatial scale (several meters) between the replicates of each site (Cohu et al., 2011; Cohu and Lemée,

2012). The important inter-sites variation, observed on a large spatial scale, from tens to hundreds of kilometers, corresponds to the results reported in temperate and tropical studies (Grzebyk et al., 1994; Chang et al., 2000; Aligizaki and Nikolaidis, 2006; Parsons and Preskitt, 2007). During the summer of 2008, blooms in the eastern sites of Villefranche-sur-Mer, Nice and Genoa appeared in late July, corresponding to the main blooming period reported for the whole Ligurian Sea (Mangialajo et al., 2011). Although Ramatuelle and Saint-Raphaël are also located in the Ligurian Sea, *Ostreopsis* abundances remained low in these sites during the whole sampling year. In Cassis, situated in the Gulf of Lyon, no bloom was recorded, while Mangialajo et al. (2011) noted important developments in nearby sites (Morgiret, Endoume and Mèjean) during mid and late August 2008. As the Cassis site was sampled twice in August, the risk of having missed bloom events in this western site seems negligible. Thus, differences in the occurrence of *Ostreopsis* blooms observed in the present study between eastern and western sites were unlikely to be due to a delay in blooms occurring.

The spatial heterogeneity of *Ostreopsis* cf. *ovata* development may therefore reflect a real preference for specific areas. Comparing the characteristics of sampled eastern and western sites, the nature of substrate (natural *vs.* artificial rocks) and hydrodynamic characteristics did not seem to have decisively affected the *Ostreopsis* distribution. However, previous studies suggested a higher *Ostreopsis* spp. growth in calm to slightly turbulent waters (Grzebyk et al., 1994; Chang et al., 2000; Vila et al., 2001b; Simoni et al., 2003; Shears and Ross, 2009).

### 4.3. Anthropogenic pressure

The population density, clearly higher in the three eastern sites of Nice, Genoa and Villefranche-sur-Mer, might be related to substantial *Ostreopsis* cf. *ovata* proliferation. Urbanization, linked to the population density, increases water pollution and modifies the coastal habitats (including macroalgal assemblages, Mangialajo et al., 2008a), which probably impacts the distribution of *O.* cf. *ovata*, as suggested in several studies. In North Line Islands, Briggs and Leff (2007) observed increasing *Ostreopsis* sp. abundances along a gradient of human disturbance, probably linked to the induced change in macroalgae cover. In the Adriatic Sea, Ungano et al. (2010) showed a positive correlation between *O. ovata* abundances and anthropogenic microbiological contamination, related to population density. Similarly, effluents from sewage-treatment plants, generally corresponding to the amount of anthropogenic pressure, seemed to promote the proliferation of some toxic dinoflagellates (Doig and Martin, 1974; Mallin et al., 2005). Harmful algal blooms are also known to be stimulated in eutrophic and confined waters, such as harbors and recreational/industrial coastal areas (Vila et al., 2001a; Vila and Maso, 2005; Maso and Garcés, 2006). Finally, other studies have also shown an important effect of urbanization and human disturbance on the abundance and species composition of dinoflagellates cysts (Saetre et al., 1997; Dale et al., 1999; Pospelova et al., 2002).

### 4.4. Depth preference

*Ostreopsis* cf. *ovata* epiphytic abundances generally decreased from 0.5 to 3 m depth. The occurrence of dense populations of *Ostreopsis* at the sub-surface indicates a resistance to high light intensities. This observation is in agreement with the results of Totti et al. (2010) in the Adriatic Sea, which showed high abundances at 1 and 2 m depths with a decrease in abundance below a 3 m depth, probably related to the decrease of light intensity. The distribution of *Ostreopsis* and the possible daily vertical migration of cells merit further investigations as it could represent the existence of vertical ecological niches.

### 4.5. Substrate preference

The maximal planktonic cell concentration measured in the present study ($68 \times 10^3$ cells $l^{-1}$) is slightly lower than the previous concentrations of *Ostreopsis* spp. reported in Monaco ($213 \times 10^3$ cells $l^{-1}$; Cohu et al., 2011) and in the Villefranche-sur-Mer Bay ($104 \times 10^3$ cells $l^{-1}$; Mangialajo et al., 2011), yet is similar to those found on the Genoa coast ($87 \times 10^3$ cells $l^{-1}$; Mangialajo et al., 2008b). In the Adriatic and Aegean seas, maximal *Ostreopsis* spp. concentrations detected in the water column were lower: $25 \times 10^3$ and $16 \times 10^3$ cells $l^{-1}$ respectively (Aligizaki and Nikolaidis, 2006; Totti et al., 2010).

The maximal epiphytic abundance of *Ostreopsis* cf. *ovata* measured in the present study ($8.54 \times 10^6$ cells $g^{-1}$ FW on *Dictyota* spp.) is one of the highest values ever measured in the NW Mediterranean Sea. In previous studies, densities of *Ostreopsis* spp. reached $7.25 \times 10^6$ cells $g^{-1}$ FW in Catalonia (Mangialajo et al., 2011), $2.77 \times 10^6$ cells $g^{-1}$ FW in Monaco (Cohu et al., 2011), $2.54 \times 10^6$ cells $g^{-1}$ FW on the Genoa coast (Mangialajo et al., 2008b) and $1.70 \times 10^6$ cells $g^{-1}$ FW in the Adriatic Sea (Totti et al., 2010). To the best of our knowledge, the maximal epiphytic abundance in the eastern Mediterranean Sea (Greece) reached $0.41 \times 10^6$ cells $g^{-1}$ FW (Aligizaki and Nikolaidis, 2006).

The positive and significant correlation between epiphytic and planktonic *Ostreopsis* cell abundances observed in this study has been repeated previously (Vila et al., 2001b; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008b, 2011; Totti et al., 2010) and seems to confirm the hypothesis of a cells resuspension from the macroalgae surface to the water column.

Our results show an important variability in the abundance of *Ostreopsis* depending on macroalgal host species, without statistically highlighting a clear substrate preference. However, *Dictyota* species seem particularly able to support very high abundances of *Ostreopsis* during blooms.

### 4.6. Concentration of oxygen and pigments

The oxygen saturation decreased between the pre-bloom and the bloom summer periods, mostly in the three eastern sites. While this depletion partly reflects a summer decrease of dissolved oxygen solubility related to the increase in seawater temperature, it might also be linked to the higher consumption of oxygen related to organic matter decomposition during *Ostreopsis* cf. *ovata* blooms, as suggested by the Spearman negative correlations obtained between *Ostreopsis* abundances and oxygen saturation. The effect of *Ostreopsis* spp. development on dissolved oxygen concentration has received little attention. However, dramatic oxygen depletions have been observed during bloom events of other harmful dinoflagellates (Zingone and Enevoldsen, 2000; Al-Ghelani et al., 2005; Ning et al., 2009).

As expected for autotrophic and mixotrophic microalgae, an increase in chlorophyll *a* concentration was related to the *Ostreopsis* blooms in the three eastern sites, corresponding to previous observations (Parsons and Preskitt, 2007; Ungaro et al., 2010).

### 4.7. Impact of temperature

The seasonal trend of *Ostreopsis* development in the Mediterranean Sea can be easily linked to the seasonal variations of seawater temperature. The present study suggests an optimum growth of *Ostreopsis* cells at temperatures between 23 and 27.5 °C. These results are in accordance with several previous studies

performed in temperate areas (Mediterranean Sea and New Zealand), where temperatures ranging from 22 °C to 30 °C seemed to constitute the best conditions for *Ostreopsis* spp. development (Chang et al., 2000; Simoni et al., 2003; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008b, 2011).

In the present work, *Ostreopsis* cells were found at 13 °C, which is one of the lowest worldwide temperatures associated with the presence of *Ostreopsis* spp. Until now, *Ostreopsis* cf. *ovata* and *Ostreopsis siamensis* have been detected at the minimal temperatures of 14 and 14.5 °C in the Mediterranean Sea (Simoni et al., 2003; Aligizaki and Nikolaidis, 2006), yet were also recorded in Peter the Great Bay (Sea of Japan) when water temperature was only 7 °C (Selina and Orlova, 2010). The presence of *O.* cf. *ovata* in this region of the Sea of Japan which is characterized by cold winter temperatures (dropping below 0 °C at the sea surface), reveals a high tolerance to extreme climates (Selina and Orlova, 2010; Selina and Levchenko, 2011).

The high fluctuations in the abundance of *Ostreopsis* cf. *ovata* during summer cannot be related to temperature variations, suggesting the impact of other physical and chemical factors, as proposed by Mangialajo et al. (2011). Moreover, the obvious seasonality of *Ostreopsis* spp. abundances in tropical climates could not entirely result from the low annual amplitude of temperatures (Ballantine et al., 1985, 1988; Morton et al., 1992; Okolodkov et al., 2007). This also suggests an important effect of other environmental parameters, such as wave activity (Okolodkov et al., 2007), nutrient availability, salinity or light intensity and photoperiod (Morton et al., 1992). Some biotic factors could also be involved, such as the availability of some seasonal macroalgal substrates and the competition with other microphytes (Okolodkov et al., 2007).

### 4.8. Impact of nutrient concentrations

During spring and summer, very high concentrations of nitrate and silicate were recorded at a depth of 0.5 m in Cassis. In this site, a fresh water discharge was observed at the subsurface, which probably provided nutrients. Since low *Ostreopsis* cf. *ovata* abundances were measured simultaneously, the increase in nitrate and silicate concentrations did not seem to impact the microalgae development. Moreover, the freshwater source should have a negative impact on *Ostreopsis* cf. *ovata* growth *via* a decrease of salinity, as observed on *Ostreopsis lenticularis* and others dinoflagellates by Delgado et al. (2006).

While nitrate is usually one of the main nitrogen sources used by microalgae, it did not seem to be a limiting factor. *Ostreopsis* spp., suspected to be a mixotroph, could obtain additional organic sources of nitrogen and other nutrients through phagotrophy and therefore be less sensitive to changes in nutrient supply ratios (Faust and Morton, 1995; Faust, 1998; Stoecker et al., 2006; Burkholder et al., 2008; Ignatiades and Gotsis-Skretas, 2010). In further studies, the role of ammonium and urea in *Ostreopsis* spp. growth should be investigated. These available forms of nitrogen are known to be used by some heterotrophic dinoflagellates, such as *Pfiesteria* spp. (Glibert et al., 2006).

Silicate did not have any clear impact on the abundance of *Ostreopsis* cf. *ovata*, as generally expected for dinoflagellates which do not specifically use silicate for their growth, in contrast to diatoms. It is interesting to note that nitrogen and silicate concentrations were negatively correlated with temperature, as expected in temperate waters where the thermocline formation prevents nutrient replenishment in surface water during summer. This decrease in nutrient concentrations during the warm season, corresponding to the *Ostreopsis* bloom period, could partly explain the observed negative or null correlations with the abundance of *Ostreopsis*.

Finally, phosphate seemed to be the only limiting nutrient for the *Ostreopsis* growth. Since phosphate is positively related to temperature, there might be an interacting or a cumulative effect of these two parameters on the *Ostreopsis* development. The slight increase in phosphate concentrations during summer was surprising and might be due to a seasonal anthropogenic pollution.

In the Mediterranean Sea, Vila et al. (2001b) noted that inorganic nitrogen, phosphate and silicate concentrations appeared to be unassociated with *Ostreopsis* sp. distribution. In the Adriatic Sea, Ungaro et al. (2010) only showed a negative link between *O. ovata* and nitrite, as shown in the present study, and a positive correlation between cell abundances and the ratio $NO_3$:$NO_2$. In Hawaii, Parsons and Preskitt (2007) observed opposing results, as nutrient positively correlated with an undetermined *Ostreopsis* species, yet was not significantly correlated with *O. ovata*. Since an increase in nutrient availability might have an indirect impact on *Ostreopsis* spp. development by inducing a shift in macroalgae substrate cover and/or composition (Briggs and Leff, 2007; Burkholder et al., 2008), the direct effect of nutrient on *Ostreopsis* growth is very difficult to assess *in situ*. Moreover, the coastal habitat of *Ostreopsis* spp. is often impacted by anthropogenic activities, especially in the NW Mediterranean Sea. Therefore *in situ* nutrient concentrations can show important spatial and temporal variations at small scales, which lead to an additional difficulty in ecological studies. It would be useful to investigate the impact of long-term changes in nutrient availability and ratios on *Ostreopsis* bloom frequency and amplitude, as suggested in more general studies (Anderson et al., 2002; Li et al., 2009; Ning et al., 2009).

## 5. Conclusion

The present study highlighted a high spatial heterogeneity in the development of *Ostreopsis* cf. *ovata* within an obvious seasonal trend. Overall, temperature was the most important factor, explaining why *Ostreopsis* growth was restricted to the summer period. Concerning the impact of nutrients, *Ostreopsis* abundance was only related to phosphate concentration. The distribution of epiphytic cells was strongly affected by depth and substrates. In conclusion, the temporal and spatial development of *O.* cf. *ovata* depends on multiple factors which are difficult to discriminate.

Further *in situ* and *in vitro* studies are needed to investigate the effects of substrate and depth on the distribution of epiphytic cells, as well as the impact of urbanization on *Ostreopsis* development.

## Acknowledgement

This work was supported by the French national program MediOs 2 (LITEAU-Ministère de l'Ecologie, Conseil Général des Alpes-Maritimes, Agence de l'Eau Rhône Méditerranée Corse)[SS].

## References

Al-Ghelani, H.M., AlKindi, A.Y.A., Amer, S., Al-Akhzami, Y.K., 2005. Harmful algal blooms: physiology, behavior, population dynamics and global impacts – a review. Journal of Science 10, 1–30.

Aligizaki, K., Nikolaidis, G., 2006. The presence of the potentially toxic genera *Ostreopsis aligialigi*and *Coolia* (Dinophyceae) in the North Aegean Sea, Greece. Harmful Algae 5, 717–730.

Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25, 704–726.

Anderson, M.J., 2005. PERMANOVA: A FORTRAN Computer Program for Permutational Multivariate Analysis of Variance. University of Auckland, Otro.

Ashton, M., Rosado, W., Govind, N.S., Tosteson, T.R., 2003. Culturable and non-culturable bacterial symbionts in the toxic benthic dinoflagellate *Ostreopsis lenticularis*. Toxicon 42, 419–424.

Ballantine, D.L., Bardales, A.T., Tosteson, T.R., Durst, H.D., 1985. Seasonal abundance of *Gambierdiscus toxicus* and *Ostreopsis* sp. in coastal waters of southwest Puerto

Rico, M. Gabriel, C., Salvat, B. (Eds.), Proc. 5th Int. Coral Reef Cong vol. 4, Tahiti,
namics and
toxicity of natural populations of benthic dinoflagellates in southwestern Puerto Rico. Journal of Experimental Marine Biology and Ecology 119, 201–212.
Barroso Garcia, P., Rueda de la Puerta, P., Parrón Carreñob, T., Marín Martínezb, P., Guillén Enríquezc, J., 2008. Brote con síntomas respiratorios en la provincia de Almería por una posible exposición a microalgas tóxicas. Gaceta Sanitaria 22, 578–584.
Battocchi, C., Totti, C., Vila, M., Masó, M., Capellacci, S., Accoroni, S., Reñé, A., Scardi, M., Penna, A., 2010. Monitoring toxic microalgae *Ostreopsis* (dinoflagellate) species in coastal waters of the Mediterranean Sea using molecular PCR-based assay combined with light microscopy. Marine Pollution Bulletin 60, 1074–1084.
Brescianini, C., Grillo, C., Melchiorre, N., Bertolotto, R., Ferrari, A., Vivaldi, B., Icardi, G., Gramaccioni, L., Funari, E., Scardala, S., 2006. Ostreopsis ovata algal blooms affecting human health in Genova, Italy, 2005 and 2006. Eurosurveillance 11, 3040.
Briggs, A., Leff, M., 2007. A comparison of toxic dinoflagellate densities along a gradient of human disturbance in the North Line Islands. Final Report, Stanford University.
Burkholder, J.M., Glibert, P.M., Skelton, H.M., 2008. Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. Harmful Algae 8, 77–93.
Carballeira, N.M., Emiliano, A., Sostre, A., Restituyo, J.A., González, I.M., Colón, G.M., Tosteson, C.G., Tosteson, T.R., 1998. Fatty acid composition of bacteria associated with the toxic dinoflagellate *Ostreopsis lenticularis* and with Caribbean *Palythoa* species. Lipids 33, 627–632.
Chang, F.H., Shimizu, Y., Hay, B., Stewart, R., Mackay, G., Tasker, R., 2000. Three recently recorded *Ostreopsis* spp. (Dinophyceae) in New Zealand: temporal and regional distribution in the upper North Island from 1995 to 1997. New Zealand Journal of Marine and Freshwater Research 34, 29–39.
Ciminiello, P., Aversano, C.D., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Grillo, C., Melchiorre, N., 2006. The Genoa 2005 Outbreak. Determination of putative palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method. Analytical Chemistry 78, 6153–6159.
Cohu, S., Thibaut, T., Mangialajo, L., Labat, J.P., Passafiume, O., Blanfuné, A., Simon, N., Cottalorda, J.M., Lemée, R., 2011. Occurrence of the toxic dinoflagellate *Ostreopsis* cf. ovata in relation with environmental factors in Monaco (NW Mediterranean). Marine Pollution Bulletin 62, 2681–2691.
Cohu, S., Lemée, R., 2012. Vertical distribution of the toxic epibenthic dinoflagellates *Ostreopsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis* in the NW Mediterranean Sea. Cahiers de Biologie Marine 53, 373–380.
Dale, B., Thorsen, T.A., Fjellsa, A., 1999. Dinoflagellate cysts as indicators of cultural eutrophication in the Oslofjord, Norway. Estuarine, Coastal and Shelf Science 48, 371–382.
Deeds, J.R., Schwartz, M.D., 2010. Human risk associated with palytoxin exposure. Toxicon 56, 150–162.
Delgado, G., Lechuga-Devéze, C.H., Popowski, G., Troccoli, L., Salinas, C.A., 2006. Epiphytic dinoflagellates associated with ciguatera in the northwestern coast of Cuba. Revista de Biologia Tropical 54, 299–310.
Doig, M.T., Martin, D.F., 1974. The response of *Gymnodinium breve* to municipal waste materials. Marine Biology 24, 223–228.
Faust, M.A., 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. Journal of Phycology 31, 996–1003.
Faust, M.A., 1998. Mixotrophy in tropical benthic dinoflagellates. In: Harmul Algae Proc. 8th Int. Conf. Harmful Algae, Vigo, Spain, Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO. pp. 390–393.
Faust, M.A., Morton, S.L., 1995. Morphology and ecology of the marine dinoflagellate *Ostreopsis labens* sp. nov. (Dinophyceae). Journal of Phycology 31, 456–463.
Faust, M.A., Morton, S.L., Quod, J.P., 1996. Further SEM study of marine dinoflagellates: the genus *Ostreopsis* (Dinophyceae). Journal of Phycology 32, 1053–1065.
Gasol, M., Del Giorgio, A., 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Scientia Marina 64, 197–224.
Glibert, P.M., Burkholder, J.M., Parrow, M.W., Lewitus, A.J., Gustafson, D.E., 2006. Direct uptake of nitrogen by *Pfiesteria piscicida* and *Pfiesteria shumwayae*, and nitrogen nutritional preferences. Harmful Algae 5, 380–394.
Grzebyk, D., Berland, B., Thomassin, B.A., Bosi, C., Arnoux, A., 1994. Ecology of ciguateric dinoflagellates in the coral reef complex of Mayotte Island (S. W. Indian Ocean). Journal of Experimental Marine Biology and Ecology 178, 51–66.
Ignatiades, L., Gotsis-Skretas, O., 2010. A review on toxic and harmful algae in Greek coastal waters (E. Mediterranean Sea). Toxins 2, 1019–1037.
Ito, E., Ohkusu, M., Yasumoto, T., 1996. Intestinal injuries caused by experimental palytoxicosis in mice. Toxicon 34, 643–652.
Lenoir, S., Ten-Hage, L., Turquet, J., Quod, J.-P., Bernard, C., Hennion, M.-C., 2004. First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in Southwestern Indian Ocean. Journal of Phycology 40, 1042–1051.
Li, J., Glibert, P.M., Zhou, M., Lu, S., Lu, D., 2009. Relationships between nitrogen and phosphorus forms and ratios and the development of dinoflagellate blooms in the East China Sea. Marine Ecology Progress Series 383, 11–26.
Lorenzen, C.J., Newton Downs, J., 1986. The specific absorption coefficients of chlorophyllide a and pheophorbide a in 90% acetone, and comments on the fluorometric determination of chlorophyll and pheopigments. Limnology and Oceanography 31, 449–452.
Mallin, M.A., McIver, M.R., Wells, H.A., Parsons, D.C., Johnson, V.L., 2005. Reversal of eutrophication following sewage treatment upgrades in the New River Estuary, North Carolina. Estuaries 28, 750–760.
Mangialajo, L., Chiantore, M., Cattaneo-Vietti, R., 2008a. Loss of fucoid algae along a gradient of urbanisation, and structure of benthic assemblages. Marine Ecology Progress Series 358, 63–74.
Mangialajo, L., Bertolotto, R., Cattaneo-Vietti, R., Chiantore, M., Grillo, C., Lemee, R., Melchiorre, N., Moretto, P., Povero, P., Ruggieri, N., 2008b. The toxic benthic dinoflagellate *Ostreopsis ovata*: quantification of proliferation along the coastline of Genoa, Italy. Marine Pollution Bulletin 56, 1209–1214.
Mangialajo, L., Ganzin, N., Accoroni, S., Asnaghi, V., Blanfuné, A., Cabrini, M., Cattaneo-Vietti, R., Chavanon, F., Chiantore, M., Cohu, S., Costa, E., Fornasaro, D., Grossel, H., Marco-Miralles, F., Masó, M., Reñé, A., Rossi, A.M., Sala, M.M., Thibaut, T., Totti, C., Vila, M., Lemée, R., 2011. Trends in *Ostreopsis* proliferation along the Northern Mediterranean coasts. Toxicon 57, 408–420.
Maso, M., Garcés, E., Pagès, F., Camp, J., 2003. Drifting plastic debris as a potential vector for dispersing harmful algal bloom (HAB) species. Scientia Marina 67, 107–111.
Maso, M., Garcés, E., 2006. Harmful microalgae blooms (HAB); problematic and conditions that induce them. Marine Pollution Bulletin 53, 620–630.
Mattos Nascimento, S., Monteiro, P.O., Leite Ferreira, C.E., González-Rodríguez, E., 2008. Ostreopsis ovata blooms on Rio de Janeiro coast. Harmful Algae News 37, 1–4.
Miraglia, M., Marvin, H.J.P., Kleter, G.A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., Santis, B.D., Dekkers, S., Filippi, L., Hutjes, R.W.A., Noordam, M.Y., Pisante, M., Piva, G., Prandini, A., Toti, L., van den Born, G.J., Vespermann, A., 2009. Climate change and food safety: an emerging issue with special focus on Europe. Food and Chemical Toxicology 47, 1009–1021.
Monti, M., Minocci, M., Beran, A., Ives, L., 2007. First record of *Ostreopsis* cfr. *ovata* on macroalgae in the Northern Adriatic Sea. Marine Pollution Bulletin 54, 598–601.
Morton, S.L., Norris, D.R., Bomber, J.W., 1992. Effect of temperature, salinity and light intensity on the growth and seasonality of toxic dinoflagellates associated with ciguatera. Journal of Experimental Marine Biology and Ecology 157, 79–90.
Ning, X., Lin, C., Hao, Q., Liu, C., Le, F., Shi, J., 2009. Long term changes in the ecosystem in the northern South China Sea during 1976–2004. Biogeosciences 6, 2227–2243.
Okolodkov, Y.B., Campos-Bautista, G., Gárate-Lizárraga, I., González-González, J.A.G., Hoppenrath, M., Arenas, V., 2007. Seasonal changes of benthic and epiphytic dinoflagellates in the Veracruz reef zone, Gulf of Mexico. Aquatic Microbial Ecology 47, 223–237.
Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., Ratsimaloto, M., Naoki, H., Yasumoto, T., 1999. Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37, 55–65.
Parsons, M.L., Preskitt, L.B., 2007. A survey of epiphytic dinoflagellates from the coastal waters of the island of Hawai'i. Harmful Algae 6, 658–669.
Parsons, M.L., Aligizaki, K., Dechraoui Bottein, M.Y., Santiago, F., Morton, S.L., Penna, A., Rhodes, L., 2012. *Gambierdiscus* and *Ostreopsis*: reassessment of the state of knowledge of their taxonomy, geography, ecophysiology and toxicology. Harmful Algae 14, 107–129.
Pearce, I., Marshall, J.A., Hallegraeff, G.M., 2001. Toxic epiphytic dinoflagellates from east coast Tasmania, Australia. Harmful Algal Blooms 54–57.
Penna, A., Vila, M., Fraga, S., Giacobbe, M.G., Andreoni, F., Riobo, P., 2005. Characterization of *Ostreopsis* and *Coolia* (Dinphyceae) isolates in the Western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. Journal of Phycology 41, 212–225.
Penna, A., Fraga, S., Battocchi, C., Casabianca, S., Giacobbe, M.G., Riobo, P., Vernesi, C., 2010. A phylogeographical study of the toxic benthic dinoflagellate genus *Ostreopsis* Schmidt. Journal of Biogeography 37, 380–841.
Pérez-Guzmán, L., Pérez-Matos, A.E., Rosado, W., Tosteson, T.R., Govind, N.S., 2008. Bacteria associated with toxic clonal cultures of the dinoflagellate *Ostreopsis lenticularis*. Marine Biotechnology 10, 492–496.
Pin, L.C., Teen, L.P., Ahmad, A., Usup, G., 2001. Genetic diversity of *Ostreopsis ovata* (Dinophyceae) from Malaysia. Marine Biotechnology 3, 246–255.
Pospelova, V., Chmura, G.L., Boothman, W.S., Latimer, J.S., 2002. Dinoflagellate cyst records and human disturbance in two neighboring estuaries, New Bedford Harbor and Apponagansett Bay, Massachusetts (USA). Science of the Total Environment 298, 81–102.
Rhodes, L., 2011. World-wide occurrence of the toxic dinoflagellate genus *Ostreopsis* Schmidt. Toxicon 57, 400–407.
Rhodes, L., Adamson, J., Suzuki, T., Briggs, L., Garthwaite, I., 2000. Toxic marine epiphytic dinoflagellates, *Ostreopsis siamensis* and *Coolia monotis* (Dinophyceae), in New Zealand. New Zealand Journal of Marine and Freshwater Research 34, 371–383.
Rhodes, L., Smith, K.F., Munday, R., Selwood, A.I., McNabb, P.S., Holland, P.T., Bottein, M.Y., 2010. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. Toxicon 56, 751–758.
Riobo, P., Paz, B., Franco, J.M., 2006. Analysis of palytoxin-like in *Ostreopsis* cultures by liquid chromatography with precolumn derivatization and fluorescence detection. Analytica Chimica Acta 566, 217–223.
Saetre, M.M.L., Dale, B., Abdullah, M.I., Saetre, G.P., 1997. Dinoflagellate cysts as potential indicators of industrial pollution in a Norwegian Fjord. Marine Environment Research 44, 167–189.
Selina, M.S., Orlova, T.Y., 2010. First occurrence of the genus *Ostreopsis* (Dinophyceae) in the Sea of Japan. Botanica Marina 53, 243–249.

Shelina, T.M., Levchenko, E.V., 2011. Species composition and morphology of dinoflagellates (Protista) epiphytic assemblages of Peter the Great Bay in the Sea of Japan. Russian Journal of Marine Biology 37, 23–37.

Shears, N.T., Ross, P.M., 2009. Blooms of benthic dinoflagellates of the genus *Ostreopsis*; an increasing and ecologically important phenomenon on temperate reefs in New Zealand and worldwide. Harmful Algae 8, 916–925.

Shears, N.T., Ross, P.M., 2010. Toxic cascades: multiple anthropogenic stressors have complex and unanticipated interactive effects on temperate reefs. Ecology Letters 13, 1149–1159.

Simoni, F., Gaddi, A., Paolo, C.D., Lepri, L., 2003. Harmful epiphytic dinoflagellate on Tyrrhenian Sea reefs. Harmful Algae News 24, 13–14.

Simoni, F., Di Paolo, C., Gori, L., Lepri, L., Mancino, A., Falaschi, A., 2004. Further investigation on blooms of *Ostreopsis ovata*, *Coolia monotis*, *Prorocentrum lima* on the macroalgae of artificial and natural reefs in the Northern Tyrrhenian Sea. Harmful Algae News 26, 6–7.

Stoecker, D., Tillmann, U., Granéli, E., 2006. Phagotrophy in harmful algae. Ecological Studies 189, 177–187.

Taniyama, S., Arakawa, O., Terada, M., Nishio, S., Takatani, T., Mahmud, Y., Noguchi, T., 2003. *Ostreopsis* sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. Toxicon 42, 29–33.

Tichadou, L., Glaizal, M., Armengaud, A., Grossel, H., Lemée, R., Kantin, R., Lasalle, J.L., Drouet, G., Rambaud, L., Malfait, P., Haro, L.d., 2010. Health impact of unicellular algae of the *Ostreopsis* genus blooms in the Mediterranean Sea: experience of the French Mediterranean coast surveillance network from 2006 to 2009. Clinical Toxicology 48, 839–844.

Tosteson, T.R., Ballantine, D.L., Tosteson, C.G., Hensley, V., Bardales, A.T., 1989. Associated bacterial flora, growth, and toxicity of cultured benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus*. Applied and Environmental Microbiology 55, 137–141.

Totti, C., Accoroni, S., Cerino, F., Cucchiari, E., Romagnoli, T., 2010. *Ostreopsis ovata* bloom along the Conero Riviera (northern Adriatic Sea): relationships with environmental conditions and substrata. Harmful Algae 9, 233–239.

Tréguer, P., Corre, P.L., 1975. Manuel d'analyse des sels nutritifs dans l'eau de mer. Utilisation de l'autoanalyseur II Technicon, 2ème édition. Université de Bretagne Occidentale Brest 110 pp.

Turki, S., 2005. Distribution of toxic dinoflagellates along the leaves of seagrass *Posidonia oceanica* and *Cymodocea nodosa* from the Gulf of Tunis. Cahiers de Biologie Marine 46, 29–34.

Ungano, N., Assennato, G., Blonda, M., Cudillo, B., Petruzzelli, M.R., Mariani, M., Pastorelli, A.M., Aliquò, M.R., D'angela, A., Aiello, C., Ranieri, S., 2010. Occurrence of the potentially toxic dinoflagellate *Ostreopsis ovata* along the Apulian coastal areas (Southern Italy) and relationship with anthropogenic pollution. Fresenius Environmental Bulletin 19, 1813–1821.

Ungaro, N., Pastorelli, A.M., Di Festa, T., Galise, I., Romano, C., Assennato, G., Blonda, V., Perrino, V., 2010. Annual trend of the dinoflagellate *Ostreopsis ovata* in two sites along the Southern Adriatic coast. Biology Marine Mediterranean 17, 183–184.

Utermöhl, H., 1958. Zur Vervoll Kommung der Quantitativen Phytoplankton-Methodik. Mitteilungen. Internationale Vereinigung fur theoretische und angewandte Limnologie 9, 1–38.

Vila, M., Camp, J., Garcés, E., Maso, M., Delgado, M., 2001a. High resolution spatio-temporal detection of potentially harmful dinoflagellates in confined waters of the NW Mediterranean. Journal of Plankton Research 23, 497–514.

Vila, M., Garcés, E., Masó, M., 2001b. Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. Aquatic Microbial Ecology 26, 51–60.

Vila, M., Maso, M., 2005. Phytoplankton functional groups and harmful algal species in anthropogenically impacted waters of the NW Mediterranean Sea. Scientia Marina 69, 31–45.

Yasumoto, T., 1998. Fish poisoning due to toxins of microalgal origins in the Pacific. Toxicon 36, 1515–1518.

Zingone, A., Enevoldsen, H.O., 2000. The diversity of harmful algal blooms: a challenge for science and management. Ocean and Coastal Management 43, 725–748.

armful Algae 28 (2013) 10–22

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

# Occurrence of palytoxins in marine organisms from different trophic levels of the French Mediterranean coast harvested in 2009

R. Biré [a], S. Trotereau [a,*], R. Lemée [b,c], C. Delpont [a], B. Chabot [a], Y. Aumond [b,c], S. Krys [a]

[a] Anses, Laboratoire de sécurité des aliments de Maisons-Alfort, 23 Avenue du Général de Gaulle, 94706 Maisons-Alfort Cedex, France
[b] Université Pierre et Marie Curie-Paris 6, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer Cedex, France
[c] CNRS, Marine Microbial Ecology and Biogeochemistry, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer Cedex, France

ARTICLE INFO

*Article history:*
Received 8 February 2012
Received in revised form 30 April 2013
Accepted 30 April 2013

*Keywords:*
Haemolytic test
LC–MS/MS
Marine organisms
Mediterranean
*Ostreopsis* cf. *ovata*
Palytoxins

ABSTRACT

Four sites located in Nice and Villefranche-sur-Mer, on the French Mediterranean coast, were monitored during the summer of 2009 for the presence of epiphytic and planktonic *Ostreopsis* cf. *ovata*, and that of palytoxin (PITX) and 2 of its analogues (ovatoxin-a (OVTX-a) and ostreocin-D (OST-D)) in different marine organisms.

Several of the 15 species that were sampled between June and September 2009 were found to be contaminated with OVTX-a as the major toxin (90% of the toxin profile) and PITX; this included fish, echinoderms, gastropods, crustaceans and cephalopods. The contamination levels varied geographically and between species, with the herbivorous species generally having higher toxin levels than carnivorous ones.

The determination of the toxin distribution between the digestive tube (DT) and the remaining tissue (RT) or roe in the case of the sea urchin *Paracentrotus lividus* showed that the toxins were sequestered in the DT. The highest toxin level ever recorded over the course of the study was of 392.2 μg for the sum of OVTX-a and PITX per kg of DT of the flathead mullet *Mugil cephalus*. No quantifiable levels of toxins were found in the roe of the sea urchins or in the RT of the other marine products. However, in several cases, the toxin level in the whole flesh of the analysed organisms was above 30 μg OVTX-a + PITX/kg, when knowing that the European food safety authority's opinion is that an adult should not ingest more than 30 μg PITX + OST-D per kg of shellfish meat to avoid putting the consumer's health at risk. This was observed for the following four species: the sea urchin *P. lividus*, the red-mouthed rock shell *Stramonita haemastoma*, the warty crab *Eriphia verrucosa* and the flathead mullet *M. cephalus*.

The collection of such data is of great importance to refine and complete the risk assessment of PITX and its analogues and has to be encouraged in order to provide reliable information for setting up a regulatory level that would protect the consumers of edible marine organisms



## 1. Introduction

Palytoxin (PITX) is a complex non-protein compound with amphiphilic properties that was first isolated from the zoanthid *Palythoa* sp. after which it was named (Moore and Scheuer, 1971). The production of PITX and PITX-like compounds by dinoflagellates of the genus *Ostreopsis* has been reported in the literature (Onuma et al., 1999). *Ostreopsis* blooms were solely reported in tropical areas until the beginning of the 2000s with the first blooms in temperate waters, especially in the Mediteranean (Ciminiello et al., 2006) and the Aegean sea (Aligizaki et al., 2008; Aligizaki and Nikolaidis, 2006). This highlighted the human risks associated with the exposure via aquatic activities and through the consumption of contaminated marine organisms.

During the summer of 2005, about 200 people sought medical treatment in Genoa, Italy, because of cutaneous and respiratory problems after being exposed to marine aerosols; Ciminiello and co-workers (2006) showed the presence of PITX associated with *Ostreopsis ovata*. In France, the presence of *Ostreopsis* spp. was responsible for similar symptoms between 2006 and 2009 reported by a few people who went swimming and diving on the Mediterranean coast (Tichadou et al., 2010).

PITX and PITX-like compounds have been reported in marine organisms collected in various tropical countries including Colombia, Madagascar, Philippines, Japan, Australia, and Micronesia (Aligizaki et al., 2011; Gleibs and Mebs, 1999; Munday, 2008) and some cases of food poisoning were reported in the literature, some being fatal (Aligizaki et al., 2011; Deeds and Schwartz, 2010;

* Corresponding author. Tel.: +33 10 49772738; fax: +33 10 49772650.
*E-mail address:* sophie.trotereau@anses.fr (S. Trotereau).

1568-9883/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.hal.2013.04.007

Monday, 2008; Tubaro et al., 2011b). Several symptoms were reported by the consumers after eating contaminated marine organisms; these included a metallic taste, gastrointestinal malaise, diarrhoea, nausea, vomiting, ataxia, dizziness, numbness of extremities, myalgia, dyspnoea, convulsions and bradicardia (Tubaro et al., 2011b).

No food poisoning has been associated with *Ostreopsis* blooms in the Mediterranean, even though the presence of PlTX-like compounds was reported in shellfish collected in the Aegean Sea (Greece) in 2003 (Aligizaki et al., 2008), in mussels and sea urchin from the Ligurian coastline (Bellocci et al., 2008b), and from the French Mediterranean coast (Amzil et al., 2009) and during this bloom of *Ostreopsis* in the French Mediterranean coast no seafood poisoning was reported by the epidemiological system.

The data reported in the literature regarding the presence of PlTX and PlTX-like compounds in edible marine organisms were obtained with different methods, including biological (mouse bioassay, haemolytic test) and chemical methods (LC–MS). The toxin levels accounted for in these reports vary, some being expressed as mouse or haemolytic units per gram or as microgram of PlTX per kilogram of specimen; this makes it difficult to compare the different sets of data and thereby does not facilitate the risk assessment. Yet, an important step has been made in that direction, as a scientific panel under the aegis of the European food safety authority (EFSA) assessed the risk associated with the presence of PlTX and its analogue ostreocin-D (OST-D) in shellfish and estimated that the toxin level should not exceed 30 μg for the sum of PlTX and OST-D per kg (μg PlTX + OST-D/kg) of shellfish meat to protect the consumers' health (EFSA, 2009). This risk assessment was performed on the basis of the scarce toxicological, occurrence and consumption data available at the time. It pointed out the need for additional information on the toxicity of the different analogues, as well as on the occurrence of these toxic compounds in marine products, other than shellfish, destined to human consumption, in order to refine and complete the EFSA risk assessment that currently only concerns the shellfish. The provision of such data can come from monitoring programmes as well as from research projects.

The present study aimed at collecting information on the occurrence and the distribution of PlTX and 2 of its analogues (OVTX-a and OST-D) in a wide variety of edible marine organisms harvested in four recreational sites located in Nice and Villefranche-sur-Mer on the French Mediterranean coast. The sampling campaign took place between June and September 2009, in locations where *Ostreopsis* cf. *ovata* had previously been observed during the same periods.

## 2. Materials and methods

### 2.1. Sampling locations

The choice of the first two sites (Fig. 1), site N (43°41′27.19″ N and 7°17′35.09″ E) located east from Nice harbour and site V1 (43°42′6.87″ N and 7°19′14.45″ E), located in Villefranche, south from "Plage de la Réserve", was based on high *Ostreopsis* cf. *ovata* abundances recorded in 2008.

Two other locations were also sampled when blooms of *Ostreopsis* occurred in the area; these are site V2 in Villefrance, which corresponds to "Plage des jeunes" (43°42′10.81″ N and 7°19′13.04″ E) and site V3, also known as "Rochambeau" (43°41′34.83″ N and 7°18′31.66″ E).

### 2.2. Sampling periods

The edible marine organisms targeted in the study as well as the planktonic and epiphytic *Ostreopsis* samples were collected between the 2nd of June (week 23) and the 31st of September 2009 (week 39), with variations depending on the site. During the course of the sampling period, samples were taken on a bimonthly basis in sites N and V1. The sampling frequency was increased to weekly when the toxic bloom was detected.

Additional sampling on a wider range of marine organisms in sites V2 and V3 was carried because *Ostreopsis* cf. *ovata* blooms occurred in these areas.

### 2.3. Sampling of marine organisms

A wide variety of marine organisms available on site were sampled in the four locations and included molluscs (gastropods and cephalopods), echinoderms, fish, and crustaceans (Table 1).

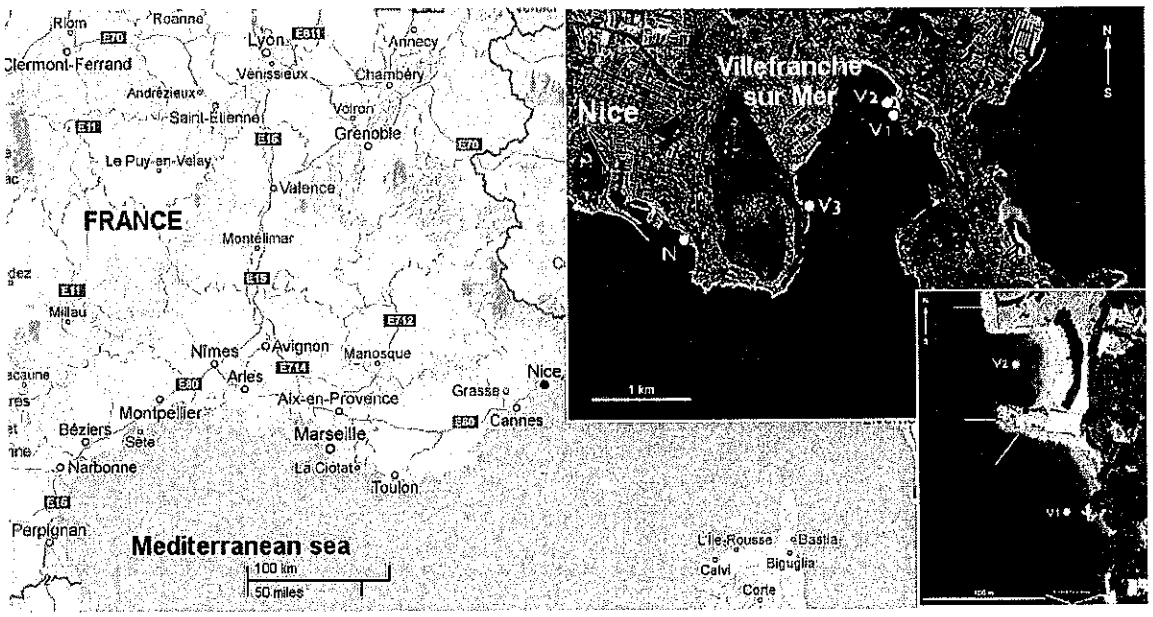



**Fig. 1.** Location of the sampling sites selected for the study. N = Nice, Plage de la Réserve; V1 = Villefranche, beach located below Plage des Jeunes; V2 = Villefranche, Plage des jeunes; V3 = Rochambeau.

Table 1
Information regarding the species sampled in the 4 selected sites.

| Group | Species | Authorities | Common name | Diet[1] | | | Sampling sites | | | | Part analysed[2] | Remarks (mainly major diet) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | H | O | C | N | V1 | V2 | V3 | | |
| Gastropods | *Patella* spp. | Linnaeus, 1758 | Limpet | * | | | * | * | * | * | WF | A mix of *Patella coerueia* (Linnaeus, 1758) and *Patella rustica* (Linnaeus, 1758) |
| Gastropods | *Stramonita haemastoma* | Linnaeus, 1767 | Red-mouthed rock shell | | | * | * | * | * | * | WF | Feeding on bivalves and other small sessile animals having a shell |
| Cephalopods | *Octopus vulgaris* | Cuvier, 1797 | Common Octopus | | | * | | | * | * | DT, RT | Feeding on crustaceans and molluscs |
| Crustaceans | *Eriphia verrucosa* | Forskål, 1775 | Warty crab/Yellow crab | | | * | * | * | | * | WF | Opportunistic that feeds on dead or alive prays |
| Crustaceans | *Maja squinado* | Herbst, 1788 | Spinous spider crab | | | * | | | | * | WF | Feeding on molluscs and small crustaceans |
| Echinoderms | *Paracentrotus lividus* | Lamarck, 1816 | Common sea urchin | * | | | * | * | * | * | DT, roe | Feeding on macroalgae |
| Fish | *Diplodus annularis* | Linnaeus, 1758 | Annular seabream | | | * | | | * | | DT, RT | Feeding on crustaceans, molluscs and echinoderms |
| Fish | *Diplodus sargus* | Linnaeus, 1758 | White seabream | | | * | | | | * | DT, RT | Feeding on crustaceans, molluscs and echinoderms |
| Fish | *Mugil cephalus* | Linnaeus, 1758 | Flathead mullet | | * | | | | * | * | DT, RT | Feeding by grazing various soft or rocky substrates and *Posidonia* leaves |
| Fish | *Mullus surmuletus* | Linnaeus, 1758 | Striped red mullet | | | * | * | * | | * | DT, RT | Feeding on worms and crustaceans found while excaving soft substrates |
| Fish | *Muraena helena* | Linnaeus, 1758 | Mediterranean moray | | | * | | | | * | DT, RT | Feeding on fish, crustaceans and cephalopods |
| Fish | *Sarpa salpa* | Linnaeus, 1758 | Seabream | * | | | * | * | * | * | DT, RT | Feeding behaviour depending on age. Adults feed on macroalgae and *Posidonia* leaves |
| Fish | *Scorpaena porcus* | Linnaeus, 1758 | Scorpionfish | | | * | * | * | * | * | DT, RT | Feeding on crabs, shrimps and fish |
| Fish | *Serranus scriba* | Linnaeus, 1758 | Painted comber | | | * | | | * | | DT, RT | Feeding on crustaceans, molluscs and fish |
| Fish | *Symphodus (Crenilabrus) tinca* | Linnaeus, 1758 | East Atlantic Peacock | | | * | | | | * | DT, RT | Feeding on worms and crustaceans |

(1) H = herbivorous; O = omnivorous; C = carnivorous.
(2) DT = digestive tube; RT = remaining tissue; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

Status of scientific names and authorities were checked in WoRMS (World Register of Marine Species; www.marinespecies.org). All these species are edible and can be eaten either directly or as an ingredient of a fish soup for instance.

### 2.4. Sampling techniques and preparation of the samples

All the species were collected by snorkelling or fishing from the shore, from the surface to 3 m depth maximum. Depending on the species, a variable number of specimens were sampled at the time to have enough quantity of matrix for toxin analysis, while making sure that there would be enough resource in the environment for the whole sampling period. Thus a preliminary sampling campaign showed that in order to obtain a minimum of 10 g of each biological matrix (either as whole animal, digestive tube [DT], roe or remaining tissue [RT]) it was necessary to collect 4 specimens of Warty crabs (*Eriphia verrucosa*), 10 sea urchins (*Paracentrotus lividus*), 15 limpets (*Patella* spp.), 8 red-mouthed rock shells (*Stramonita haemastoma*), 4 seabreams (*Sarpa salpa*) and 3 scorpionfish (*Scorpaena porcus*).

The molluscs and crustaceans were analysed as whole flesh (WF), after removing the shell of the molluscs or the exoskeleton of the crustaceans. For all the other marine organisms that were sampled (cephalopods, echinoderms and fish), the DT was separated from the RT or roe in the case of the sea urchins, and each part was analysed separately. The toxin concentration in the whole flesh, composed of the DT and the roe for sea urchins and DT and RT for the other species (except molluscs and crustacean), was then calculated as the sum of the toxin concentration of the different tissues pondered by the weight of the corresponding tissues.

All the samples were stored at −20 °C until being analysed by the haemolytic test and LC–MS/MS.

### 2.5. Ostreopsis *planktonic and epiphytic abundances*

#### 2.5.1. Planktonic cells

To estimate the abundances of *Ostreopsis* cf. *ovata* planktonic cells in the water column of the four selected sites, 250 mL of sea water were collected in a plastic flask at 0.3 m depth and 0.2 m above the macroalgae that were subsequently sampled to estimate the epiphytic abundances of the dinoflagellate. Three different sampling points were selected for each site; and the abundances of the planktonic cells were determined as the average of the 3 counts.

The water samples were fixed in acidic lugol solution (2%; v/v) and stored at 4 °C in the dark. A 50 mL volume of each water sample was settling during 24 h before counting planktonic *Ostreopsis* cells with an inverted microscope (Utermöhl method, 1958). Concentrations were then reported as number of cells per litre (cells $l^{-1}$).

#### 2.5.2. Epiphytic cells

To estimate the abundances of *Ostreopsis* cf. *ovata* epiphytic cells, ca. 10 g fresh weight (FW) of the macroalgae *Halopteris scoparia* (*Linnaeus*) *Sauvageau* (sampling sites V1, V2 and V3) or *Corallina* spp. (site N; *H. scoparia* was not available) were carefully sampled in a 250 mL plastic flask with the surrounding seawater, avoiding as much as possible any loss of microalgae. Macroalgae samples were vigorously shaken and passed through a 500 μm meshed filter to separate the macroalgae from the water containing the microalgae. The macroalgae were rinsed twice

with 100 mL of 0.2 μm filtered seawater to recover a maximum of microalgae, then dried on absorbent paper and weighed (±0.01 g). Epibenthic cell abundances in the filtered water were evaluated with an optical microscope, using calibrated squared chambers (1 mL; Sedgwick Rafter) and then reported as number of cells per gram of fresh weight of macroalgae (cells/g FW).

### 2.6. Determination of the toxin content of the marine products

#### 2.6.1. Reagents used

All the reagents were of analytical grade unless otherwise specified.

PITX was purchased from Wako Chemical GmbH (Neuss, Germany) as a 100 μg lyophilised powder. The toxin quantity was determined gravimetrically. This standard was 90% pure as estimated chromatographically.

De-ionised water (18.2 mΩ) was obtained using a Milli-Q® purification system (Millipore, Molsheim, France).

Methanol (MeOH) and acetonitrile (MeCN) were of HPLC grade and were purchased from Fisher Scientific (Illkirch, France).

Mouse blood containing lithium heparinate as anti-blotting agent was purchased from Charles River Laboratories (L'Arbresle, France).

#### 2.6.2. Haemolytic test

The haemolytic test is based on the capacity of PITX and analogues to convert the $Na^+/K^+$-ATPase pump into a non specific channel, leading to ion imbalance and delayed haemolysis of mammalian erythrocytes (Habermann et al., 1981). This characteristic haemolytic activity was the basis for the development of variants of haemolytic tests to determine the PITX-group toxin content of marine organisms extracts (Bignami, 1993; Riobó et al., 2008; Taniyama et al., 2002).

In this study, the haemolytic test was used as a first level test to screen a large number of samples. Therefore, we considered the sensitivity of the test for the PITX-group toxin as the most important aspect and consequently decided to use mouse red blood cells due to their high sensitivity to PITXs haemolytic activities (Habermann et al., 1981). Furthermore, each positive sample was tested for specificity with ouabain acting as an ATPase blocker and was finally confirmed by an LC–MS/MS analysis.

*2.6.2.1. Extraction procedure.* A 2.5 g portion of marine product homogenate was extracted with 7.5 mL of 50% aqueous MeOH. The mixture was homogenised at 12,000 rpm for 2 min using an Ultra-turrax. After centrifuging the extract at 3700 × *g* for 10 min, the supernatant was transferred to a 25 mL volumetric flask, while the pellet was re-extracted with 7.5 mL of 50% aqueous MeOH. The extract was centrifuged and the supernatant was transferred to the same volumetric flask. The volume was made up to 25 mL using Milli-Q water, bringing the MeOH concentration down to 30% and the final extract was filtered through a 0.45 μm PET filter (Chromafil Xtra PET 45/25 Macherey-Nagel, Hoerd, France).

*2.6.2.2. Haemolytic test.* A series of 11 calibration points was prepared using the PITX standard, with concentrations ranging from 0.0 (blank) to 5.0 ng/mL (1.9 pM). Similarly, the sample extracts had to be diluted several folds to enable the quantification of the toxin content. The dilution factors applied were comprised between 1/2 and 1/256. All dilutions were made in 30% aqueous MeOH.

A 50 μL aliquot of the diluted extract or calibration point was added to 950 μL of phosphate buffer saline (PBS) containing 0.5% (v/v) of mouse erythrocytes obtained from Charles River Laboratories (L'Arbresle, France). The number of erythrocytes in the PBS buffer containing 0.5% of mouse erythrocytes was checked as a quality control; the average count over 131 determinations was $5.7 \times 10^7$ cells (relative standard deviation = 9.7%). The solution was incubated at 37 °C for 4 hours and centrifuged at 1000 × *g* for 10 min. A 200 μL aliquot of each extract or calibration point was put in triplicate in a 96-well plate (Nunc microwell F96 PS NST, Fisher Scientific) and the optical density (OD) was read at 450 nm with a spectrophotometer (MRX 3100, Dynex Technologies). The percentage of lysis is a linear function of the OD (ratio of the difference between OD and the blank OD (OD min) and the difference between the maximum OD and the blank OD). The relationship between the percentage of lysis and the concentration of palytoxin is a sigmoïdal curve that is transformed in a linear curve by using the logit/log model. The percentage of lysis of an extract was determined from the OD obtained for each dilution of this extract. The toxin concentration in the extract was determined using the logit/log model and was calculated using the linear part of the curve Logit (% lysis) = $a \log X + b$, giving the following equations:

$$\text{concentration } X \text{ (in ng/mL)} = 10^{(\text{Logit}-b)/a} \times \text{dilution factor}$$

with

$$\text{Logit} = \ln \frac{\%\,\text{lysis}}{1 - \%\,\text{lysis}}$$

and

$$\%\,\text{lysis} = \frac{OD - OD_{min}}{OD_{max} - OD_{min}} = \text{slope}_{\text{calibration curve}} \times OD + \text{intercept}_{\text{calibration curve}}$$

As the haemolytic potency was estimated using PITX as a calibrant, the results were expressed in microgram of PITX equivalent per kilogram of the analysed matrix (μg PITX eq./kg).

The limit of quantification (LOQ) of the haemolytic test was determined as 1.2 μg PITX eq./kg.

The specificity of the haemolytic activity of PITX was confirmed on the positive samples by adding 50 μL aliquot of the diluted extract or calibration point to 950 μl phosphate buffer saline containing 0.5% (v/v) of mouse erythrocytes and 500 μM of ouabain pre-incubated at 37 °C for 1 h. In the presence of PITX and ouabain a shift in the response curve compared to the curve without ouabain was observed.

#### 2.6.3. Tandem mass spectrometry (LC–MS/MS) analysis

*2.6.3.1. Extraction procedure.* A 2 g portion of marine product homogenate was extracted with 10 mL of 80% aqueous MeOH. This solvent was shown to be the most efficient by Ciminiello and co-workers in 2011 (2011a).The mixture was homogenised at 10,000 rpm for 2 min using a Polytron and then centrifuged at 4000 × *g* for 8 min. The supernatant was transferred to a 20 mL volumetric flask, while the pellet was re-extracted with 9 mL of 80% aqueous MeOH. After being centrifuged the second extract was transferred to the same 20 mL volumetric flask and the volume was completed to the mark using 80% aqueous MeOH. The extract was then filtered using a 0.45 μm PTFE filter (Chromafil Xtra PTFE 45/25 Macherey-Nagel, Hoerd, France).

*2.6.3.2. Solid-phase extraction (SPE) cleanup.* Prior to LC–MS/MS analysis, the extracts were purified using StrataX (60 mg/3 mL) cartridges (Phenomenex, France).

The cartridges were placed on a Manifold and conditioned with 3 mL of MeOH and 3 mL of 30% aqueous MeOH.

In a 15 mL tube, 4.5 mL of extract were added to 7.5 mL of Milli-Q water to bring the MeOH concentration down to 30%. This solution was then loaded and passed through the cartridge. A

washing step was carried out, using 3 mL of 10% aqueous MeOH and the cartridge was run dry. The elution was performed with 3 mL of 90% aqueous MeOH containing 0.5% of formic acid and the cartridge was run dry to collect the elutant in a 5 mL amber tube.

*2.6.3.3. LC–MS/MS analysis.* The LC–MS/MS analyses were performed on a Dionex Ultimate 3000 (Dionex, France) coupled to an API4000 Qtrap mass spectrometer, a triple quadrupole – linear ion trap hybrid (AB Sciex, Les Ulis, France), equipped with an electrospray ionisation source. The toxins were separated on a Phenomenex Gemini C18 column (150 mm × 2 mm; 3 μm) equipped with a guard column (20 mm × 2 mm; 3 μm) of the same phase. A gradient of mobile phase A (100% $H_2O$ with 30 mM acetic acid) and B (95% aqueous MeCN with 30 mM acetic acid) was run at 200 μL/min starting at 0% B and rising to 100% B in 12 min. The gradient was held at 100% B for 5 min and was decreased to 0% B in 0.1 min. This composition was held for 7.9 min, until the end of the run.

PITX and palytoxin-like compounds, OVTX-a and OST-D, were analysed by using an ESI triple-quadrupole MS instrument in positive mode with an ionspray voltage of 5.5 kV. MRM experiments were carried out by selecting three transitions (precursor ion → - product ion). For palytoxin, the selected transitions were *m/z* 1340.9 → 327.3 ($[M+2H]^{2+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$), *m/z* 1331.9 → 327.3 ($[M+2H\text{-}H_2O]^{2+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$) and *m/z* 876.2 → 327.3 ($[M+^3H\text{-}^3H_2O]^{3+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$). The analogues share this same fragmentation behaviour with respectively for OVTX-a *m/z* 1324.9 → 327.3, *m/z* 1315.9 → 327.3 and *m/z* 865.5 → 327.3 and for OST-D *m/z* 1318.9 → 327.3, *m/z* 1309.9 → 327.3 and *m/z* 861.5 → 327.3. The most intense transition was used for quantification purpose ($[M+2H\text{-}H_2O]^{2+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$).

The toxin concentrations in the analysed samples were determined from a PITX external calibration curve. OVTX-a and OST-D were quantified using PITX as calibrant, assuming an equimolar response for all these 3 toxins.

The limit of detection (LOD) and LOQ of the LC–MS/MS method were determined as 7.4 and 24.5 μg PITX/kg, respectively.

## 3. Results

### 3.1. Monitoring of the site N (Nice)

The site N located in Nice was sampled from week 24 to 36 of the year 2009 (Table 2). The epiphytic *Ostreopsis* cf. *ovata* cells started blooming in week 28 and reached a maximum concentration of about 120,000 cells/g FW in 3 weeks (Fig. 2). The toxin levels determined by the haemolytic test in the sampled marine products followed the same trend as the *Ostreopsis* cells with the highest concentrations observed in week 31 for the Warty crab *Eriphia verrucosa* whole flesh (38.4 μg PITX eq./kg) and the seabream *Sarpa salpa* (29.7 μg/kg). The toxin concentration in the whole flesh of the sea urchin *Paracentrotus lividus* reached a maximum of 61.6 μg/kg in week 32; this organism had the highest toxin concentrations recorded on Nice coast. The red-mouthed rock shell *Stramonita haemastoma* was also found to be contaminated with a maximum level of 34.1 μg/kg in the whole flesh, in week 34. From all the sampled marine products, limpets belonging to the genera *Patella* were the only animals for which the highest toxin concentration (7.5 μg/kg) was recorded in week 30 that is before the maximum of the *O. cf. ovata* epibenthic abundance. Over the course of the sampling period, the scorpionfish *Scorpaena porcus* and the striped red mullet *Mullus surmuletus* had toxin levels below the limit of quantification (LOQ) of the haemolytic test (1.2 μg PITX eq./kg).

From the 7 species that were sampled in site N and analysed, only three had toxin levels in the whole flesh above 30 μg PITX eq./kg; this echoes to the EFSA opinion reporting that the toxin level in the particular case of shellfish should not exceed 30 μg PITX + OST-D/kg in order to protect the consumers' health. These species are respectively the Warty crab *Eriphia verrucosa* (week 31), the sea urchin *Paracentrotus lividus* (weeks 32 and 34) and the red-mouthed rock shell *Stramonita haemastoma* (week 34). The toxin concentration in the seabream *Sarpa salpa* in week 31 was equal to 29.7 μg PITX eq./kg of whole flesh.

The analysis of the different tissues (DT, roe, RT) of the sampled marine products showed that the highest toxin concentration was found in the seabream DT (230 μg/kg) in week 31 (Fig. 3); this toxin level in the DT brought the toxin level in the whole flesh to 29.7 PITX eq./kg. On two other occasions (weeks 30 and 34), this species (*Sarpa salpa*) had toxin levels in its DT of 66.5 and 99.0 μg/kg respectively. In weeks 31, 32 and 34, the toxin levels found in the sea urchin DT were respectively 41.9, 97.6 and 50.7 μg PITX eq./kg. However, it is important to notice that despite high toxin levels in the DT of some of the sampled species, the roe of the sea urchins or RT of the other animals were not contaminated (levels below the LOQ). Trace amounts of PITX-like compounds were found in the DT of the scorpionfish in week 26 (3.6 μg PITX eq./kg) and the striped red mullet DT in week 34 (3.3 μg PITX eq./kg).

**Table 2**
Palytoxins concentration (μg/kg) determined by the haemolytic test in different parts of fishery products harvested in Nice (site N) and *Ostreopsis* cf. *ovata* abundances from week 24 to week 36.

| Common name | Latin name | wk 24 | wk 26 | wk 28 | wk 30 | wk 31 | wk 32 | wk 34 | | wk 36 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Nice (N)* | | | | | | | | | | |
| **Patella WF** | *Patella spp* | <LOQ | <LOQ | <LOQ | **7.5** | **1.2** | **2.6** | **2.6** | – | <LOQ |
| **Red-mouthed rock shell WF** | *Stramonita haemastoma* | <LOQ | <LOQ | <LOQ | **2.9** | **6.1** | **13.0** | **34.1** | – | **5.1** |
| **Sea urchin roe** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 1.4 | <LOQ | – | <LOQ |
| **Sea urchin DT** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **24.4** | **41.9** | **97.6** | **50.7** | – | **5.8** |
| **Sea urchin WF** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **14.6** | **26.6** | **61.6** | **34.7** | – | 3.0 |
| **Warty crab WF** | *Eriphia verrucosa* | <LOQ | <LOQ | <LOQ | **1.1** | **38.4** | **3.2** | **9.5** | – | <LOQ |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | – | <LOQ |
| **Scorpionfish DT** | *Scorpaena porcus* | <LOQ | **3.6** | <LOQ | **1.4** | **2.2** | **1.2** | **1.9** | – | <LOQ |
| Scorpionfish WF | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | – | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| **Seabream DT** | *Sarpa salpa* | <LOQ | <LOQ | **3.9** | **66.5** | **230.0** | **26.2** | **15.6** | **99.0** | **1.2** |
| Seabream WF | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | **12.1** | **29.7** | **5.5** | **2.9** | **21.2** | <LOQ |
| Red mullet RT | *Mullus surmuletus* | – | – | – | – | – | – | <LOQ | – | – |
| **Red mullet DT** | *Mullus surmuletus* | – | – | – | – | – | – | **3.3** | – | – |
| Red mullet WF | *Mullus surmuletus* | – | – | – | – | – | – | <LOQ | – | – |
| *Ostreopsis* cf. *ovata* | **(cells/g fresh weight)** | **189** | **166** | **4749** | **20,786** | **120,736** | **18,054** | **9325** | | **4004** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

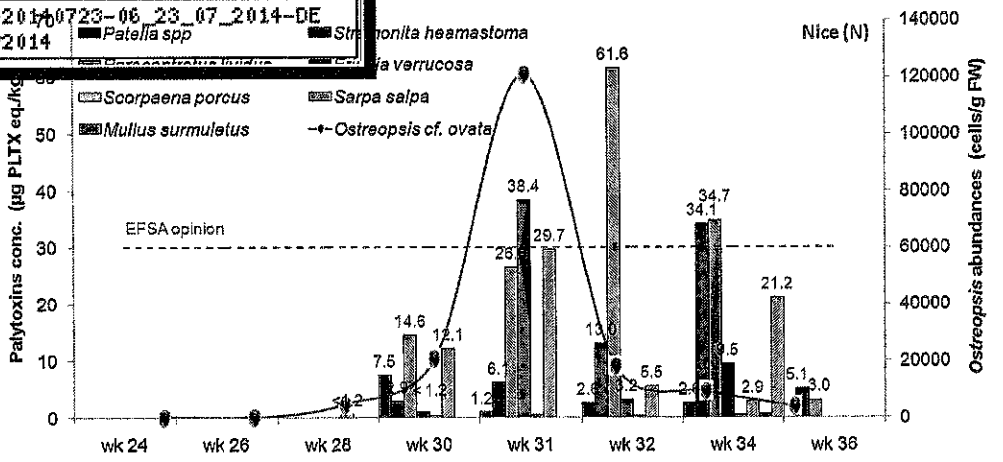



Fig. 2. Palytoxins concentration determined by the haemolytic test in the whole flesh of the fishery products harvested in Nice (site N) from week 24 to week 36. The dashed line represents the threshold of 30 µg PITX + OST-D/kg proposed by the EFSA and specifically applicable to shellfish. The blue curve represents the *Ostreopsis* abundances on the same period. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Similarly to the other species, no toxin was detected in the RT of both fish species (*Scorpaena porcus*, *Mullus surmuletus*).

### 3.2. Monitoring of the site V1 (Villefranche)

The site V1 located in Villefranche was sampled from week 23 to week 39 of the year 2009 (Table 3). Similar to what was observed in Nice, the highest *Ostreopsis* cell abundances were recorded in week 31 but with somewhat lower concentrations (ca. 39,000 Cells/g FW). The highest toxin concentration was detected in the whole flesh of the seabream *Sarpa salpa* in week 31 with 16.4 µg PITX eq./kg. Inversely to what was observed in Nice, the highest toxin concentrations in limpets of the genus *Patella* (5.4 µg/kg) coincided with the highest cell counts in week 31. The concentrations of equivalent PITX in the whole flesh of the sea urchin *Paracentrotus lividus* ranged from 1.4 to 5.5 µg/kg in weeks 29 and 30, respectively. The toxin levels measured in the red-mouthed rock shell *Stramonita haemastoma* were comprised between 1.2 and 3.4 µg PITX eq./kg. All the samples of the Warty crab *Eriphia verrucosa*, the scorpionfish *Scorpaena porcus* and the red mullet *Mullus surmeletus* had PITX levels below the LOQ of the haemolytic test.

The analysis of the different tissues of the species showed that the toxins were sequestered in the DT of the sea urchins (7.4 µg PITX eq./kg) and the seabream *Sarpa salpa* (79.4 µg PITX eq./kg), while the corresponding roe or RT did not contain quantifiable PITX levels.

### 3.3. Monitoring of the site V2 (Villefranche – Plage des Jeunes)

The results of the monitoring carried out on the Plage des Jeunes (site V2), in Villefranche, are presented in Table 4. A wider panel of species was sampled from week 30 to week 35 of the year 2009, with notably more fish samples. The cell abundances of the epiphytic *Ostreopsis* cf. *ovata* showed that the dinoflagellate reached a maximum concentration in week 31 with ca. 336,000 cells/g FW. This is by far the highest cell abundances recorded in the four sites sampled during this study.

In week 31 and 33, the whole flesh of the sea urchin *Paracentrotus lividus* had equivalent PITX concentrations of 67.5 and 33.7 µg/kg, respectively (Table 4). All the other species had much lower PITX concentrations, the red-mouthed rock shell *Stramonita haemastoma* being the second highest concentrated sample with 10.8 µg PITX eq./kg (week 33), followed by the spinous spider crab *Maja squinado* (8.3 µg PITX eq./kg in week 33), by the seabream *Sarpa salpa* (6.3 µg PITX eq./kg in week 33), by the limpets *Patella* spp. (4.8 µg PITX eq./kg in week 33) and by *Octopus vulgaris* (2.8 µg PITX eq./kg in week 35). All the other fish species

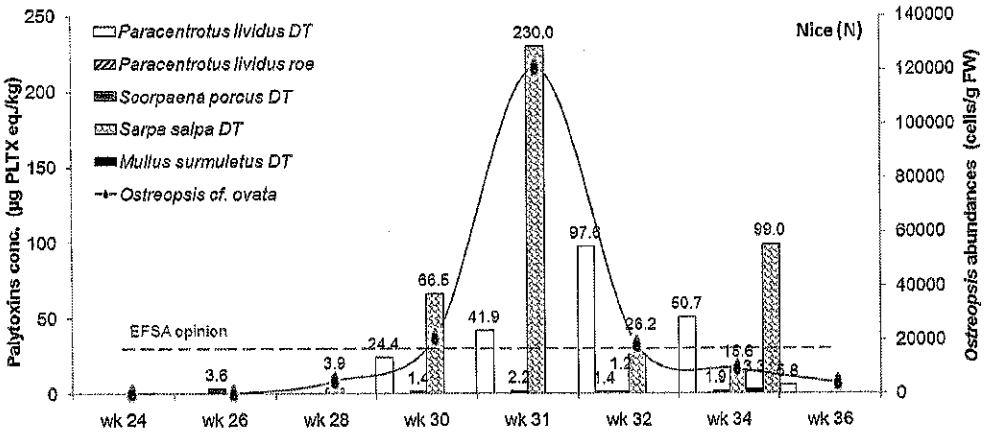



Fig. 3. Palytoxins concentration determined by the haemolytic test in the different parts of the fishery products harvested in Nice (site N) from week 24 to week 36. DT = digestive tube; RT = remaining tissue. The dashed line represents the threshold of 30 µg PITX + OST-D/kg proposed by the EFSA and specifically applicable to shellfish. The blue curve represents the *Ostreopsis* abundances on the same period.

Table 3
Palytoxins concentration (μg/kg) determined by the haemolytic test in different parts of fishery products harvested in Villefranche (site V1) and *Ostreopsis* cf. *ovata* abundances from week 23 to week 39.

| Common name | Latin name | wk 23 | wk 25 | wk 27 | wk 29 | wk 30 | wk 31 | wk 33 | wk 35 | wk 37 | wk 39 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Villefranche (V1)* | | | | | | | | | | | |
| **Patella WF** | *Patella spp* | <LOQ | <LOQ | <LOQ | **1.2** | **1.8** | **5.4** | **4.9** | <LOQ | <LOQ | <LOQ |
| **Red-mouthed rock shell WF** | *Stramonita haemastoma* | <LOQ | <LOQ | <LOQ | <LOQ | **2.6** | **1.2** | **3.4** | <LOQ | <LOQ | <LOQ |
| Sea urchin roe | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| **Sea urchin DT** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **2.0** | **7.4** | **2.1** | <LOQ | <LOQ | <LOQ | <LOQ |
| **Sea urchin WF** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **1.4** | **5.5** | **1.6** | <LOQ | <LOQ | <LOQ | <LOQ |
| Warty crab WF | *Eriphia verrucosa* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Scorpionfish DT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Scorpionfish WF | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Seabream DT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | **8.7** | **79.4** | **4.1** | <LOQ | **1.2** | <LOQ |
| Seabream WF | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | **1.9** | **16.4** | <LOQ | <LOQ | <LOQ | <LOQ |
| Red mullet RT | *Mullus surmuletus* | – | – | – | – | – | – | – | <LOQ | – | – |
| Red mullet DT | *Mullus surmuletus* | – | – | – | – | – | – | – | <LOQ | – | – |
| Red mullet WF | *Mullus surmuletus* | – | – | – | – | – | – | – | <LOQ | – | – |
| *Ostreopsis* cf. *ovata* | **(Cells/g fresh weight)** | **167** | **60** | **3580** | **5813** | **14,836** | **38,922** | **4577** | **2054** | **1919** | **1913** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

(*Scorpaena porcus*, *Mullus surmuletus*, *Serranus scriba*, *Mugil cephalus* and *Diplodus annularis*) had toxin levels below the LOQ of the haemolytic test (1.2 μg PITX eq./kg).

The results regarding the distribution of the toxins between tissues of the species are presented in Table 4. The DT is again the most contaminated part of the sea urchin (*Paracentrotus lividus*) and the octopus (*Octopus vulgaris*), with respectively 110.7 and 6.4 μg PITX eq./kg; however, for both species lower toxin levels were detected in the roe or the RT with respectively 6.8 and 2.5 μg PITX eq./kg. As for the fish samples of scorpionfish (*Scorpaena porcus*), seabream (*Sarpa salpa*), and annular seabream (*Diplodus annularis*), toxins were detected in the DT with values ranging from 3.2 (*S. porcus*) to 25.6 μg PITX eq./kg (*S. salpa*), while the RT were not contaminated.

### 3.4. Monitoring of the site V3 (Rochambeau)

The site of Rochambeau (V3) was monitored bimonthly from week 32 to 36 of the year 2009; the maximum *Ostreopsis* cf. *ovata* abundances were recorded in week 32 with ca. 52,700 cells/g FW (Table 5) and rapidly declined in the following weeks reaching roughly 8800 and 4300 cells/g FW in week 34 and 36 respectively. The maximum toxin concentrations in the whole flesh were

**Table 4**
Palytoxins concentration (μg/kg) determined by the haemolytic test in different parts of fishery products harvested in Villefranche Plage des jeunes (site V2) and *Ostreopsis* cf. *ovata* abundances from week 30 to week 35.

| Common name | Latin name | wk 30 | wk 31 | wk 33 | wk 35 |
|---|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Villefranche plage des jeunes (V2)* | | | | | |
| **Spinous spider crab WF** | *Maja squinado* | – | – | **8.3** | – |
| **Red-mouthed rock shell WF** | *Stramonita haema stoma* | – | – | **10.8** | **2.0** |
| **Patella WF** | *Patella spp* | – | **4.5** | **4.8** | **1.2** |
| Flathead mullet RT | *Mugil cephalus* | – | – | <LOQ | – |
| Flathead mullet DT | *Mugil cephalus* | – | – | <LOQ | – |
| Flathead mullet WF | *Mugil cephalus* | – | – | <LOQ | – |
| Sea urchin roe | *Paracentrotus lividus* | – | **6.8** | <LOQ | <LOQ |
| Sea urchin DT | *Paracentrotus lividus* | – | **110.7** | **53.0** | **4.5** |
| Sea urchin WF | *Paracentrotus lividus* | – | **67.5** | **33.7** | **2.6** |
| **Octopus RT** | *Octopus vulgaris* | – | – | – | **2.5** |
| **Octopus DT** | *Octopus vulgaris* | – | – | – | **6.4** |
| **Octopus WF** | *Octopus vulgaris* | – | – | – | **2.8** |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ |
| **Scorpionfish DT** | *Scorpaena porcus* | **3.7** | <LOQ | **3.2** | <LOQ |
| Scorpionfish WF | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ |
| Red mullet RT | *Mullus surmuletus* | – | – | – | <LOQ |
| Red mullet DT | *Mullus surmuletus* | – | – | – | <LOQ |
| Red mullet WF | *Mullus surmuletus* | – | – | – | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | – | <LOQ | <LOQ |
| **Seabream DT** | *Sarpa salpa* | **7.6** | – | **25.6** | <LOQ |
| **Seabream WF** | *Sarpa salpa* | < LOQ | – | 6.3 | <LOQ |
| Painted comber RT | *Serranus scriba* | – | – | <LOQ | – |
| Painted comber DT | *Serranus scriba* | – | – | <LOQ | – |
| Painted comber WF | *Serranus scriba* | – | – | <LOQ | – |
| Annular seabream RT | *Diplodus annularis* | – | – | <LOQ | – |
| **Annular seabream DT** | *Diplodus annularis* | – | – | 10.6 | – |
| Annular seabream WF | *Diplodus annularis* | – | – | <LOQ | – |
| **Ostreopsis cf. ovata** | **(cells/g fresh weight)** | **100,615** | **336,124** | **23,131** | **18,487** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

Table 4
Palytoxins concentration (μg/kg) determined by the haemolytic t in different parts of fishery products harvested in Rochambeau (site V3) and *Ostreopsis* cf. *ovata*

| Common name | Latin name | wk 32 | wk 34 | wk 36 |
|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Rochambeau (V3)* | | | | |
| **Patella WF** | *Patella spp* | **12.7** | **3.8** | **1.2** |
| **Red-mouthed rock shell WF** | *Stramonita heamastoma* | **13.8** | **4.3** | **3.4** |
| **Warty crab WF** | *Eriphia verrucosa* | **4.9** | <LOQ | <LOQ |
| **spinous spider crab WF** | *Maja squinado* | – | **2.0** | – |
| Sea urchin roe | *Paracentrotus lividus roe* | <LOQ | <LOQ | <LOQ |
| **Sea urchin DT** | *Paracentrotus lividus* | **256.6** | **4.5** | **5.2** |
| **Sea urchin WF** | *Paracentrotus lividus* | **179.6** | **2.6** | **3.2** |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ |
| **Scorpionfish DT** | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ |
| **Scorpionfish WF** | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ |
| Seabream DT | *Sarpa salpa* | **25.1** | **8.4** | **1.2** |
| Seabream WF | *Sarpa salpa* | **4.8** | **1.3** | <LOQ |
| East Atlantic peacock wrasse RT | *Symphodus tinca* | <LOQ | <LOQ | – |
| East Atlantic peacock wrasse DT | *Symphodus tinca* | **1.9** | **1.8** | – |
| East Atlantic peacock wrasse WF | *Symphodus tinca* | <LOQ | <LOQ | – |
| Flathead mullet RT | *Mugil cephalus* | <LOQ | <LOQ | – |
| **Flathead mullet DT** | *Mugil cephalus* | **392.2** | **72.8** | – |
| **Flathead mullet WF** | *Mugil cephalus* | **53.5** | **13.2** | – |
| Mediterranean moray RT | *Muraena helena* | – | <LOQ | – |
| Mediterranean moray DT | *Muraena helena* | – | <LOQ | – |
| Mediterranean moray WF | *Muraena helena* | – | <LOQ | – |
| **Octopus RT** | *Octopus vulgaris* | – | **18.5** | – |
| **Octopus DT** | *Octopus vulgaris* | – | **1.3** | – |
| **Octopus WF** | *Octopus vulgaris* | – | **16.9** | – |
| **White seabream RT** | *Diplodus sargus* | <LOQ | **1.2** | – |
| **White seabream DT** | *Diplodus sargus* | **5.1** | **1.4** | – |
| **White seabream WF** | *Diplodus sargus* | <LOQ | **1.2** | – |
| **Ostreopsis cf. ovata** | **(cells/g fresh weight)** | **52,734** | **8771** | **4345** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

obtained in week 32: the sea urchin *Paracentrotus lividus* was by far the most contaminated species with 179.6 μg PlTX eq./kg, followed by the flathead mullet *Mugil cephalus* (53.5 μg PlTX eq./kg), the red-mouthed rock shell *Stramonita haemastoma* (13.8 μg PlTX eq./kg) the limpet *Patella* spp. (12.7 μg PlTX eq./kg), the Warty crab *Eriphia verrucosa* (4.9 μg PlTX eq./kg) and the seabream *Sarpa salpa* (4.8 μg PlTX eq./kg). In the following weeks, the toxin levels decreased but some species showed contamination with a maximum of 16.9 μg PlTX eq./kg (*Octopus vulgaris*). During the sampling period, some species never showed contamination in the whole flesh: this includes the scorpionfish (*Scorpaena porcus*), the East Atlantic peacock (*Symphodus tinca*), the Mediterranean moray (*Muraena helena*) and the white seabream (*Diplodus sargus*).

The results regarding the distribution of the toxins between the different tissues of the marine organisms are presented in Table 5. Similar to what was observed on the other sites, the DT was the most contaminated tissue with the highest contamination levels being observed for the flathead mullet *Mugil cephalus* and the sea urchin *Paracentrotus lividus* (392.2 and 256.6 μg PlTX eq./kg, respectively). *Octopus vulgaris* was the only species having a toxin concentration higher in the RT (18.5 μg PlTX eq./kg) than in the DT (1.3 μg PlTX eq./kg), contrary to what was observed for the same species in Villefranche – Plage des jeunes (site V2).

### 3.5. Comparison of the toxin levels determined by haemolytic test and LC–MS/MS

All the samples analysed by the haemolytic test and that gave a result above the LC–MS/MS LOQ (24.5 μg PlTX/kg) were analysed using the latter technique to determine the toxin profile.

As shown in Fig. 4, the LC–MS/MS analyses showed that the toxin profile of the samples was composed of OVTX-a as the major

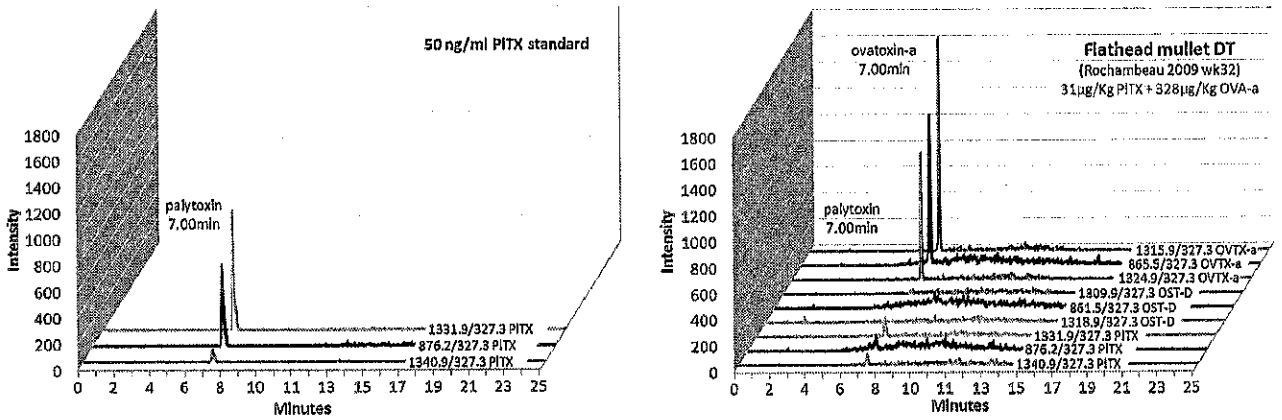



Fig. 4. LC–MS/MS chromatograms of palytoxin standard solution and flathead mullet contaminated sample by OVTX-a and PlTX.

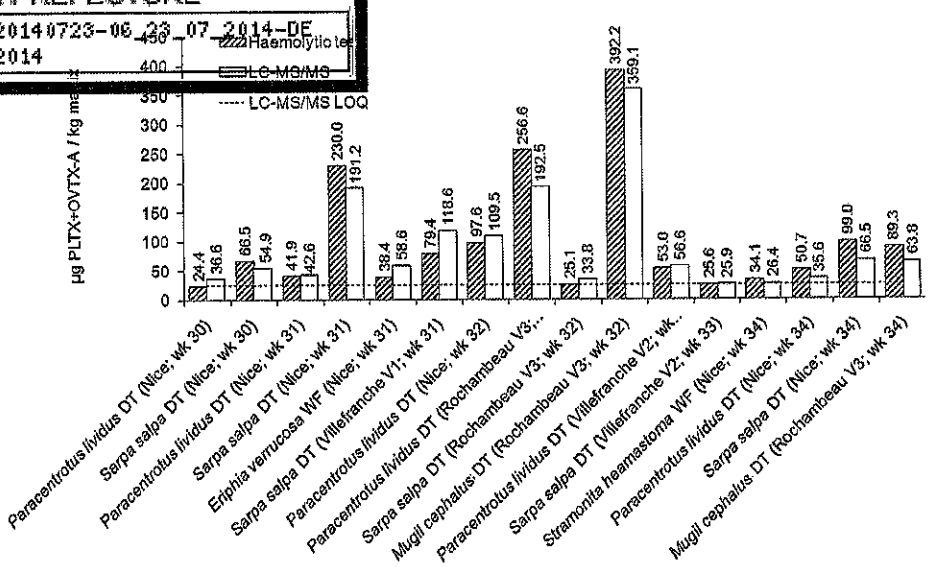



**Fig. 5.** Comparison of the Palytoxins levels determined by the haemolytic test and by LC–MS/MS analysis in the marine products harvested in the four different sampling sites. WF = whole flesh; DT = digestive tube.

compound (90% of the toxin profile) while PITX only represented 10%. No trace of OST-D was detected in any of the samples.

The results obtained using both methods (haemolytic test and LC–MS/MS) are presented in Fig. 5. As shown in Fig. 6, there is a good agreement between both sets of results whatever the species and the nature of the sample (whole flesh or digestive tube), with a $R^2$ of 0.9533.

The ratio calculated by normalising the LC–MS/MS result with that of the haemolytic test ranges from 0.7 to 1.5 with the median value being comprised between 0.9 and 1.0.

## 4. Discussion

### 4.1. Ostreopsis *abundances and monitoring*

To evaluate the cell abundances in the four sites sampled as part of the present study, it was decided to rely on the epiphytic cells rather than the planktonic ones. This choice was based on the fact that benthic cells are considered to be more conservative as being less susceptible to hydrodynamics disturbances (Mangialajo et al., 2011). The planktonic cell abundances were yet determined for

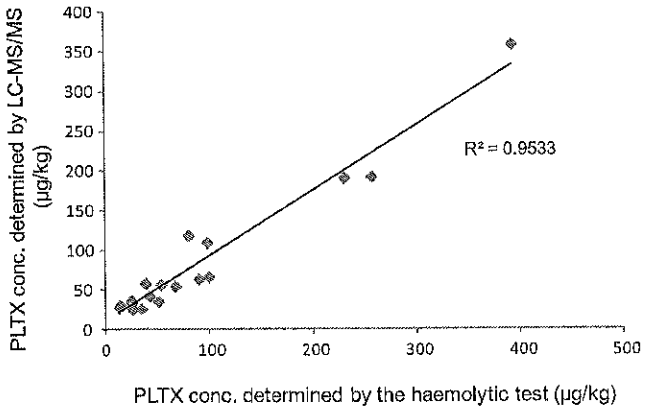



**Fig. 6.** Relationship between the PITX concentrations determined using the LC–MS/MS method and the haemolytic test. For the LC–MS/MS method, the toxin concentration was determined as the sum of OVTX-a and PITX, both detected in the samples.

informative purposes in Nice (N), Villefranche (Sites V1 and V2) and Rochambeau (V3) and the maximum values coincided temporally with the corresponding benthic cell abundances (data not shown). This correlation between epiphytic and planktonic cell abundances has already been reported in the literature (Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008; Vila et al., 2001) and this goes along with the hypothesis of the epiphytic cells being the stock of the available *Ostreopsis* cf. *ovata* biomass (Mangialajo et al., 2011). Some authors reported a delay between the benthic and the planktonic cell abundances as a result of different phenomena such as cell re-suspension due to wave action (Totti et al., 2010), to human activities (boat traffic, human trampling; (Mangialajo et al., 2011)) or to vertical migration on a daily cycle (Vila et al., 2001). Mangialajo and co-workers (2011) hypothesised that there may be a link between vertical migration and a "potential critical density threshold" above which the epiphytic cells would move up and down in the water column, thus turning into a planktonic state.

The *Ostreopsis* cell abundances recorded in the four sites that were sampled in 2009 varied greatly with the highest values being found in Villefranche – Plage des jeunes (site V2) with more than 330,000 cells/g macroalgae FW, followed by Nice (ca. 120,000 cells/g FW). The two remaining sites had much lower cell abundances with ca. 53,000 and 39,000 cells/g FW for Rochambeau (V3) and Villefranche (V1), respectively. In all sites except for Rochambeau, the maximum cell abundances were recorded in week 31; in site V3 the highest abundance was recorded in week 32. However, data collected as part of another research programme, MediOs (Méditerranée *Ostreopsis*), showed that the *Ostreopsis* abundances in Rochambeau ranged from ca. 193,000 to ca. 794,000 cells/g FW between week 29 and week 31 in 2009, with the highest counts being recorded in week 30 (data not shown).

Despite general characteristics of the sites (e.g. rocky type, conformation of the coast, macroalgal communities, wave exposure) that can be considered as comparable, some sites seem to be more prone to *Ostreopsis* development than others (Mangialajo et al., 2011). The influence of environmental factors on *Ostreopsis* growth in comparable sites (e.g. shallow waters and rocky areas) is not clear and is a subject of controversy. It makes it difficult to select a monitoring point that would be representative of the cell abundances in a larger area.

As the epiphytic *Ostreopsis* cells are more conservative and represent the stock of available biomass, Mangialajo and co-workers (2011) recommend to policy makers and managers to primarily monitor benthic abundances and to increase sampling frequency during high season (July in NW Mediterranean). According to the data collected as part of our study, the high season would spread from mid-July to mid-August, as the highest cell abundances occurred during this time frame.

Because PITX and its analogues are not regulated yet, either within the EU or internationally, the determination of *Ostreopsis* cell abundances sometimes relies on research projects that are limited in time when it should be included in perennial monitoring programmes. The latter should specifically be dealing with the risk associated with the direct exposure to *Ostreopsis*, via the water (recreational activities) and/or with the consumption of contaminated food organisms.

In France, shellfish production areas located on the coastlines are monitored as part of the Rephy network of Ifremer, an official network mandated by the Ministry of agriculture aiming at monitoring the phytoplankton and the phycotoxins. In the Mediterranean areas at risk regarding *Ostreopsis*, a preventive system has been put in place, with a threshold of 4000 *Ostreopsis* planktonic cells per litre triggering the flesh testing in shellfish present in the area or close by (Ifremer, 2011), even though there is no shellfish production areas in the sites known to be affected by blooms of *Ostreopsis*. The professional or leisure harvesting of sea urchins on the Mediterranean coast is forbidden by local regulation, every year generally from the 1st of May to the 31st of October; therefore there is no monitoring of this species for the presence of PITX during this timeframe. However, during the winter the monitoring is carried out by Ifremer (French Research Institute for Exploitation of the Sea) on a few selected sites, a month before and during the harvesting season (generally from the 1st of November to the 31st of March). The phytoplankton is not used as a trigger but the analysis of sea urchins is directly performed by LC–MS/MS, on a monthly basis; this frequency can be increased to weekly when the contamination levels in PITX and analogues are above the LC–MS/MS LOQ (ca. 25 μg/kg), and will be brought back to monthly as soon as the toxin levels drop below the LOQ (Ifremer, 2011). Although there is no PITX regulation in France, the competent authorities have already foreseen risks management measures to protect human health. The recommendations to be applied between the 15th of June and the 15th of September associated with the consumption of marine organisms coming from leisure activities and from locations where *Ostreopsis* is present are the following: (i) the fish should be eviscerated prior to consumption, (ii) there is a reminder about the period during which the harvesting of sea urchins is forbidden, (iii) all the other marine organisms should not be eaten (DGS, 2010).

Putting in place an efficient monitoring system is not an easy task as many parameters cannot be controlled. For example the toxicity of *Ostreopsis* has to be taken into account, as the sites having the highest abundances may not relate to the most contaminated marine products. This was observed as part of the present study, where the analysis of the water and macroalgae samples collected in the four sites revealed that the site V2 (Villefranche – Plage des jeunes) had cell abundances ca. six folds higher than those in V3 (Rochambeau), and yet the animal species from the latter site had the highest contamination levels.

### 4.2. PITX levels in the marine products

In the present study, herbivorous, omnivorous and carnivorous species were sampled in the four selected sites. There seems to be a relation between the contamination levels of the different animals and their feeding behaviour, as the highest levels were generally found in the herbivorous or omnivorous species. These species, that is the limpet *Patella* spp., the sea urchin *Paracentrotus lividus*, and the fish *Sarpa salpa* and *Mugil cephalus*, were found to be contaminated as determined by the haemolytic test. This can be explained by the grazing behaviour of the animals that feed directly from substrates covered with epiphytic *Ostreopsis* cells. The case of the seabream *S. salpa* is interesting as this fish adapts its feeding behaviour; juveniles (<5 cm) can feed on planktonic aggregates of *Ostreopsis* cf. *ovata* in the water column while the sub-adults and adults have an herbivorous diet and graze on macroalgae colonised by *Ostreopsis*. Whatever its life stage, in the event of *Ostreopsis* blooms, this fish is likely to be contaminated and would be a good sentinel species.

A wider range of carnivorous marine animals (11 different species) was sampled and not all of them were contaminated. While some species such as the mollusc *Stramonita haemastoma* and the crustacean *Eriphia verrucosa* had the highest toxin levels, sometimes close to those of the herbivorous species, other predators especially fish species were never found to be contaminated: the Mediterranean moray *Muraena helena*, the East Atlantic peacock *Symphodus tinca*, the red mullet *Mullus surmuletus* and the painted comber *Serranus scriba*. This could be explained by the fact that the molluscs and the crustacean mentioned above feed on prays that lay on the bottom and are therefore indirectly exposed to the high abundances of *Ostreopsis* cells whereas the fish feed on prays present in the water column with lower cell counts. Wachi and Hokama (2001) reported that both herbivorous (*Acanthurus olivaceus*, *A. scandvicensis*, *Zanclus cornutus*) and carnivorous Hawaiian reef fish (*Myripristis brendti*, *Khulia scandvicensis*) from Barber's Point, in Oahu, were contaminated with PITX.

Fifteen marine species were sampled as part of the study reported herein, but PITX and analogues have been found in a much wider variety of animals including shellfish (*Mytilus galloprovencialis* (Aligizaki et al., 2008; Bellocci et al., 2008a, 2008b; Espina et al., 2009); *Venus verrucosa* and *Modiolus barbatus* (Aligizaki et al., 2008); *Arca imbricate*, *Artina* sp., *Barbatia candida* and *Chama macerophylla* (Gleibs and Mebs, 1999)), crustaceans (*Demania alcalai* and *Lophozozimus pictor* (Alcala et al., 1988; Yasumoto et al., 1986); *Demania reynaudii* (Alcala et al., 1988); *Platypodiella spectabilis* (Gleibs and Mebs, 1999)), fish (*Melichtys vidua* (Fukui et al., 1987); *Decapterus macrosoma* (Kodama et al., 1989); *Scarus ovifrons* (Taniyama et al., 2003); *Chaetodon* spp. (Gleibs and Mebs, 1999); *Herklotsichthys quadrimaculatus* (Onuma et al., 1999)), cephalopods (octopus (A. Milandri personal communication; cited in Aligizaki et al. (2011)), echinoderms (*Paracentrotus lividus* (Amzil et al., 2009; Bellocci et al., 2008a, 2008b); *Acanthaster planci* (Gleibs & Mebs, 1999)), sponges (*Amphimedon*, *Crella*, *Dactylospongia* spp., *Liosina paradoxa*; (Gleibs and Mebs, 1999)), gorgonians (*Briareum* sp. (Gleibs and Mebs, 1999)) and soft corals (*Sarcophyton*, *Sinularia* spp. (Gleibs and Mebs, 1999)).

In our study, the toxin distribution between the different tissues of the organisms was determined for the echinoderms, fish, crustaceans and cephalopods. These marine organisms were dissected in DT and RT or roe for the sea urchins and each tissue was analysed separately. The toxin contribution in the contaminated samples solely came from the DT while the RT was not contaminated whatever the species considered. There is only one case in which the roe of the sea urchin *Paracentrotus lividus* collected in Villefranche (site V2) showed quantifiable levels of PITX as determined by the haemolytic test. The fact that the DT of the sea urchin sample had a high contamination level might have resulted in a cross contamination of the roe while dissecting the different parts. This hypothesis is strengthened by the fact that the sea urchin harvested in week 32 in Rochambeau (site V3) was even more contaminated (ca. 250 μg PITX eq./kg) and yet the roe had a

contamination level below the LOQ of the haemolytic test. This shows how easy it is to cross-contaminate the roe during the dissection process and addresses the question of the risk associated with this procedure when performed by the consumers themselves and not by professionals. This point can be extended to all the marine organisms for which a dissection process would be involved.

During the course of the study, the toxin concentration in the RT was higher than in the DT in only one occasion; this was for the cephalopod *Octopus vulgaris* harvested in site V3 in week 34. An explanation to this phenomenon might be that after being sequestered in the DT, the toxin was absorbed and incorporated to the RT. Yet, the octopus harvested in site V2 in week 35 did not show the same trend with the DT being the most contaminated part. Another hypothesis might be that there were *Ostreopsis* cf. *ovata* cells on the tentacles of the common octopus when it was sampled and subsequently analysed, explaining why the toxin level in the RT (including the tentacles) was higher than in the DT.

Some papers reported the presence of PlTX and its analogues in carnivorous and herbivorous fish (Gleibs and Mebs, 1999; Wachi and Hokama, 2001). These studies had a closer look at the toxin distribution and reported that toxins were sequestered in different tissues including the skin, gills, eggs, muscle, viscera, and intestine, the latter part generally being the most contaminated. In these studies, the toxins are not sequestered in the DT, contrarily to what we observed, but are also found in several other tissues, which is the sign that an absorption process took place leading to the transfer of the toxins. Several hypotheses can be made to explain the differences: the origin of fish, from tropical to temperate areas, could lead to differences in the modalities of contamination; the duration of the blooms or the abundances of *Ostreopsis* could also be involved. It would be interesting to have a closer look at the absorption mechanisms to determine the level of absorption and establish whether the recurring exposure to high abundances of *Ostreopsis* blooms would lead to different sequestration patterns with the toxins being absorbed in the DT and transferred to the other organs.

According to Aligizaki and co-workers (2011) one must differentiate the marine organisms that solely carry the toxin in their DT (possibly in the form of the toxigenic macroalgae) that is not further absorbed, from the species that are capable of absorbing and incorporating the toxin in their tissues. In our study, the hypothesis is mostly in favour of a passive carry of toxins in the DT, for the *Ostreopsis* abundances reported, and the absorption seems to be absent or low.

Although no regulation has been put in place yet for PlTX and its analogues, either at the European level or internationally, a significant step has been made in this direction with the EFSA opinion reporting that the toxin level in the shellfish should not exceed 30 μg for the sum of PlTX and OST-D per kg (EFSA, 2009). This scientific opinion is based on the data available at the time and notably the toxicological study from Ito and Yasumoto (2009) on PlTX and OST-D. In all the sites sampled as part of the present study the toxin levels found in the DT of three species (*Sarpa salpa*, *Paracentrotus lividus*, *Mugil cephalus*) were above 30 μg PlTX eq./kg. When reported to the whole flesh, the contamination levels dropped but were still above 30 μg PlTX eq./kg except in Villefranche (site V1). Because the ESFA opinion has been established for PlTX and OST-D and is based on shellfish, for which the portion size of the consumption might be very different from that of other marine organisms such as echinoderms, gastropods and fish, one cannot strictly drive any conclusion from the sanitary status of the marine products tested in this study. Indeed, in the process of risk assessment, the safety threshold determined for a specific contaminant is calculated using the acute reference dose (ARfD), which varies according to the contaminants considered in the toxicological studies, and using the portion size. If we make the assumption that the sanitary threshold for PlTX and OVTX-a, the toxins found in the present study, determined for marine organisms is similar to the threshold recommended by EFSA, the consumption of the three species mentioned above would endanger human health and the preventative measures consisting in eviscerating the fish and not consuming other marine organisms as recommended by the French General Directorate for Health (DGS, 2010) would protect the consumers. However, some meals require the use of the entire fish, as the DT confers a specific taste; similarly, the DT of sea urchins can be eaten along with the roe as some consumers dip pieces of bread in the watery intestinal mixture as a delicacy. In some other cases, it is a matter of consumption habits dictated by practical reasons for instance with the animals being too small to be transformed (e.g. evisceration).

The mollusc *Stramonita haemastoma* and the crustacean *Eriphia verrucosa* collected in Nice were analysed as whole flesh and were also found to have toxin levels above the recommended threshold. In this case, no evisceration process can be proposed as we did not collect information about toxin distribution in the different parts and also because evisceration by consumers is not realistic, furthermore, these species are eaten as WF.

When referring to the EFSA opinion to know whether the marine organisms might be at risk, it is important to recall the limits of this opinion. First of all, the experts that were consulted based their work on the limited data available at the time, as only three countries (Greece, France and Italy) answered to the call for data of occurrence. Secondly, the data were obtained from shellfish sampled in contaminated areas and not intended for human consumption; therefore these data do not represent levels of PlTX and analogues in shellfish that currently could reach the market. Thirdly, this opinion is about PlTX and its analogue OST-D, but the toxin profiles can be markedly different. Indeed, the OVTX analogues constitute up to 90% of the toxin profile of the marine organisms analysed in our study and they represent up to 99% in *Ostreopsis ovata* cells in Italy (2011b; Ciminiello et al., 2010b, 2011c). Additional information is required regarding the toxic potential of PlTX analogues; a further step has been done by Tubaro and co-workers (2011a) as they evaluated the oral toxicity in mice of 42-hydroxy PlTX isolated from the cnidarians zoanthid *Palythoa tuberculosa*. Fourthly, the portion size taken into account for shellfish is 400 g of meat. For other marine organisms such as fish, crustaceans, sea urchins and cephalopods, the consumption portion size might be significantly different.

### 4.3. Comparison of the haemolytic test and LC–MS/MS results

Although the haemolytic test and the LC–MS/MS methods do not rely on the same detection principles, the quantitative results found using both methods are in agreement.

Ciminiello and co-workers (2010a,b, 2011b) reported the discovery of OVTX-a analogues called OVTX-b, -c, -d and -e, which account for ca. 45% of the *Ostreopsis ovata* toxin profile. As these new analogues were not searched for in the present study and yet the haemolytic test and LC–MS/MS results are in agreement, this could suggest that (i) these analogues were not present in the marine organisms; although the OVTX-a analogues have been detected in Italy in the *Ostreopsis* cells analysed by Ciminiello and co-workers, the French strain of *Ostreopsis* cf. *ovata*, responsible for the contamination of the marine organisms is likely to have a different toxin composition.; (ii) the analogues were present but they have an haemolytic potential markedly lower than that of PlTX and OVTX-a, explaining why the results of the haemolytic test were not higher than those of LC–MS/MS. (iii) they were initially present in the dinoflagellate but were metabolised by the marine organisms and were transformed into other compounds with no or

low haemolytic activity. Beyond the considerations mentioned above, the haemolytic test and LC–MS/MS are good candidates as the methods to be implemented in a monitoring programme for PITX and its analogues. The haemolytic test is less expensive and more sensitive but takes more time, thus leading to a longer sample turn-around, especially when a confirmatory step using ouabain has to be performed. Ciminiello and co-workers (2011c) reported a LOQ of 168 μg/kg for their LC–MS/MS method but the sensitivity of the method can be improved by including a cleanup step such as solid phase extraction (SPE). This would have the advantage of reducing if not eliminating the matrix effect and it would enable the addition of a concentration step increasing the sensitivity of the method. The LC–MS/MS method used in our study included an SPE cleanup resulting in a lower LOQ of 24.5 μg/kg meat, and yet the sensitivity of this method would be border line if the EFSA recommended threshold of 30 μg PITX + OST-D/kg meat was adopted for shellfish.

## 5. Conclusions

This study aimed to complete the database of marine products contamination in France (Amzil et al., 2012). We made such an inventory of the contamination levels of *Ostreopsis* toxins found during the summer 2009 in different species covering a wide variety of edible marine organisms, sampled in four different sites located on the French Mediterranean coast.

The toxin levels determined by the haemolytic test in the marine organisms followed the same trend as the *Ostreopsis* cell abundances in the sampled sites. The toxin profile of the marine organisms determined by LC–MS/MS showed the presence of OVTX-a as the major compound (90% of the profile) and PITX.

The results presented herein showed that herbivorous species are more likely to be contaminated and at higher levels, although some omnivorous and carnivorous species were also found to be contaminated. Independently of the feeding behaviour, the toxins were sequestered in the DT suggesting only a passive carry of the toxins and a low absorption rate, in the conditions of our study.

A few samples were found to have toxin concentrations in the whole flesh above 30 μg PITX eq./kg, which echoes to the EFSA opinion reporting that the toxin level in shellfish should not exceed 30 μg PITX + OST-D/kg to protect the consumer.

The process of risk assessment is entirely dependent on the availability of data from different nature and origin. Thus, it is necessary to produce toxin occurrence data and particularly to confirm that the evisceration of fish eliminates the contamination even for longer blooms and/or higher abundances, and for other species not collected in our study. For organisms that are entirely eaten, more occurrence data are needed.

Furthermore, the EFSA opinion is based on a 400 g portion size of shellfish meat, and the question that must be addressed is the determination of the portion size of the species other than shellfish.

The availability of toxicological data is also crucial as for now there is a lack of information on this matter.

The collection of additional data would enable in the end the regulation of PITX and its analogues in marine products destined to human consumption and thereby protecting the consumers.

## Conflicts of interest

The authors declare that there is no conflict of interest.

## Acknowledgements

This work was funded by the General Directorate for Health (French Ministry of Labour, Employment and Health) as part of the programme "Changement climatique et santé humaine" and was also supported by the French national programme MediOs 2 (LITEAU-Ministère de l'Ecologie, Conseil Général des Alpes-Maritimes, Agence de l'Eau Rhône Méditerranée Corse).

The authors wish to thank Sylviane Dragacci for her help and her valuable input while writing this article.[SS]

## References

Alcala, A., Alcala, L., Garth, J., Yasumura, D., Yasumoto, T., 1988. Human fatality due to ingestion of the crab *Demania reynaudii* that contained a palytoxin-like toxin. Toxicon 26 (1) 105–107.

Aligizaki, K., Katikou, P., Milandri, A., Diogène, J., 2011. Occurrence of palytoxin-group toxins in seafood and future strategies to complement the present state of the art. Toxicon 57 (3) 390–399.

Aligizaki, K., Katikou, P., Nikolaidis, G., Panou, A., 2008. First episode of shellfish contamination by palytoxin-like compounds from Ostreopsis species (Aegean Sea, Greece). Toxicon 51 (3) 418–427.

Aligizaki, K., Nikolaidis, G., 2006. The presence of the potentially toxic genera Ostreopsis and Coolia (Dinophyceae) in the North Aegean Sea, Greece. Harmful Algae 5 (6) 717–730.

Amzil, Z., Sibat, M., Chomerat, N., Grossel, H., Marco-Miralles, F., Lemee, R., Nezan, E., Sechet, V., 2012. Ovatoxin-a and Palytoxin Accumulation in Seafood in Relation to Ostreopsis cf. ovata Blooms on the French Mediterranean Coast. Marine Drugs 10 (2) 477–496.

Amzil, Z., Sibat, M., Séchet, V., Lemée, R., Kantin, R., Grossel, H., 2009. First report on palytoxin-like detection in seafood in relation with *Ostreopsis* spp. bloom on French Mediterranean coasts.In: 7th International Conference on Molluscan Shellfish Safety, Nantes.

Bellocci, M., Ronzitti, G., Milandri, A., Melchiorre, N., Grillo, C., Poletti, R., Yasumoto, T., Rossini, G.P., 2008a. Addendum to A cytolytic assay for the measurement of palytoxin based on a cultured monolayer cell line [Anal. Biochem. 374 (2008) 48–55] Analytical Biochemistry 381 (1) 178.

Bellocci, M., Ronzitti, G., Milandri, A., Melchiorre, N., Grillo, C., Poletti, R., Yasumoto, T., Rossini, G.P., 2008b. A cytolytic assay for the measurement of palytoxin based on a cultured monolayer cell line. Analytical Biochemistry 374 (1) 48–55.

Bignami, G., 1993. A rapid and sensitive hemolysis neutralization assay for palytoxin. Toxicon 31 (6) 817–820.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Tartaglione, L., Rossi, R., Soprano, V., Capozzo, D., Serpe, L., 2011a. Palytoxin in seafood by liquid chromatography tandem mass spectrometry: Investigation of extraction efficiency and matrix effect. Analytical and Bioanalytical Chemistry 401 (3) 1043–1050.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., 2010a. Palytoxins: a still haunting Hawaiian curse. Phytochemistry Reviews 9 (4) 491–500.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., 2011b. A 4-decade-long (and still ongoing) hunt for palytoxins chemical architecture. Toxicon 57 (3) 362–367.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Grillo, C., Melchiorre, N., 2006. The Genoa 2005 outbreak. Determination of putative palytoxin in Mediterranean Ostreopsis ovata by a new liquid chromatography tandem mass spectrometry method. Anal Chem 78 (17) 6153–6159.

Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., Guerrini, F., Pistocchi, R., 2010b. Complex palytoxin-like profile of Ostreopsis ovata. identification of four new ovatoxins by high-resolution liquid chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry 24 (18) 2735–2744.

Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E., Forino, M., Tartaglione, L., 2011c. LC–MS of palytoxin and its analogues: State of the art and future perspectives. Toxicon 57 (3) 376–389.

Deeds, J.R., Schwartz, M.D., 2010. Human risk associated with palytoxin exposure. Toxicon 56 (2) 150–162.

DGS, 2010. Note de service DGS/EA3/EA4 no 2010-238 du 30 juin 2010 relative à la surveillance sanitaire et environnementale et aux modalités de gestion des risques sanitaires pour la saison balnéaire 2010. liés à la présence de la microalgue toxique Ostreopsis spp. dans les eaux de baignade en méditerranée et à la contamination par ses toxiques des produits de la mer issus de la pêche de loisir. Bulletin Officiel Santé – Protection sociale – Solidarité (2010/8) 261–298.

EFSA, 2009. Scientific opinion on marine biotoxins in shellfish – palytoxin group. EFSA Journal 7 (12) 1393–1430.

Espina, B., Cagide, E., Louzao, M.C., Fernandez, M.M., Vieytes, M.R., Katikou, P., Villar, A., Jaen, D., Maman, L., Botana, L.M., 2009. Specific and dynamic detection of palytoxins by in vitro microplate assay with human neuroblastoma cells. Bioscience Reports 29 (1) 13–23.

Fukui, M., Murata, M., Inoue, A., Gawel, M., Yasumoto, T., 1987. Occurrence of palytoxin in the trigger fish *Melichtys vidua*. Toxicon 25 (10) 1121–1124.

Gleibs, S., Mebs, D., 1999. Distribution and sequestration of palytoxin in coral reef animals. Toxicon 37 (11) 1521–1527.

Habermann, E., Ahnert-Hilger, G., Chhatwal, G.S., Beress, L., 1981. Delayed haemolytic action of palytoxin general characteristics. Biochimica et Biophysica Acta (BBA) – Biomembranes 649 (2) 481–486.

Ifremer, 2011. Cahier de procédures et de programmation REPHY 2011. Ifremer, Nantes.

K., Yasumoto, T., 2008. Toxicological studies on palytoxin and ostreocin-D administered into mice by three different routes. Toxicon 54 (3) 24–251.

laboratory findings implicating palytoxin as cause of ciguatera poisoning due to *Decapterus macrosoma* (mackerel). Toxicon 27 (9) 1051–1053.

Mangialajo, L., Bertolotto, R., Cattaneo-Vietti, R., Chiantore, M., Grillo, C., Lemee, R., Melchiorre, N., Moretto, P., Povero, P., Ruggieri, N., 2008. The toxic benthic dinoflagellate Ostreopsis ovata: quantification of proliferation along the coastline of Genoa, Italy. Marine Pollution Bulletin 56 (6) 1209–1214.

Mangialajo, L., Ganzin, N., Accoroni, S., Asnaghi, V., Blanfuné, A., Cabrini, M., Cattaneo-Vietti, R., Chavanon, F., Chiantore, M., Cohu, S., Costa, E., Fornasaro, D., Grossel, H., Marco-Miralles, F., Masó, M., René, A., Rossi, A.M., Sala, M.M., Thibaut, T., Totti, C., Vila, M., Lemée, R., 2011. Trends in Ostreopsis proliferation along the Northern Mediterranean coasts. Toxicon 57 (3) 408–420.

Moore, R.E., Scheuer, P.J., 1971. Palytoxin: a new marine toxin from a coelenterate. Science 172, 495–498.

Munday, R., 2008. Occurrence and toxicology of palytoxins. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins – Pharmacology, Physiology, and Detection. second ed. CRC Press, Boca Raton, pp. 693–713.

Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., Ratsimaloto, M., Naoki, H., Yasumoto, T., 1999. Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37 (1) 55–65.

Riobó, P., Paz, B., Franco, J.M., Vázquez, J.A., Murado, M.A., 2008. Proposal for a simple and sensitive haemolytic assay for palytoxin: toxicological dynamics, kinetics, ouabain inhibition and thermal stability. Harmful Algae 7 (4) 415–429.

Taniyama, S., Arakawa, O., Terada, M., Nishio, S., Takatani, T., Mahmud, Y., Noguchi, T., 2003. Ostreopsis sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. Toxicon 42 (1) 29–33.

Taniyama, S., Mahmud, Y., Terada, M., Takatani, T., Arakawa, O., Noguchi, T., 2002. Occurrence of a food poisoning incident by palytoxin from a serranid Epinephelus sp. in Japan. Journal of Natural Toxins 11 (4) 277–282.

Tichadou, L., Glaizal, M., Armengaud, A., Grossel, H., Lemée, R., Kantin, R., Lasalle, J.L., Drouet, G., Rambaud, L., Malfait, P., De Haro, L., 2010. Health impact of unicellular algae of the Ostreopsis genus blooms in the Mediterranean Sea: Experience of the French Mediterranean coast surveillance network from 2006 to 2009. Clinical Toxicology 48 (8) 839–844.

Totti, C., Accoroni, S., Cerino, F., Cucchiari, E., Romagnoli, T., 2010. Ostreopsis ovata bloom along the Conero Riviera (northern Adriatic Sea): relationships with environmental conditions and substrata. Harmful Algae 9 (2) 233–239.

Tubaro, A., Del Favero, G., Beltramo, D., Ardizzone, M., Forino, M., De Bortoli, M., Pelin, M., Poli, M., Bignami, G., Ciminiello, P., Sosa, S., 2011a. Acute oral toxicity in mice of a new palytoxin analogue: 42-hydroxy-palytoxin. Toxicon 57 (5) 755–763.

Tubaro, A., Durando, P., Del Favero, G., Ansaldi, F., Icardi, G., Deeds, J.R., Sosa, S., 2011b. Case definitions for human poisonings postulated to palytoxins exposure. Toxicon 57 (3) 478–495.

Utermöhl, H., 1958. Zur Vervoll Kommung der Quantitativen Phytoplankton-Methodik. Mitteilungen Internationale Vereinigung fur theoretische und angewandte. Limnologie 9, 1–38.

Vila, M., Garcés, E., Masó, M., 2001. Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. Aquatic Microbial Ecology 26, 51–60.

Wachi, K.M., Hokama, Y., 2001. Diversity of marine biotoxins in the near-shore ocean area: presence of a palytoxin-like entity at Barber's Point Harbor, Oahu. Journal of Natural Toxins 10 (4) 317–333.

Yasumoto, T., Yasumura, D., Ohizumi, Y., 1986. Palytoxin in two species of xanthid crab from the Philippines. Agricultural and Biological Chemistry 50 (1) 163–167.

**Annexe 6**

Municipalité de Villefranche-sur-Mer
Département des Alpes-Maritimes

# Surveillance de la qualité des eaux de baignade : recherche et suivi du développement des dinoflagellés toxiques du genre *Ostreopsis* au niveau du littoral de la commune de Villefranche-sur-Mer

# Eté 2014

N° enregistrement :



Réf UPMC : C13556

CONTRAT DE PRESTATION

Entre

**La commune de Villefranche-sur-Mer**
représentée par .....

D'UNE PART,

Et :

**Le Département des Alpes-Maritimes**, représenté par le Président du Conseil général en exercice, M. Eric CIOTTI, domicilié, en cette qualité, au centre administratif départemental, route de Grenoble, B.P. 3007, 06201 Nice cedex 3, agissant en vertu d'une délibération de la Commission permanente du Conseil général en date du ..... 2014.

désigné ci-après : « le Département »

DE DEUXIEME PART,

**L'Université Pierre et Marie Curie (Paris 6)**, Etablissement Public à Caractère Scientifique, culturel et professionnel, ayant son siège 4, place Jussieu - 75252 Paris Cedex 05, France, représentée par son Président, Monsieur Jean Chambaz,
ci-après désigné par l' «UPMC », et

**Le Centre National de la Recherche Scientifique**, Etablissement public à caractère scientifique et technologique, ayant son siège 3, rue Michel Ange 75794 PARIS Cedex
16, représenté par son Président, Monsieur Alain Fuchs, ci-après désigné par « CNRS »

L'UPMC et le CNRS agissant tant en leur nom que pour le compte du Laboratoire d'Océanographie de Villefranche, unité mixte de recherche de l'UPMC et du CNRS n° 7093, dirigé par Monsieur Antoine SCIANDRA, ci-après désigné par « LOV »,

L'UPMC et le CNRS étant ci-après désignés par les «ETABLISSEMENTS ».

L'UPMC a reçu pour le présent contrat mandat du CNRS pour le signer, en son nom et pour son compte.

DE TROISIEME PART

La **Commune** de Villefranche-sur-Mer, le Département et les ETABLISSEMENTS étant ci-après désignés individuellement et/ou collectivement par « Partie(s) ».

**Préambule :**

Le projet objet du présent contrat s'insère dans le cadre de la surveillance de la qualité des eaux de baignade et concerne le suivi du développement des dinoflagellés toxiques du genre *Ostreopsis* au niveau des plages de Villefranche-sur-Mer.

Depuis une dizaine d'années, des algues marines du genre *Ostreopsis* (Dinoflagellé) se développent parfois de façon très importante sur les côtes méditerranéennes de l'Italie, de la France et de l'Espagne. Ces algues unicellulaires, qui mesurent environ 50 µm de longueur, se développent préférentiellement à très faible profondeur, sur d'autres végétaux (macroalgues ou phanérogames) ou directement sur le substrat. Lorsque les conditions sont favorables (période estivale), ces microalgues peuvent proliférer et se retrouver en suspension dans d'eau.

Ces microalgues contiennent des toxines (palytoxine et dérivés) et leur prolifération peut avoir des conséquences néfastes, aussi bien pour la santé humaine que pour l'écosystème. Les conditions deviennent hypoxiques, voir anoxiques, avec une importante mortalité des invertébrés (principalement des oursins et des coquillages).

Au niveau de la santé humaine, les réactions sont diverses : irritations cutanées, affections respiratoires, conjonctivites, fièvre. Les microalgues étant probablement transportées par les embruns marins, même les personnes n'étant pas en contact direct avec l'eau de mer peuvent être atteintes (en particulier les vacanciers sur les plages).

- **A Gênes**, durant l'été 2005, 200 personnes ont ressenti ces différents symptômes et 20 personnes ont été hospitalisées. La plage atteinte a été interdite au public pendant plus d'une semaine, en plein été, avec des conséquences économiques certaines.

- **En Espagne**, plusieurs habitants d'un immeuble situé en bordure d'une plage ont été intoxiqués.

- **Durant l'été 2006 , une espèce du genre *Ostreopsis* a été trouvée à Marseille**, dans une calanque de l'île du Frioul. La baignade a été interdite le 10 août.

- **Durant les étés 2007, 2008, 2009, 2010, 2011, 2012 et 2013, *Ostreopsis* cf. *ovata* a été trouvée à Monaco dans les anses du Larvotto mais également sur les zones des communes françaises comme Villefranche-sur-Mer et Nice.**

Les conditions qui permettent la prolifération de cette algue sont encore peu connues, mais tous les pays riverains de la Méditerranée nord-occidentale prennent au sérieux ce problème et commence à envisager la potentialité d'un développement important de cette algue.

**Ceci exposé, les Parties ont convenu ce qui suit :**

## ARTICLE 1. OBJET DU CONTRAT.

L'objectif de ce contrat est de suivre la présence et le développement des algues du genre *Ostreopsis* sur le littoral de la commune de Villefranche-sur-Mer durant l'été 2014.

## ARTICLE 2. CONDITIONS GENERALES D'EXECUTION.

L'exécution du contrat sera réalisée par le Dr. Rodolphe Lemée, responsable scientifique de cette étude, Laboratoire Océanographique de Villefranche (LOV, CNRS/UPMC UMR 7093).

Ce contrat consiste à analyser les prélèvements qui seront effectués de début juillet à fin août 2014 dans la Baie de Villefranche. Plusieurs zones de baignade ont été identifiées comme potentiellement à risque pour le développement des algues du genre *Ostreopsis* (conditions écologiques, qualité des eaux) ce qui peut présenter un risque sanitaire important en raison de la fréquentation importante de cette plage.

## ARTICLE 3. CONDITIONS PARTICULIERES.

Le LOV devra obtenir auprès de la commune de Villefranche-sur-Mer ou des services concernés toutes les autorisations nécessaires, ainsi que, le cas échéant, les éléments et conditions de son intervention.
Les Etablissements feront leur affaire du matériel et des équipements de protection de leur personnel.
Les Etablissements assureront toutes les responsabilités inhérentes à la bonne exécution du présent contrat conformément aux règles de droit en la matière.

## ARTICLE 4. METHODE ET DUREE DE L'ETUDE.

L'étude objet du présent contrat sera réalisée uniquement de début juillet à début septembre 2014, période la plus propice au développement des algues du genre *Ostreopsis*.
Si une prolifération de ces algues était observée, une alerte serait donnée le plus rapidement possible à la Commune de Villefranche-sur-Mer et au Département, **au maximum 48 heures après les récoltes**, sous forme synthétique des résultats consignés dans un « Bulletin de surveillance » transmis par courriel.

## Méthode :

Dénombrement au microscope des cellules d'*Ostreopsis* spp. présentes sur des macroalgues et dans l'eau au niveau de 5 sites dans la Baie de Villefranche durant l'été 2014. Les travaux objet du présent contrat comprennent les récoltes effectuées sur place, le dénombrement des microalgues au LOV ainsi que l'envoi, au maximum 48 heures après les récoltes, des résultats par courriel sous forme synthétique dans un « Bulletin de surveillance ».

Pour le dénombrement des microalgues, différents types de substrats biotiques (différentes espèces de macroalgues) seront prélevés. Les microalgues seront détachées de ce substrat par agitation, l'eau de mer sera récupérée puis filtrée pour enlever les grosses particules. Les microalgues seront énumérées via l'utilisation de microscopes inversés et droits. La concentration algale sera rapportée à la masse humide et sèche des substrats biotiques.

Les échantillons seront récoltés et analysés en 4 sites chaque semaine. Ces 5 sites sont :
1) La plage des jeunes
2) La plage des Marinières, côté est
3) La plage des Marinières, côté ouest
4) La plage du port de la Darse, côté nord
5) La plage devant l'Observatoire Océanologique de Villefranche-sur-Mer

Ces sites sont connus pour être propice au développement d'*Ostreopsis* cf. *ovata*.

Pour information, le Site Rochambeau fera l'objet d'un suivi très particulier durant l'été 2014 dans le cadre d'un projet européen (projet ENPI M3HABs), dans lequel le Conseil Général des Alpes-Maritimes ainsi que la Commune de Villefranche sont impliqués en tant que partenaires. Les résultats du suivi effectué dans le cadre de ce projet européen seront transmis dans le Bulletin de surveillance hebdomadaire, comme étant le site n° 6, mais ces analyses au site Rochambeau ne sont bien sûr pas facturées à la Commune de Villefranche ou au Conseil Général des Alpes-Maritimes.

## Normes :

Il n'existe actuellement aucun aspect réglementaire concernant le développement des espèces du genre *Ostreopsis*.

Dans ce projet, et en appliquant le principe de précaution, toutes les espèces du genre *Ostreopsis* seront comptées. La détermination au niveau spécifique n'est pas réalisable en microscopie optique et pose également des problèmes en microscopie électronique. Des données très récentes montrent que seule une analyse moléculaire poussée permet de déterminer les taxons au niveau spécifique, mais ce genre d'analyse n'est prévu dans ce contrat.

En tenant compte des seuils empiriques utilisés en France en 2009 et 2010 par l'Ifremer et l'Institut de Veille Sanitaire Française, les seuils de pré-alerte et d'alerte retenus sont les suivants :

- Le seuil de pré-alerte est considéré comme atteint lorsque les prélèvements d'eau de mer révèlent des quantités supérieures à **30 000 cellules par litre d'eau**.
- Le seuil d'alerte est considéré comme atteint lorsque les prélèvements d'eau de mer révèlent des quantités supérieures à **100 000 cellules par litre d'eau de mer**.

Si les seuils de pré-alerte et d'alerte français changent durant l'été 2014, la Commune de Villefranche-sur-Mer et le Département en seront avisés le plus rapidement possible par R. Lemée.

**Durée :**

5 analyses (site 1 à 5) par semaine, sur une période de 10 semaines, du 2 juillet au 3 septembre 2014, sont prévues.
Le présent contrat viendra à échéance à la remise du rapport final mentionné à l'article 5 ci-dessous.

## ARTICLE 5. DOCUMENTS A REMETTRE PAR LE LOV

L'étude fera l'objet d'un rapport final (avant fin décembre 2014), incluant les connaissances acquises et les propositions de suivi pour les années à venir, conforme aux conditions d'exécution de la mission.

Tous les documents photographiques, cartographiques et le rapport final seront remis en trois exemplaires originaux en tirage papier et sur CD-Rom.

## ARTICLE 6. PROPRIETE DES RESULTATS DE L'ETUDE ET UTILISATION DES DOCUMENTS.

Les documents rendus par le LOV dans le cadre du présent contrat et notamment le rapport final et les « Bulletin de surveillance » engagent sa responsabilité et sa crédibilité scientifique. Ils ne peuvent être modifiés sans son accord.

Indépendamment de la remise du rapport final, le LOV s'engage à fournir, si nécessaire, un complément d'information concernant les résultats de l'étude.

Les résultats de la présente étude sont l'entière propriété de la Commune de Villefranche-sur-Mer et du Département. Ils jouissent du droit de faire reproduire librement, selon tous procédés connus et inconnus à ce jour, et sans frais, tout ou partie de ces résultats.

Cependant, les données acquises par les chercheurs du LOV pourront être utilisées par ces derniers dans le cadre classique de la diffusion de travaux scientifiques (congrès nationaux et internationaux, publications) sous réserve que la Commune de Villefranche- sur-Mer et le Département soit mentionnés.

## ARTICLE 7. REMUNERATION ET MODALITES DE PAIEMENT.

Le prix de la double analyse : une analyse du nombre d'*Ostreopsis* spp. sur macroalgue associée à une analyse de la concentration d'*Ostreopsis* spp. dans l'eau au-dessus de cette macroalgue, est de 250 Euros TTC. En considérant 5 sites par semaine pendant 10 semaines, le montant de l'étude est arrondi à 12 000 euros TTC (douze milles euros TTC). Ce prix n'est ni actualisable, ni révisable.

La participation du Département à ce contrat s'élève à 6 000 € comme celle de la Commune de Villefranche-sur-Mer. Le montant total du contrat sera versé par la Commune de Villefranche-sur-Mer et par le Département sur le compte de l'UPMC au nom de son Agent Comptable

RECETTE GENERALE DES FINANCES DE PARIS
94 RUE REAUMUR
75002 PARIS
COMPTE 10071 75000 00001005793 64

sur présentation de factures aux échéances suivantes :

- Un premier versement (50 %) dès notification du présent contrat.
- Un second versement (50%) à la remise rapport final tel que prévu à l'article 5.

Les factures doivent être envoyées en 1 exemplaire à l'adresse suivante sous la référence d'achat ....

Commune de Villefranche-sur-Mer
Mme ...

Département des Alpes Maritimes
Mr ...

## ARTICLE 8. RESPONSABILITE ET ASSURANCES

### 8.1. RESPONSABILITE

Les Etablissements sont responsables, pendant l'exécution de ce contrat de tous dommages qu'eux-mêmes, leur personnel, leur matériel, leurs sous-traitants, fournisseurs et/ou prestataires de service, pourraient causer à l'autre partie et/ou à tout tiers.

### 8.2. FORCE MAJEURE

Aucune des Parties n'est tenue pour responsable en cas de non-respect de l'une quelconque des clauses du présent contrat en cas de force majeure. Sont notamment considérés comme cas de force majeure : les cas d'incendie, explosion, inondation, guerre déclarée ou non, grèves de longue durée, émeute, blocus ou embargo, restriction ou interdiction gouvernementale. La Partie frappée par la force majeure doit en aviser immédiatement l'autre Partie par tout moyen, confirmé par lettre recommandée avec accusé de réception. Il est entendu que les deux Parties ne peuvent invoquer la force majeure que pendant la durée de son effet. Elles s'engagent à faire tous leurs efforts pour en limiter au maximum les conséquences. La force majeure n'emporte pas le non-paiement des factures émises.

### 8.3. ASSURANCES

Les Etablissements déclarent avoir souscrit, en tant que de besoin auprès d'une Compagnie d'Assurance, une assurance de responsabilité civile et professionnelle couvrant notamment, entre autres garanties, les conséquences pécuniaires qu'ils peuvent encourir dans le cas d'accidents causés aux tiers et engageant leur responsabilité.

## ARTICLE 9. RESILIATION.

Le contrat pourra être résilié sans autre forme ni indemnité, à l'initiative de la Commune de Villefranche-sur-Mer ou du Département, en cas de manquement grave aux obligations du présent contrat, et ce 30 jours après une mise en demeure restée infructueuse par lettre recommandée avec avis de réception.

Le LOV s'oblige, en cas de résiliation, à remettre à la Commune de Villefranche-sur-Mer et au Département tous les résultats de l'étude et les documents en sa possession, nécessaires à la poursuite par un autre des missions qui lui sont confiées.

## ARTICLE 10. REGLEMENT DES LITIGES.

Les Parties se déclarent d'accord pour qu'en cas de conflit sur l'interprétation et/ou l'exécution du présent contrat, toutes les possibilités de solutions amiables, entres-elles, soient considérées.

Fait en trois exemplaires originaux à Villefranche-sur-Mer, le

La Commune de Villefranche sur-Mer

Le Président de l'UPMC

Jean CHAMBAZ

Le Département des Alpes-Maritimes

Eric CIOTTI

Visas

Antoine Sciandra, Directeur du Laboratoire

Rodolphe LEMEE, responsable scientifique

**Annexe 6 bis**

# Surveillance de la qualité des eaux de baignade : recherche et suivi du développement des dinoflagellés toxiques du genre *Ostreopsis* au niveau du littoral de Villefranche-sur-Mer. Eté 2013

**Rodolphe Lemée**

Laboratoire d'Océanographie de Vi'' ' UMR 7093,
Observatoire Océanol- -Mer
Université .
Contact : l

Annexe 1

Remerciements :

Un grand merci aux étudiantes du LOV de l'été 2013 : Kraïma Narjis, Marne Segond et Adeline Williams (Licence 3, Université de Nice Sophia Antipolis).

**Sommaire**

**Titre** : Surveillance de la qualité des eaux de baignade : recherche et suivi du développement des dinoflagellés toxiques du genre *Ostreopsis* au niveau du littoral de Villefranche-sur-Mer. Eté 2013

**Etat de l'art** : Le changement climatique à l'échelle planétaire et ses répercussions aux échelles locales n'ont pas seulement des effets sur la température moyenne de l'air et de l'eau, ou sur la fréquence et l'intensité des évènements météorologiques remarquables. Tous ces paramètres peuvent influencer les aires de répartition de nombreuses espèces animales et végétales. Il est possible, par exemple, de voir proliférer sous des latitudes tempérées des organismes parasites ou toxiques qui étaient en limite de leurs aires de répartition. C'est peut-être le cas de certaines espèces du genre *Ostreopsis* (dinoflagellés benthiques), souvent associées aux espèces toxiques du genre *Gambierdiscus*, dont le développement est généralement limité aux zones tropicales. Ces espèces vivent généralement entre 35° N et 35° S de latitude, à l'exception de *Ostreopsis siamensis* et *Ostreopsis ovata* qui ont été inventoriées relativement récemment en Méditerranée. Des espèces du genre *Ostreopsis* se développent donc en Méditerranée depuis plusieurs dizaines d'années, mais leurs proliférations, impliquant des effets néfastes aux niveaux écologiques, sanitaires et socio-économiques, sont beaucoup plus récentes et limitées pour l'instant au bassin occidental et à l'Adriatique.

Le genre *Ostreopsis* est connu sous les tropiques pour être à l'origine d'intoxications alimentaires (souvent mortelles) suite à l'accumulation de la palytoxine et de ses dérivés dans des crabes, des oursins ou des poissons. La palytoxine est une macromolécule (2680 Daltons) hydrosoluble complexe polyhydroxylée dont la structure chimique a été caractérisée dans les années 1980. Quelle que soit son origine, il existe très peu de différences entre les structures chimiques de la palytoxine et ses analogues. La palytoxicose est caractérisée par les symptômes d'intoxication suivants : hypersalivation, crampes abdominales, nausée, forte diarrhée, paresthésie des extrémités, spasmes musculaires et difficultés respiratoires suivi de décès dans les cas les plus graves. La forte toxicité de la palytoxine chez les mammifères en fait une des substances d'origine marine les plus toxiques connues. A titre d'exemple, la dose létale 50 (DL50) de la palytoxine chez la souris par voie intrapéritonéale est de 0,75 µg/kg. Au niveau cellulaire, la palytoxine provoque un large éventail d'effets pharmacologiques qui incluent : un efflux de potassium et un influx de sodium ; une dépolarisation des membranes excitables et l'activation secondaire des canaux calcium ; une augmentation et une mobilisation du calcium intracellulaire ; l'activation de l'échangeur sodium-calcium ; une contraction des muscles striés, lisses et cardiaques et une augmentation de la libération de neuromédiateurs au niveau des terminaisons nerveuses.

En Méditerranée, la concentration de toxines dans la chaîne alimentaire suite à des proliférations d'*Ostreopsis* spp. a été suggérée dans des poissons et des mollusques (bivalves et gastéropodes) et confirmée par des études récentes. Une intoxication par contact direct est également possible. Les espèces du genre *Ostreopsis* se développent préférentiellement à très faible profondeur, sur d'autres végétaux (macroalgues ou phanérogames) ou directement sur le substrat. Lorsque les conditions sont favorables (période estivale), ces microalgues peuvent proliférer et se retrouver en suspension dans l'eau, formant parfois des agrégats relativement

importants. Suite à la lyse des cellules ou à une exsudation, les toxines se retrouvent dans l'eau de mer, dans les agrégats et même dans les aérosols dispersés par les vents. Les conséquences peuvent alors être néfastes, aussi bien pour la santé humaine que pour l'écosystème. Les conditions deviennent hypoxiques, voir anoxiques, avec une importante mortalité des invertébrés (principalement des oursins et des coquillages). Au niveau de la santé humaine, les réactions sont diverses : irritations cutanées, affections respiratoires, conjonctivites et fièvre. La toxine ou les cellules étant transportées par les aérosols marins, même les personnes n'étant pas en contact direct avec l'eau de mer peuvent être atteintes (en particulier les vacanciers sur les plages). A Gênes, durant l'été 2005, plusieurs dizaines de personnes ont ressenti ces différents symptômes et plusieurs dizaines d'entre elles ont été hospitalisées durant plusieurs jours. Les plages atteintes ont été interdites au public pendant plus d'une semaine, en plein été, avec des conséquences économiques certaines. En Espagne, plusieurs habitants d'un immeuble situé en bordure de plage ont été intoxiqués. En Algérie, plusieurs centaines de personnes ont été légèrement intoxiquées.

**Objectifs :** A la demande de la commune de Villefranche-sur-Mer et du Conseil Général des Alpes-Maritimes, un suivi du développement d'*Ostreopsis* cf. *ovata* a été prévu durant l'été 2013 dans la baie de Villefranche. Ce suivi, qui a fait l'objet d'une convention, présente un avantage très important : une analyse rapide (moins de 48h) permettant d'émettre un « Bulletin de surveillance » hebdomadaire envoyé par mail. Seule cette rapidité dans les analyses permet de faire un travail préventif efficace.

## Matériel et Méthodes

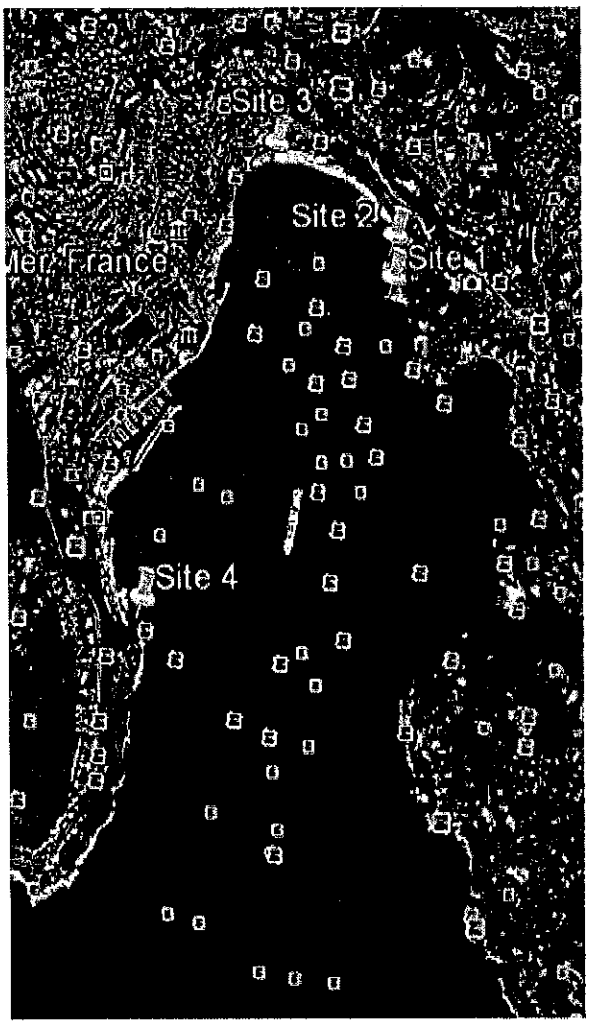
Stratégie d'échantillonnage : Nous avons réalisé des prélèvements une fois par semaine, entre **le 19 juin et 26 août 2013,** au niveau de la baie de Villefranche-sur-Mer. Quatre zones (sites 1, 2, 3 et 4) de prélèvements ont été définies (cf. Carte 1) pour ce suivi hebdomadaire. Au niveau du site 4, 3 sous-sites ont été analysés (4a, 4b et 4c), afin de mesurer la variabilité à petite échelle de l'abondance d'*Ostreopsis* cf. *ovata* (analyse du 19 juin au 21 août).

**Localisation des sites**
**Site 1** : plage des jeunes, côté nord de la digue rocheuse artificielle sud
**Site 2** : Marinières, côté ouest de la digue rocheuse artificielle à l'est du poste de secours
**Site 3** : Marinières, côté est des rochers au niveau des barrières d'entrée du parking
**Site 4** : Rochambeau, zone interne de la crique, juste en face de l'immeuble sud-est
**Sites 4a, 4b et 4c** : Au niveau du site 4, du sud (4a) au nord (4c). Distance entre les sites : environ 10 m.

Méthode de récolte : Au niveau de tous les sites, des prélèvements d'eau ont été effectués à 30 cm de profondeur et à 20 cm du substrat. Ils ont été utilisés pour évaluer le nombre de microalgues dans l'eau de mer (prélèvement dans des flacons en polypropylène de 250 ml). Un autre prélèvement a permis de récolter des macroalgues (à 50 cm de profondeur) et l'eau environnante afin de déterminer la quantité de microalgues épiphytes (flacons de 250 ml).

Comptage des microalgues dans l'eau de mer : Environ une heure après les prélèvements d'eau, nous avons ajouté 2 % de lugol acide pour fixer et préserver les échantillons. Ils ont ensuite été conservés à 4°C dans l'obscurité jusqu'à l'analyse. Pour évaluer le nombre de cellules de microalgues dans l'eau, nous avons utilisé la méthode d'Utermöhl en faisant sédimenter 50 ml de prélèvement. Les microalgues ont ensuite été déterminées et comptées à l'aide d'un microscope inversé (Axiovert 35).

Comptage des microalgues sur les macroalgues : Environ 30 minutes après les prélèvements des macroalgues avec l'eau environnante, nous avons ajouté 2 % de lugol acide afin de préserver les échantillons. De retour au laboratoire, nous avons vigoureusement agité les échantillons pour détacher les microalgues des macroalgues, puis nous avons tamisé ces échantillons sur un filtre de 500 µm de vide de maille en acier inoxydable. Les macroalgues ont ensuite été rincées 2 fois avec 100 ml d'eau de mer filtrée sur 0.2 µm (avec une agitation vigoureuse à chaque rinçage) afin de décrocher la totalité des microalgues. Les macroalgues (sur le tamis) ont alors été identifiées puis pesées (Poids Frais = PF). Elles ont ensuite été séchées 48 h à 60 °C dans une étuve afin de déterminer le Poids Sec (PS). Le filtrat (sous le filtre de 500 µm) est recueilli. Une fraction de ce dernier est observée au microscope (Alphaphot 2-YS2, Nikon) à l'aide de lames calibrées (1 ml ou 100 µl, en fonction de la concentration en cellules).




**Carte 1** : Sites de prélèvement d'*Ostreopsis* cf. *ovata* dans la baie de Villefranche-sur-Mer au cours de la saison estivale 2013. Carte Google Earth.

**Résultats et Discussion :** Un bilan des analyses hebdomadaires a été envoyé par mail (1) à la Mairie de Villefranche-sur-Mer et (2) au Conseil Général des Alpes-Maritimes, sous forme de « Bulletins de Surveillance ».

Evolution du nombre de dinoflagellés toxiques sur substrats rocheux et au niveau de la plage : Au niveau de tous les sites, nous avons prélevé les macroalgues majoritaires de chacun des sites (soit *Halopteris scoparia* (= *Stypocaulon scoparium*), soit *Padina pavonica* , soit des Dictyotales) pour suivre l'évolution d'*Ostreopsis* spp. sur la macroflore. Les figures 1 et 2 présentent l'évolution pendant l'été 2013 du nombre d'*Ostreopsis* spp. sur les macroalgues et dans l'eau pour les sites 1 à 4. Au niveau du site 4, c'est toujours le sous-site 4b qui est présenté dans ces premiers graphes.

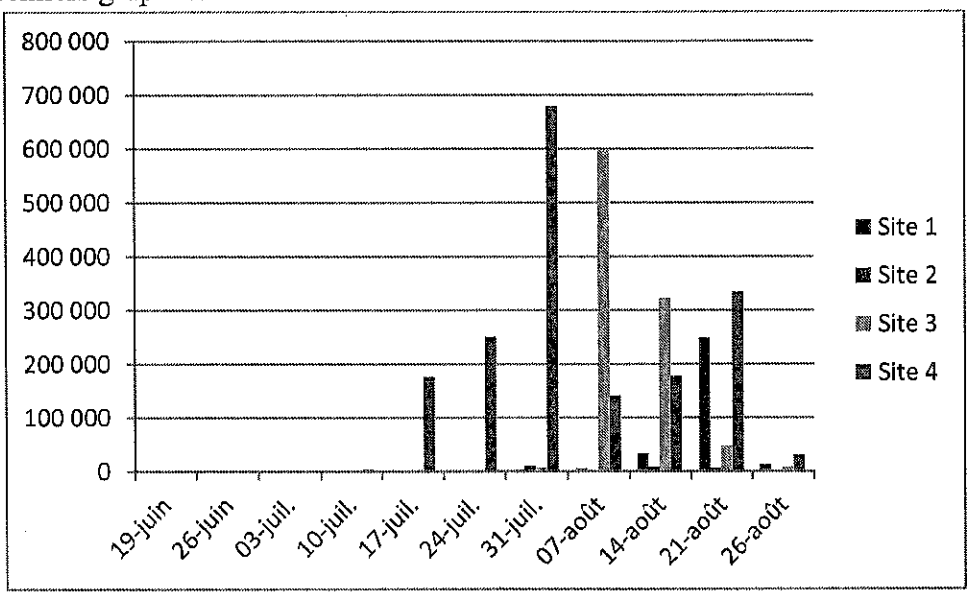



**Figure 1** : Evolution de la quantité d'*Ostreopsis* cf. *ovata* par gramme de Poids Frais de macroalgues au niveau des sites 1, 2, 3 et 4b dans la baie de Villefranche au cours de la saison estivale 2013.

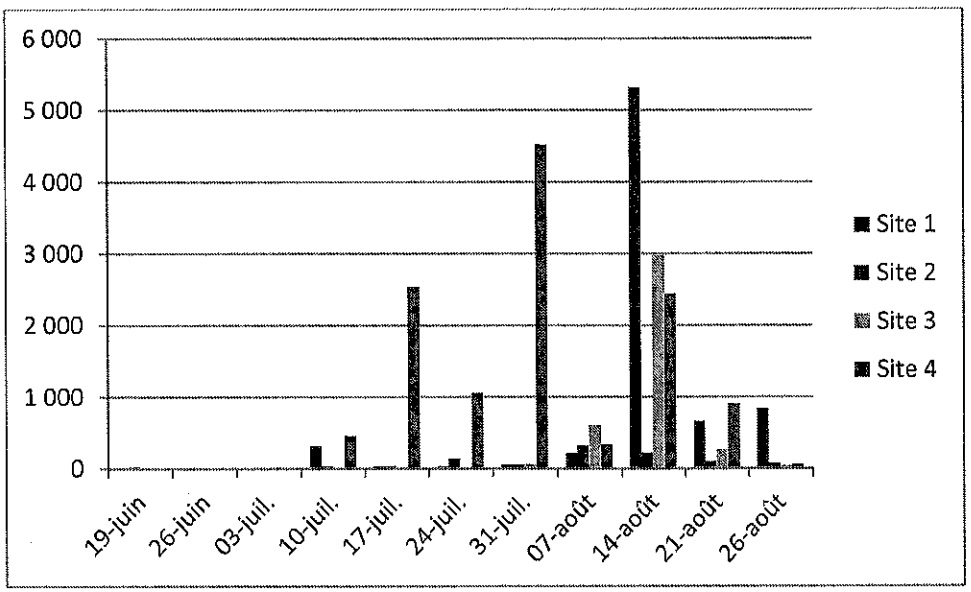



**Figure 2** : Evolution de la quantité d'*Ostreopsis* cf. *ovata* par litre d'eau de mer au niveau des sites 1, 2, 3 et 4b dans la baie de Villefranche au cours de la saison estivale 2013.

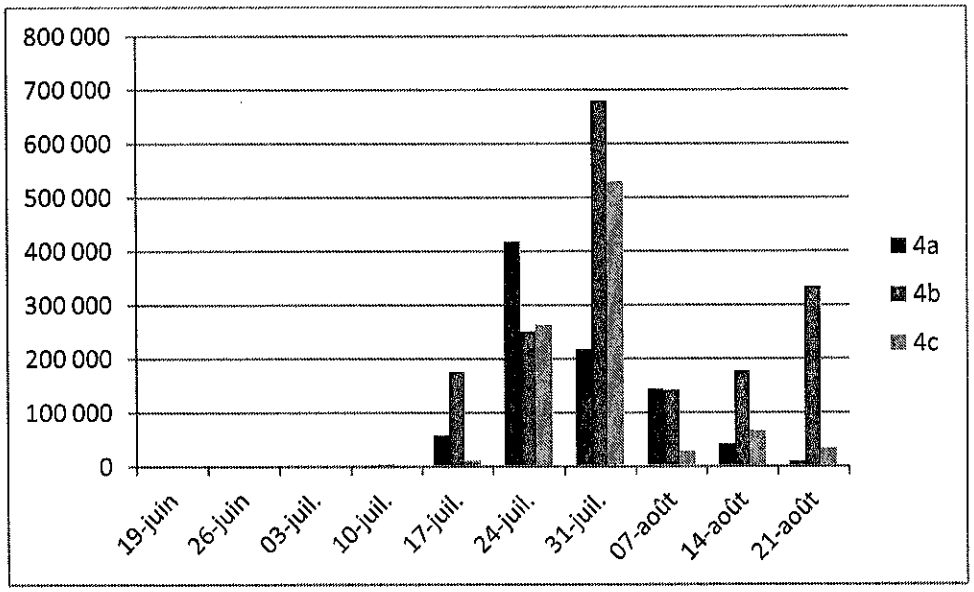



**Figure 3** : Quantité d'*Ostreopsis* cf. *ovata* par gramme de Poids Frais de macroalgues au niveau des sites 4a, 4b et 4c dans la baie de Villefranche durant l'été 2013.

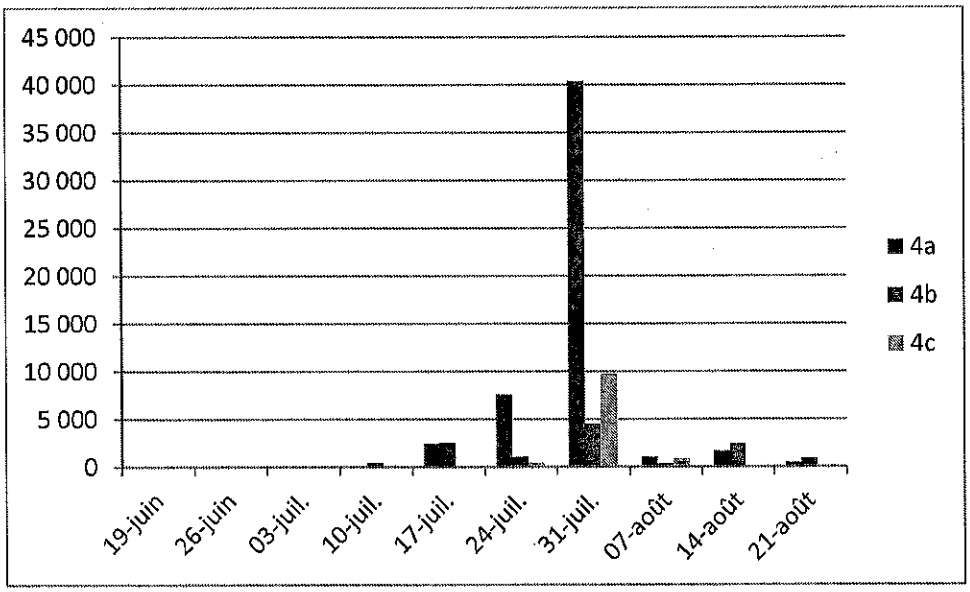



**Figure 4** : Quantité d'*Ostreopsis* cf. *ovata* par litre d'eau de mer au niveau des sites 4a, 4b et 4c dans la baie de Villefranche durant l'été 2013.

Comme durant l'été 2012, c'est le site 4 (Rochambeau) qui présente les plus fortes concentrations en *Ostreopsis* cf. *ovata*, dépassant légèrement les valeurs benthiques observées au site 3 (début de la plage des Marinières). De nombreuses fleurs d'eau ont été observées au site 4 début août (figure 5).

Contrairement à 2012, on commence à retrouver en 2013 des quantités non négligeables d'*Ostreopsis* à la Plage des Jeunes (site 1). Ce site était connu pour avoir de très fortes abondances de cette microalgue en 2008 et 2009, puis presque aucun développement de l'algue en 2012, lors des travaux de réaménagement (pas de suivi en 2010 et 2011).

Concernant la période du développement d'*Ostreopsis* cf. *ovata*, elle présente un léger décalage comparer à celle de 2012 (environ 1 semaine de retard), probablement en relation avec le printemps relativement froid dans notre région en 2013.

**Figure 5** : Fleurs d'eau à *Ostreopsis* cf. *ovata* observées au site 4 durant l'été 2013. On remarque également la présence de méduses (*Pelagia noctiluca*).

## Conclusion et recommandations pour les années futures :

En France, durant l'été 2008, le seuil de pré-alerte était de 4 000 cellules d'*Ostreopsis* cf. *ovata* par litre d'eau de mer et le seuil d'alerte était de 30 000 cellules/litre (sources : ARS PACA). Depuis l'été 2009, les seuils officiels sont plus élevés : «pré-alerte» à 30 000 cellules/litre et «alerte» à 100 000 cellules/litre. Ces seuils n'ont pas évolué depuis 2009.

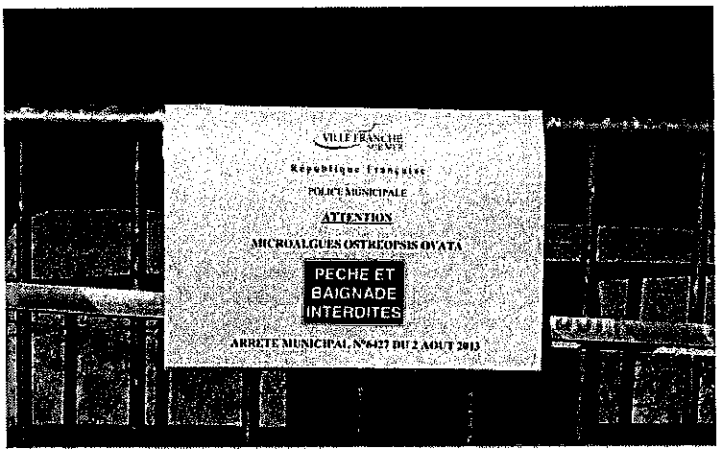



**Figure 6** : Affiche présentant l'interdiction de pêche et de baignade au site 4 durant l'été 2013.

L'été 2013 a été caractérisé par un fort développement d'*Ostreopsis* cf. *ovata* dans la baie de Villefranche, aussi bien sur les macroalgues que dans l'eau. Les quantités de microalgues planctoniques ont dépassé le seuil d'alerte de 2008 et le seuil de pré-alerte de 2009 (ARS). Un arrêté municipal a été pris afin d'interdire la baignade et la pêche pendant une semaine, à partir du 2 août (figure 6) au niveau du site 4 (Rochambeau).

Les sites 3 et 4 sont toujours des sites à risque. Le site 1 (plage de jeunes) semble également redevenir une zone propice au développement de la microalgue toxique.

Enfin l'étude des 3 sous-sites (4a, 4b et 4c) montre bien la nécessité d'effectuer plus d'un prélèvement afin d'avoir une bonne estimation de la quantité d'*Ostreopsis* cf. *ovata* dans une zone.

Il est toujours très difficile de prévoir l'intensité et la période de prolifération d'*Ostreopsis* cf. *ovata*, en liaison avec le climat (à différentes échelles de temps). Par contre, se sont souvent les mêmes sites qui sont touchés d'une année sur l'autre. Nous conseillons de focaliser les efforts de surveillance sur ces zones sensibles, surtout lorsqu'elles sont très fréquentées et/ou bordés d'immeubles.

**Publications du responsable du projet, en relation avec le sujet.**
**Ces publications sont disponibles sur simple demande.**


Mangialajo L, Bertolotto R, Cattaneo Vietti R, Chiantore M, Grillo C, **Lemée R**, Melchiorre N, Moretto P, Povero P, Ruggieri N. (2008). The toxic benthic dinoflagellate *Ostreopsis ovata*: Quantification of proliferation along Genoa coastline (Italy, NW Mediterranean Sea) in the summer of 2006. Marine Pollution Bulletin, 56 (6): 1209-1214.

Tichadou L, Glaizal M, Armengaud A, Grossel H, **Lemée R**, Kantin R, Lasalle JL, Drouet G, Rambaud L, Malfait P, de Haro L. (2010). Health impact of unicellular algae of the *Ostreopsis* genus blooms in the Mediterranean Sea: experience of the French Mediterranean Coast Surveillance Network from 2006 to 2009. Clinical Toxicology, 48: 839-844.

Mangialajo L, Ganzin N, Accoroni S, Asnaghi V, Blanfuné A, Cabrini M, Cattaneo-Vietti R, Chavanon F, Chiantore M, Cohu S, Costa E, Fornasaro D, Grossel H, Marco-Mirailles F, Maso M, Rene A, Rossi AM, Sala MM, Thibaut T, Totti C, Vila M, **Lemee R.** (2011). Trends in *Ostreopsis* proliferation along the Northern Mediterranean coasts. Toxicon, 57(3): 408-420.

Cohu S, Thibaut T, Mangialajo L, Labat JP, Passafiume O, Blanfuné A, Simon N, Cottalorda JM & **Lemée R.** (2011). Occurence of the toxic dinoflagellate *Ostreopsis* cf. *ovata* in relation with environmental factors in Monaco (NW Mediterrean). Mar. Poll. Bull. 62 (12): 2681-2691.

Amzil Z, Sibat M, Chomerat N, Grossel H, Mirrales F, **Lemee R**, Nezan E & Sechet V. (2012). Ovatoxin-a and Palytoxin accumulation in seafood in relation with *Ostreopsis* cf. *ovata* blooms on the French Mediterranean coast. Mar. Drugs 10: 477-496.

Cohu S & **Lemée R.** (2012). Vertical distribution of the toxic epibenthic dinoflagellates *Ostreopsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis* in the NW Mediterranean Sea. Cahier de Biologie Marine 53: 373-380.

**Lemée R**, Mangialajo L, Cohu S, Amzil Z, Blanfuné A, Chomerat N, Ganzin N, Gasparini S, Grossel H, Guidi-Guilvard L, Hoareau L, le Duff F, Marro S, Simon N, Nezan E, Pedrotti ML, Sechet V, Soliveres O, Thibaut T (2012). Interactions between scientists, managers and policy makers in the framework of the French MediOS project on *Ostreopsis* (2008-2010). Cryptogamie Algologie, 33 (2): 137-142.

Guidi-Guilvard L, Gasparini S & **Lemée R.** (2012). The negative impact of *Ostreopsis* cf. *ovata* on phytal meiofauna from the coastal NW Mediterranean. Cryptogamie Algologie, 33 (2): 121-128.

Blanfuné A, Cohu S, Mangialajo L, **Lemée R** & Thibaut T (2012). Preliminary assessents of the impact of *Ostreopsis* cf. *ovata* (Dinophyceae) development on macroinvertebrates in the North Western Mediterranean Sea. Cryptogamie Algologie, 33 (2): 129-136.

Sechet V, Sibat M, Chomérat N, Nézan E, Grossel H, Lehebel-Peron JB, Jauffrais T, Ganzin N, Marci-Miralles F, **Lemée R** & Amzil Z (2012). *Ostreopsis* cf. *ovata* in the French Mediterranean coast: molecular characterization and toxin profile. Cryptogamie Algologie, 33 (2) : 89-98. 3)

**Lemée R.**, Chiantore M. & Mangialajo L. (2012). Proceedings of the International Congress on *Ostreopsis* Development (ICOD, April 2011, France). Cryptogamie Algologie, 33 (2): 79-80.

ICOD (2012). Round Table 1 of the International. Conference of *Ostreopsis* development: Secondary metabolites and toxicity of *Ostreopsis*, Cryptogamie Algologie 33(2): 81-84.

ICOD (2012). Round Table 2 of the International Conference on *Ostreopsis* Development: Environmental, Health and Economic management, state of the art and perspectives, Cryptogamie Algologie, 33(2): 85-87.

Cohu S, Mangialajo L, Thibaut T, Blanfuné A, Marro S & **Lemée R.** (2013). Proliferation of the toxic dinoflagellates *Ostreopsis* cf. *ovata* in relation with depth, biotic substrate and environmental factors in NW Mediterranean Sea. Harmful Algae, 24: 32-44.

Biré R, Trotereau S, **Lemée R**, Delpont C, Chabot B, Aumond Y, Krys S. (2013). Occurrence of palytoxines in marine organisms from different trophic levels harvested on the French Mediterrean coast in 2009. Harmful Algae, 28: 10-22.

Les 2 dernières publications sont annexées à ce rapport.

Toutes les publications issues des communications orales ou affichées lors du congrès international ICOD de 2011 à Villefranche sont disponibles sur le site internet de l'Accord International RAMOGE (en bas de la page : http://www.ramoge.org/fr/ostreopsis_ovata.aspx).

Pour les gestionnaires du littoral, je recommande les publications suivantes :

ICOD (2012). Round Table 2 of the International Conference on *Ostreopsis* Development: Environmental, Health and Economic management, state of the art and perspectives, Cryptogamie Algologie, 33(2): 85-87.

et

Lemée R, Mangialajo L, Cohu S, Amzil Z, Blanfuné A, Chomerat N, Ganzin N, Gasparini S, Grossel H, Guidi-Guilvard L, Hoareau L, le Duff F, Marro S, Simon N, Nezan E, Pedrotti ML, Sechet V, Soliveres O, Thibaut T (2012). Interactions between scientists, managers and policy makers in the framework of the French MediOS project on *Ostreopsis* (2008-2010). Cryptogamie Algologie, 33 (2): 137-1

Harmful Algae 24 (2013) 32–44

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

# Proliferation of the toxic dinoflagellate *Ostreopsis* cf. *ovata* in relation to depth, biotic substrate and environmental factors in the North West Mediterranean Sea

S. Cohu [a,b,c], L. Mangialajo [c], T. Thibaut [c], A. Blanfuné [a,b,c], S. Marro [a,b], R. Lemée [a,b,*]

[a] Université Pierre et Marie Curie-Paris 6, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer cedex, France
[b] CNRS, Marine Microbial Ecology and Biogeochemistry, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer cedex, France
[c] Université Nice-Sophia Antipolis, Laboratoire Ecosystèmes Côtiers Marins et Réponse aux Stress, EA 4228, Parc Valrose, 06108 Nice Cedex2, France

ARTICLE INFO

*Article history:*
Received 10 December 2011
Received in revised form 11 January 2013
Accepted 11 January 2013

*Keywords:*
Dinoflagellate
HAB
Mediterranean Sea
*Ostreopsis*

ABSTRACT

In recent decades, the North West Mediterranean Sea has been seriously affected by the development of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata*, which is associated with harmful effects on human health and the environment. The present work aims to provide a large overview of the multiple environmental factors assumed to regulate or influence the growth of *Ostreopsis*. An intensive sampling campaign over a full annual cycle was performed along the French and Italian coasts (in six sites from Cassis to Genoa), to determine patterns of temporal and spatial distributions of both *O.* cf. *ovata* epiphytic and planktonic cells. Results highlighted substantial seasonal variations in the abundance of *Ostreopsis*. These variations correlated to seawater temperature, with an optimum growth temperature ranging from 23 °C to 27.5 °C. Phosphate concentration, rather than nitrogen or silicate, was also positively associated with *Ostreopsis*. Decreases in oxygen and increases in chlorophyll *a* concentrations were recorded during the summer blooming period. The maximal *Ostreopsis* epiphytic abundance was generally higher on *Dictyota* spp. than on the other two sampled macroalgae (up to $8.54 \times 10^6$ cells $g^{-1}$ FW), even though statistical analysis did not support a clear substrate preference. Epiphytic abundances were significantly higher at a very shallow depth (0.5 m), than at 1 and/or 3 m depths. High anthropogenic pressure (related to population density) seems to have promoted the occurrence of blooms in urbanized areas, which could partly explain the strong demarcation in *Ostreopsis* development between Western and Eastern sampling sites. The ecological niche of *Ostreopsis* cf. *ovata* needs precise definition, which will require further *in situ* and *in vitro* experimental studies, to determine the relative importance of distinct environmental parameters.



## 1. Introduction

Blooms of the dinoflagellate *Ostreopsis* J. Schmidt represent an increasing and worrying issue throughout the world. These harmful benthic microalgae grow in tropical and subtropical areas (Ballantine et al., 1988; Morton et al., 1992; Grzebyk et al., 1994; Faust, 1995; Faust et al., 1996; Parsons and Preskitt, 2007; Mattos Nascimento et al., 2008). However, in the last few decades the frequency of *Ostreopsis* proliferations has increased in temperate areas. Coastal zones of the Mediterranean Sea are particularly affected, with the highest abundance recorded in the North Western part (Mangialajo et al., 2008b, 2011; Totti et al., 2010; Cohu et al., 2011; Parsons et al., 2012). Blooms have also been reported in temperate areas of New Zealand (Chang et al., 2000; Rhodes et al., 2000; Shears and Ross, 2009, 2010), Tasmania and South Australia (Pearce et al., 2001; Rhodes et al., 2010), the islands of Kyushu, Shikoku and Okinawa in Japan (Taniyama et al., 2003; Rhodes, 2011) and Peter Great Bay in Eastern Russia (Selina and Orlova, 2010; Selina and Levchenko, 2011).

The proliferation of *Ostreopsis* is a complex phenomenon linked to many interacting factors. As is the case for other harmful algae, human activities might be involved. Indeed, various factors have possibly contributed to the expansion of HABs. These include: transport of resting cysts in ballast waters or on floating debris (Maso et al., 2003; Vila and Maso, 2005); coastal water eutrophication (Maso and Garcés, 2006); building harbors which increase water residence-time (Vila et al., 2001a; Vila and Maso, 2005); global climate change (Maso and Garcés, 2006; Miraglia et al., 2009).

*Ostreopsis* bloom events may have important environmental and health consequences. *Ostreopsis* species produce potent palytoxin and its derivatives (Onuma et al., 1999; Taniyama et al., 2003; Lenoir et al., 2004; Riobo et al., 2006), which are

* Corresponding author at: Université Pierre et Marie Curie-Paris 6, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer cedex, France.
*E-mail address:* lemee@obs-vlfr.fr (R. Lemée).

1568-9883/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.hal.2013.01.002

thought to be responsible for tropical Neurotoxic Shellfish Poisonings *via* the consumption of contaminated seafood (Ito et al., 1996; Yasumoto, 1998; Deeds and Schwartz, 2010). Around the Mediterranean Sea, blooms of *Ostreopsis* cf. *ovata* Fukuyo frequently cause cases of skin and eye irritations and less frequently respiratory distress (Simoni et al., 2003; Brescianini et al., 2006; Ciminiello et al., 2006; Barroso Garcia et al., 2008; Tichadou et al., 2010). *Ostreopsis* proliferation can also cause significant ecological damage: mass mortalities of invertebrates occurred during blooms in New Zealand (Shears and Ross, 2009, 2010), Brazil (Mattos Nascimento et al., 2008) and the Mediterranean Sea (Simoni et al., 2003; Totti et al., 2010).

The abundance of *Ostreopsis* shows high seasonal variation, mostly in temperate areas (Vila et al., 2001a,b; Simoni et al., 2003; Turki, 2005; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011) and an important spatial heterogeneity at different scales, *i.e.* from hundreds of meters (Okolodkov et al., 2007; Shears and Ross, 2009; Cohu et al., 2011) to several kilometers (Grzebyk et al., 1994; Chang et al., 2000; Aligizaki and Nikolaidis, 2006; Parsons and Preskitt, 2007). In the Mediterranean Sea, the seasonal trend of *Ostreopsis* spp. clearly follows the wide range in variations of temperature (Vila et al., 2001a,b; Aligizaki and Nikolaidis, 2006; Ungaro et al., 2010). However, the impact of temperature during the summer period seems less evident due to strong interactions with other factors. The marked heterogeneity in the distribution of *Ostreopsis* spp. among several substrates (*i.e.* macroalgae, sea grasses, dead biotic substrates, rock and sand) could partially explain the spatial variability of epibenthic cells (Grzebyk et al., 1994; Faust et al., 1996; Turki, 2005; Aligizaki and Nikolaidis, 2006; Parsons and Preskitt, 2007; Mangialajo et al., 2008b; Totti et al., 2010). Moreover, the 3-dimensional structure of macroalgae hosts and/or the production of secondary metabolites may affect *Ostreopsis* colonization (Parsons and Preskitt, 2007; Totti et al., 2010). Depth (as related to light intensity) could also strongly restrict *Ostreopsis* development, although most observations have been limited to a depth between 0.2 and 5 m (*e.g.* Aligizaki and Nikolaidis, 2006; Monti et al., 2007; Battocchi et al., 2010; Cohu et al., 2011). In addition, the distribution of *Ostreopsis* cells in deeper waters is unknown. The influence of nutrient availability has been poorly studied and contrasting results have been reported for tropical and temperate areas (Vila et al., 2001b; Parsons and Preskitt, 2007; Ungaro et al., 2010). Likewise, the relationships between the development of *Ostreopsis* and other parameters such as oxygen saturation and concentrations of algal pigments (*e.g.* chlorophyll *a* and pheophytin) are poorly understood. While several *in vitro* experiments have highlighted bacterial species associated with the toxicity of *Ostreopsis* spp. (Tosteson et al., 1989; Carballeira et al., 1998; Ashton et al., 2003; Pérez-Guzmán et al., 2008), the impact of *Ostreopsis* blooms on bacterial abundance in the natural environment has not been examined.

The present study aims to provide a base for our understanding of *Ostreopsis* ecology in the Mediterranean Sea. An intensive, wide-ranging sampling campaign documenting the development of *O.* cf. *ovata* in relation to a suite of environmental factors (substrate, depth, temperature, nutrients, oxygen, chlorophyll, pheophytin and bacterial abundance) was conducted over one year along the South-Eastern French and Ligurian coasts, from Cassis (France) to Genoa (Italy).

## 2. Materials and methods

### 2.1. Ostreopsis *cf.* ovata

From February 2008 to February 2009, sampling was conducted by snorkeling and diving in six sites along the Gulf of Lions and Ligurian coasts: Cassis, Ramatuelle, Saint-Raphaël, Nice, Villefranche-sur-Mer and Genoa (Fig. 1). Each site was sampled once per season in Winter, Spring and Fall, and four times in Summer (Table 1). For each site, the hierarchical sampling design described below was conducted at 0.5, 1 and 3 m depths in three randomly chosen sub-sites, nearly 10 m apart, in order to constitute triplicates.

Concentrations of planktonic *Ostreopsis* were determined from seawater samples collected in a 250 ml plastic flask, at about 20 cm above the rocky substrate. To determine epiphytic cell abundance, three macroalgae species were collected on rocky substrate: the bush-like Phaeophyceae *Halopteris scoparia* (Linnaeus) Sauvageau, the flat-ribbon Phaeophyceae *Dictyota* spp. (mostly *Dictyota dichotoma*) and the articulated corallinales (Rhodophyceae) *Corallina elongata* (Ellis & Solander) or rarely *Jania rubens* (Linnaeus). Each macroalga was carefully sampled in a 250 ml plastic flask with the surrounding seawater, avoiding any loss of

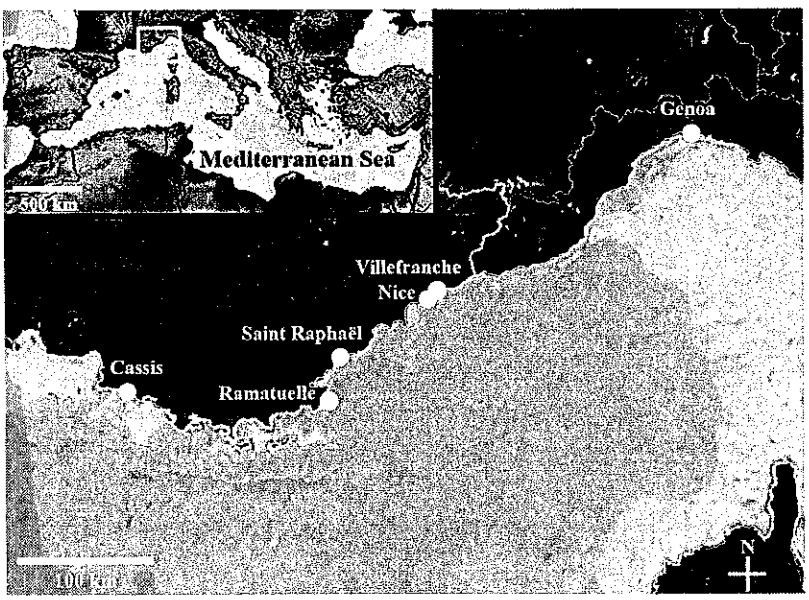



**Fig. 1.** The Mediterranean Sea showing the positions of the six sampled sites, Cassis, Ramatuelle, Saint Raphaël, Nice, Villefranche-sur-Mer and Genoa along the Gulf of Lions and Ligurian coasts.

**Table 1**
Characteristics of the sampled sites: dates of sampling, geographical coordinates, nature of substrate and hydrodynamic mode (+ = sheltered, ++ = moderate, +++ = exposed). The maximal epiphytic abundances and planktonic concentrations of *Ostreopsis* cf. *ovata* are indicated for each site with the depth, the substrate and the season at which they were measured.

| Time (code) | Cassis | Ramatuelle | Saint Raphaël | Nice | Villefranche-sur-Mer | Genoa |
|---|---|---|---|---|---|---|
| Winter (W) | 13th March 2008 | 4th March 2008 | 20th February 2008 | 2nd February 2008 | 14th February 2008 | 20th March 2008 |
| Spring (Sp) | 7th May 2008 | 25th April 2008 | 23th April 2008 | 18th April 2008 | 15th April 2008 | 14th May 2008 |
| Summer1 (Su1) | 2nd July 2008 | 6th June 2008 | 9th June 2008 | 2nd June 2008 | 2nd June 2008 | 26th June 2008 |
| Summer2 (Su2) | 24th July 2008 | 4th July 2008 | 16th July 2008 | 18th July 2008 | 7th July 2008 | 11th July 2008 |
| Summer3 (Su3) | 6th August 2008 | 29th July 2008 | 29th July 2008 | 30th July 2008 | 30th July 2008 | 31st July 2008 |
| Summer4 (Su4) | 22nd August 2008 | 25th August 2008 | 25th August 2008 | 27th August 2008 | 27th August 2008 | 19th August 2008 |
| Fall/Winter (F/W) | 5th December 2008 | 17th November 2008 | 3rd February 2009 | 16th January 2009 | 14th November 2008 | 17th February 2009 |
| GPS coordinates | 43°12.75′N | 43°11.26′N | 43°25.20′N | 43°41.45′N | 43°42.16′N | 44°23.29′N |
| | 5°32.11′E | 6°38.73′E | 6°51.55′E | 7°17.59′E | 7°19.19′E | 8°59.61′E |
| Nature of substrate | Artificial | Natural | Natural | Natural | Artificial | Natural |
| Hydrodynamic mode | +++ | + | ++ | +++ | + | ++ |
| Population density (pers/km$^2$) | 290 | 65 | 380 | 4795 | 1279 | 2496 |
| Max. epiphytic abundance (cells g$^{-1}$ FW) | $0.06 \times 10^6$ on *Dictyota* spp. | $0.01 \times 10^6$ on *Dictyota* spp. | $0.02 \times 10^6$ on *Corallina elongata* | $1.98 \times 10^6$ on *Dictyota* spp. | $8.54 \times 10^6$ on *Dictyota* spp. | $2.81 \times 10^6$ on *Dictyota* spp. |
| Max. planktonic concentration (cells l$^{-1}$) | $1 \times 10^3$ | $3 \times 10^3$ | $0.4 \times 10^3$ | $12 \times 10^3$ | $7 \times 10^3$ | $68 \times 10^3$ |

microalgae as much as possible. Depending on their growth cycle and the environmental disturbance, some macroalgae species were missing in some sites/depths, particularly during winter, and therefore could not be collected. *Ostreopsis* cells were fixed by adding acidic Lugol at 1% (vol./vol.) in water samples and 2% (vol./vol.) in macroalgae samples. Concentrations of planktonic cells in the water samples were evaluated with an inverted microscope using the Utermöhl method (Utermöhl, 1958) and settling 50 ml of sea water. *Ostreopsis* concentrations were recorded as the number of cells per liter (cells l$^{-1}$). Macroalgae samples were vigorously shaken and passed through a 500 μm meshed filter to separate macroalgae and water containing microalgae. Macroalgae were rinsed twice with 100 ml of 0.2 μm filtered seawater to recover a maximum of microalgae, then dried on absorbent paper and weighed (±0.01 g). Epiphytic cell abundances in the rinsed water were determined in 1 ml volume Sedgwick Rafter cells using a standard light microscope and recorded as the number of cells per gram of fresh weight of macroalgae (cells g$^{-1}$ FW).

### 2.2. Measurement of environmental factors

#### 2.2.1. Temperature and oxygen

Seawater temperature (±0.1 °C) and oxygen saturation (±0.1%) were measured in each site at both 0.5 and 3 m depths during sampling sessions, using an oxymetric/temperature sensor (Oxy1971, WTX). The oxygen saturation is the ratio, expressed in percentage, between the measured oxygen concentration and the theoretical solubility calculated from the seawater temperature.

#### 2.2.2. Nutrient and pigments

In order to quantify nutrients and concentrations of chlorophyll *a* (Chl *a*) and pheophytin, samples of seawater were collected at 0.5 and 3 m depths in triplicate, following the same method used for planktonic cells. Samples intended for nutrient analysis were fixed with 0.06% of $HgCl_2$ solution and kept in the dark at 4 °C. Concentrations of nitrite, nitrate, phosphate and silicate were measured using an automatic analysis chain (EV2-Alliance Instrument) according to the methodology of Tréguer and Corre (1975). Samples for Chl *a* and pheophytin quantification were filtered (1 l per sample) on a Whatman GF/F. Filters were kept frozen (−20 °C) before extraction in a 90% acetone solution. Chl *a* and pheophytin concentrations were measured using fluorometry, according to the Lorenzen method (Lorenzen and Newton Downs, 1986).

#### 2.2.3. Bacteria

Bacterial abundance was determined from water samples collected at 0.5 and 3 m depths, in triplicate. Samples (1.5 ml) were fixed in glutaraldehyde (0.65% final concentration), kept in the dark for 15 min at ambient temperature, flash frozen in liquid nitrogen and stored at −80 °C. Concentrations of heterotrophic bacteria were determined by flow cytometry (Becton Dickinson, FACSCalibur) after staining with SYBRGreen I (Molecular Probes) as described by Gasol and Del Giorgio (2000). Fluorescent 1 μm latex beads ($10^5$ beads ml$^{-1}$) were systematically added to the bacterial samples as an internal quality standard (Polyscience Inc., Europe).

### 2.3. Statistical analyses

As data on the abundance of *Ostreopsis* did not show a normal distribution, the effects of factors such as site, time, substrate and depth on both epiphytic and planktonic abundances were tested by separate PERMANOVAs (Anderson, 2005), following the orthogonal design: site (3 levels, fixed), time (4 levels, fixed, crossed), substrate (3 levels, fixed, crossed), depth (3 levels, fixed, crossed), with $n = 3$ replicates per combination of factors. This statistical

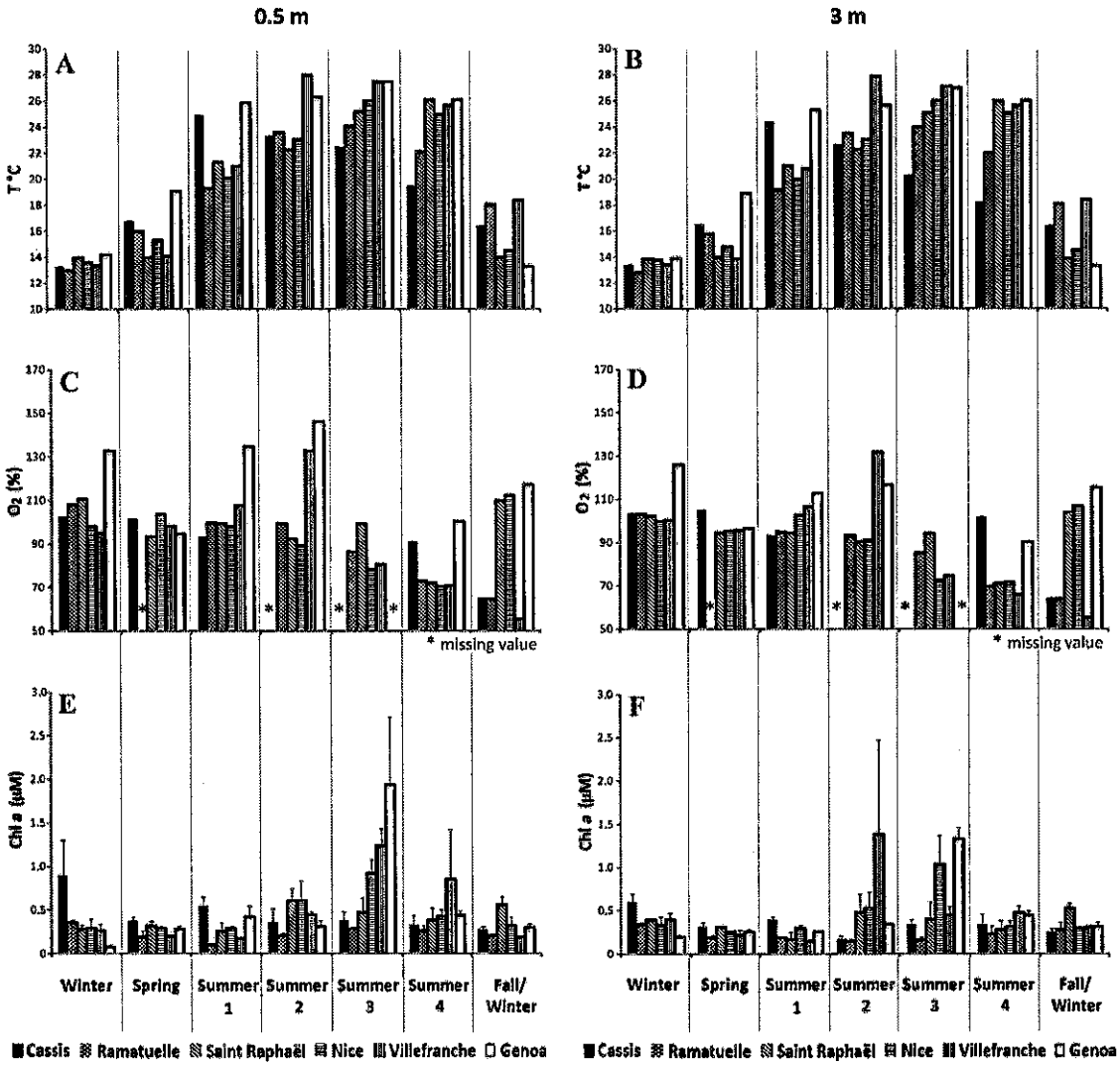



Fig. 2. Temperature (a and b), oxygen saturation (c and d) and mean chlorophyll a concentration ± SE (e and f) measured at 0.5 and 3 m depths in the six studied sites during the seven sampling periods.

design was restricted to the data collected in the eastern sites of Nice, Villefranche-sur-Mer and Genoa during the bloom summer period ("Summer1" to "Summer4") in order to avoid a bias in results induced by the frequent null abundances recorded during the other seasons and in the three western sites. PERMANOVAs were performed with 9999 random permutations, using the PERMANOVA+ program of PRIMER 6 (PRIMER-E Ltd.). Analyses were based on Euclidean distance-transformed data. Significant terms were examined individually using pair-wise tests.

The non-parametric Spearman correlation test (STATISTICA 7.0, Stat Soft, Inc.) was applied on the complete data set in order to highlight eventual relationships between *Ostreopsis* abundance and environmental parameters, as well as between the parameters themselves.

## 3. Results

### 3.1. Environmental characteristics of sites

All the sampled sites were natural rocky areas, except Cassis and Villefranche-sur-Mer which were composed of artificial rocky substrate (Table 1). Cassis and Nice sites were highly exposed to hydrodynamic conditions. The towns of Nice, Genoa and Villefranche-sur-Mer have the highest population densities respectively (data in Table 1 from the last 2008 French population census: http://www.recensement.insee.fr and from the Genoa town hall: http://www.comune-italia.it/comune-genova.html).

Trends of environmental parameters measured in the six sites at 0.5 and 3 m depths are reported in Figs. 2 and 3. Each environmental parameter showed approximately the same range of values at both sampled depths, except for nitrate and silicate concentrations which were consistently more abundant at the depth of 0.5 m than at 3 m, in Cassis.

The total temperature range encountered varied from 12.8 to 28 °C, in Ramatuelle and Villefranche-sur-Mer respectively (Fig. 2a and b). Oxygen saturation ranged from 55.2% in Villefranche-sur-Mer to 146.2% in Genoa (Fig. 2c and d). Chlorophyll *a* concentrations varied from 0.03 µM in Saint-Raphaël to 3.19 µM in Genoa (Fig. 2e and f). Concerning nutrients, Nitrite followed a typical summer depletion (Fig. 3a and b), varying from 0.01 µM in Ramatuelle to 0.35 µM in Genoa. Nitrate concentrations were often non-detectable in Genoa and Ramatuelle, and reached 20.55 µM in Cassis (Fig. 3c and d). Phosphate concentrations ranged between 0.01 µM in Villefranche-sur-Mer and 0.56 µM in Saint-Raphaël (Fig. 3e and f). Silicate varied between 0.59 and 25.08 µM, both values measured in Cassis (Fig. 3g and h). As a general rule, a longitudinal gradient of nutrient concentrations was not observed between the Eastern and Western sites. The total

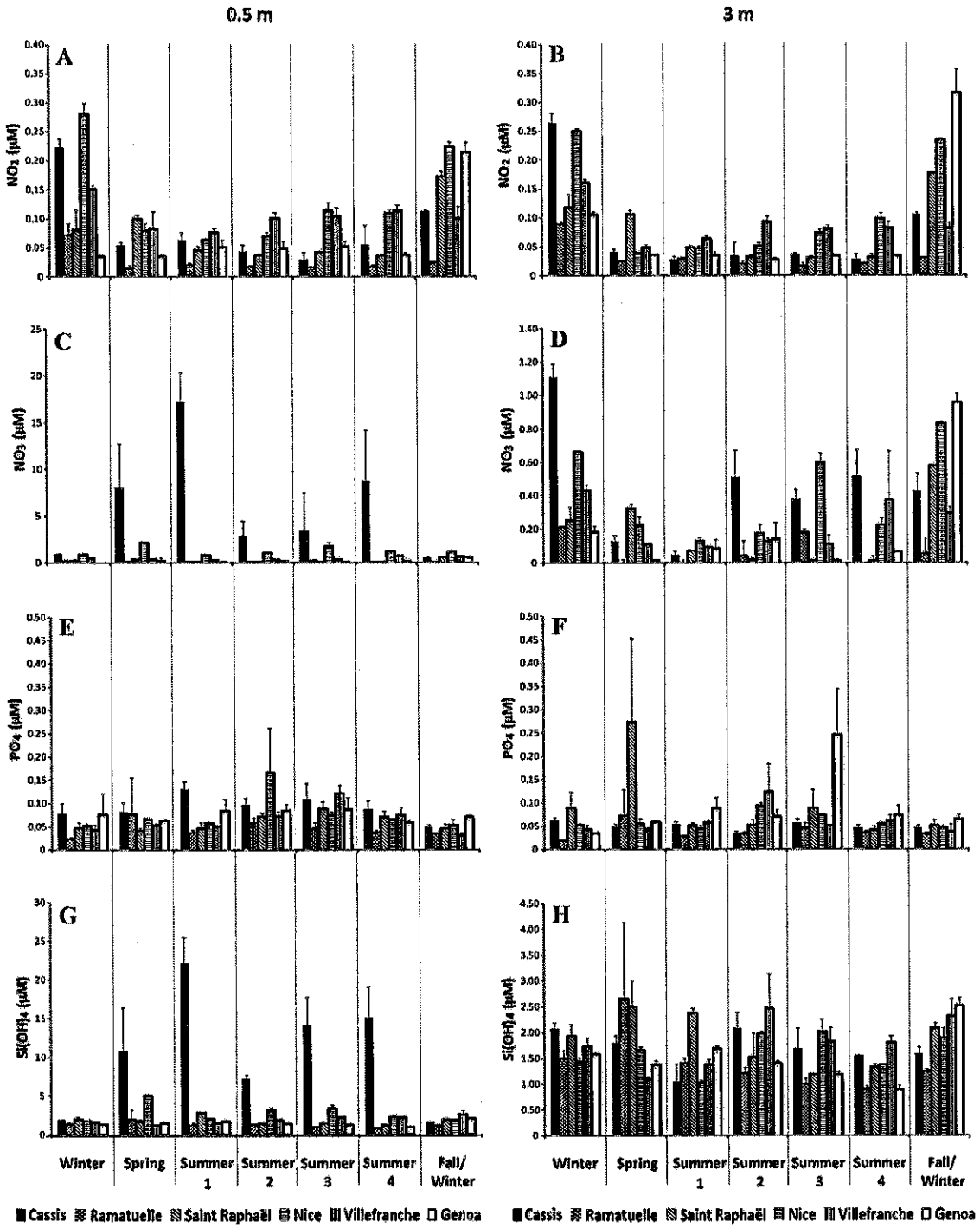



Fig. 3. Mean nutrient concentrations ± SE measured at 0.5 and 3 m depths in the six studied sites during the seven sampling periods: nitrites (a and b), nitrates (c and d), phosphates (e and f) and silicates (g and h).

bacterial abundance varied from $0.02 \times 10^6$ cells $l^{-1}$ in Nice to $2.33 \times 10^6$ cells $l^{-1}$ in Saint-Raphaël.

### 3.2. Temporal and spatial variation in Ostreopsis abundance

The presence of epiphytic *Ostreopsis* cf. *ovata* was observed for the first time on March 4th 2008 (Winter) at 0.5 m in Ramatuelle. Planktonic cells were detected later, on April 18th (Spring), at 1 m in Nice. The first epiphytic bloom was measured mainly on *Dictyota* spp. on July 11th in Genoa (Fig. 4, during Summer2, up to $1.32 \times 10^6$ cells $g^{-1}$ FW at 0.5 m), simultaneously with the first registered peak in planktonic concentrations (up to $8 \times 10^3$ cells $l^{-1}$ at 0.5 m). Subsequently, high population abundances of *Ostreopsis* were observed during the last two weeks of July, in the three eastern sites (Nice, Villefranche-sur-Mer and Genoa). The maximal epiphytic abundance ($8.54 \times 10^6$ cells $g^{-1}$ FW) was recorded at a depth of 1 m on *Dictyota* spp., on July 30th (Summer3) in Villefranche-sur-Mer. The maximum planktonic concentration ($68 \times 10^3$ cells $l^{-1}$) was recorded on July 31st, in Genoa at 0.5 m. From mid-August, epiphytic and planktonic abundances remained low or below the limit of detection. The presence of *Ostreopsis* cells in samples was detected up to December 5th (Fall/Winter) in Cassis. The abundance

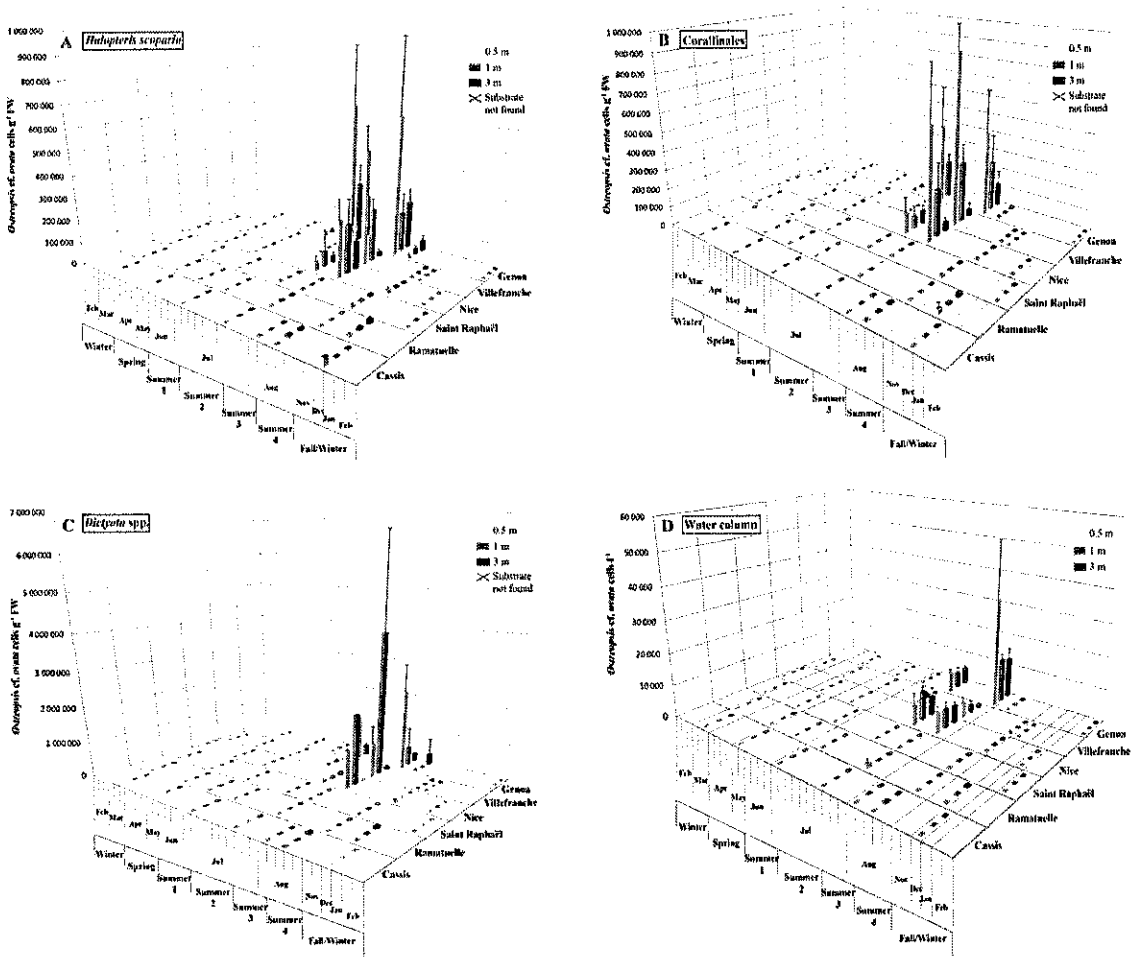



**Fig. 4.** Mean *Ostreopsis* epiphytic abundances ± SE measured on (a) *Halopteris scoparia* ($N = 360$), (b) *Corallinales* ($N = 366$), (c) *Dictyota* spp. ($N = 270$) and (d) mean *Ostreopsis* planktonic concentrations ± SE ($N = 378$) at 0.5, 1 and 3 m depths during the seven sampling periods. For clarity, the annual time scale is variable, each division on the *x*-axis corresponding to one sampling date.

of *Ostreopsis* showed a standard seasonal development, with the highest values found from June to August.

In addition to the temporal variation, a high spatial heterogeneity was clearly evident. Indeed, epiphytic and planktonic *Ostreopsis* abundances exhibited high spatial variation at a small scale within each site, as shown by the important standard errors (SE) related to the triplicates (for each substrate and depth, Fig. 4). In addition, a high heterogeneity was observed at a larger spatial scale between the sampled sites, mostly during summer. Over the whole bloom season (from Summer1 to Summer4), the mean epiphytic abundances (all substrates and depths included) reached $0.24 \times 10^6$ (± SE of $0.09 \times 10^6$) cells $g^{-1}$ FW in Genoa, $0.21 \times 10^6$ ($\pm 0.02 \times 10^6$) cells $g^{-1}$ FW in Villefranche-sur-Mer and $0.11 \times 10^6$ ($\pm 0.06 \times 10^6$) cells $g^{-1}$ FW in Nice. In the western sites, the measured mean abundances were lower: $0.03 \times 10^6$ ($\pm 0.02 \times 10^6$) in Cassis, $0.03 \times 10^6$ ($\pm 0.01 \times 10^6$) in Saint Raphaël, and $0.01 \times 10^6$ ($\pm 0.01 \times 10^6$) in Ramatuelle. Planktonic concentrations followed the same trend with higher mean values (all depths included) recorded in Genoa ($6 \times 10^3 \pm 4 \times 10^3$ cells $l^{-1}$), Nice ($3 \times 10^3 \pm 1 \times 10^3$ cells $l^{-1}$) and Villefranche-sur-Mer ($0.8 \times 10^3 \pm 0.5 \times 10^3$ cells $l^{-1}$). Very low mean concentrations ($\leq 0.1 \times 10^3$ cells $l^{-1}$) were observed in Cassis, Saint Raphaël and Ramatuelle. For both epiphytic and planktonic cells, blooms were only recorded in Genoa, Villefranche-sur-Mer and Nice, which indicates a geographical distinction between eastern and western sites.

The amplitude of epiphytic blooms strongly differed between the substrates, although the abundance of *Ostreopsis* on the three collected macroalgae and in the water column were positively correlated (Spearman test, Table 2).

Results of the four-way PERMANOVAs performed on *Ostreopsis* cf. *ovata* epiphytic and planktonic abundances during summer in the three eastern sites (periods/sites of blooms occurrence) are shown in Table 3a. Since the main effects of the factors tested individually are not considered meaningful in the presence of significant interactions (Anderson, 2005), only significant interactions using pair-wise tests are detailed (results in Table 3b).

Concerning epiphytic data, results of PERMANOVAs indicated a significant effect of the interaction time × substrate ($p = 0.03$, Table 3a). It showed that the distribution of *Ostreopsis* between the three collected substrates varied according to the sampling period. However, significant differences in the abundance of *Ostreopsis* between macroalgae were only observed during Summer1, with a higher abundance on Corallinales than *Halopteris scoparia* (pair-wise test, Table 3b). Inversely, the temporal evolution of epiphytic *Ostreopsis* cells depended on the substrate: abundances on Corallinales and *H. scoparia* were very variable during summer with significantly higher values recorded in Summer3 (late July–early August). Abundances on *Dictyota* spp. exhibited less temporal variation, without any significant differences between Summer3, Summer2 and Summer4 (higher values), as well as between Summer1 and Summer4.

**Table 2**
Results of the Spearman test performed on the complete data set (coefficients $r_S$ of Spearman, bold when significant with $p < 0.05$, bold and underlined when significant with $p < 0.01$).

| | O g$^{-1}$ Hal. | O g$^{-1}$ Cor. | O g$^{-1}$ Dic. | O l$^{-1}$ | $NO_3$ | $NO_2$ | $PO_4$ | $Si(OH)_4$ | Ntotal/P | $PO_4/Si(OH)_4$ | Chl a | Pheo. | $T$ (°C) | $O_2$ | Bacteria |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| O g$^{-1}$ Hal. | 1.000 | **0.901** | **0.893** | **0.890** | −0.061 | **−0.160** | **0.349** | −0.093 | **−0.191** | **0.362** | **0.412** | **0.193** | **0.738** | **−0.392** | 0.067 |
| O g$^{-1}$ Cor. | | 1.000 | **0.907** | **0.894** | **−0.149** | **−0.179** | **0.277** | **−0.181** | **−0.258** | **0.403** | **0.392** | **0.153** | **0.737** | **−0.362** | 0.119 |
| O g$^{-1}$ Dic. | | | 1.000 | **0.849** | −0.095 | **−0.151** | **0.322** | −0.093 | **−0.215** | **0.385** | **0.367** | 0.119 | **0.722** | **−0.295** | 0.015 |
| O l$^{-1}$ | | | | 1.000 | **−0.157** | **−0.207** | **0.362** | **−0.150** | **−0.290** | **0.450** | **0.394** | **0.197** | **0.749** | **−0.332** | 0.070 |
| $NO_3$ | | | | | 1.000 | **0.673** | **0.211** | **0.632** | **0.927** | **−0.372** | **0.236** | **0.243** | **−0.307** | 0.049 | **−0.264** |
| $NO_2$ | | | | | | 1.000 | 0.115 | **0.478** | **0.656** | **−0.176** | **0.288** | **0.404** | **−0.413** | **0.216** | **−0.231** |
| $PO_4$ | | | | | | | 1.000 | **0.396** | −0.106 | **0.459** | **0.495** | **0.407** | **0.358** | 0.100 | 0.026 |
| $Si(OH)_4$ | | | | | | | | 1.000 | **0.525** | **−0.516** | **0.284** | **0.266** | **−0.138** | 0.087 | **−0.125** |
| Ntotal/P | | | | | | | | | 1.000 | **−0.583** | 0.104 | **0.149** | **−0.440** | 0.063 | **−0.245** |
| $PO_4/Si(OH)_4$ | | | | | | | | | | 1.000 | **0.190** | **0.151** | **0.409** | 0.012 | 0.061 |
| Chl a | | | | | | | | | | | 1.000 | **0.711** | **0.256** | −0.018 | 0.103 |
| Pheo. | | | | | | | | | | | | 1.000 | 0.067 | **0.172** | −0.116 |
| $T$ (°C) | | | | | | | | | | | | | 1.000 | **−0.296** | 0.091 |
| $O_2$ | | | | | | | | | | | | | | 1.000 | 0.091 |
| Bacteria | | | | | | | | | | | | | | | 1.000 |

*Abbreviations*: O g$^{-1}$ Hal = *Ostreopsis* cf. *ovata* cells g$^{-1}$ FW of *Halopteris scoparia*; O g$^{-1}$ Cor = *Ostreopsis* cf. *ovata* cells g$^{-1}$ FW of Corallinales; O g$^{-1}$ Dic = *Ostreopsis* cf. *ovata* cells g$^{-1}$ FW of *Dictyota* spp.; O l$^{-1}$ = *Ostreopsis* cf. *ovata* cells l$^{-1}$; Chl a = chlorophyll a; Pheo = pheophytine.

PERMANOVAs also highlighted a significant effect of the interaction time × depth on both epiphytic ($p = 0.02$) and planktonic ($p = 0.03$) abundances (Table 3a). Epiphytic abundances were higher overall at 0.5 m compared to 3 m, with intermediate abundances at 1 m, except during Summer4 when no significant differences appeared between the three depths (Table 3b). In contrast, planktonic concentrations were not significantly related to depth, except during Summer1 with higher values recorded at 0.5 and 1 m than at 3 m, following the epiphytic distribution at the same period. Conversely, the temporal changes in both epiphytic and planktonic *Ostreopsis* cells varied with depth. The temporal variation in epiphytic abundances seemed to decrease with depth, with all sampling periods significantly different at 0.5 m, three significantly different periods at 1 m, and two significantly different periods at 3 m (Table 3b). Planktonic concentrations did not follow this marked trend and the highest temporal variability appeared at 1 m depth. Overall, regardless of depth, both epiphytic and planktonic abundances were higher in Summer3 and lower in Summer1, with or without significant differences with Summer4 or Summer2.

The interaction sites × time was only significant with regard to the planktonic concentrations ($p < 0.001$, Table 3a). In the three sites, higher values were recorded in Summer3 – late July/early August and in Summer2 – early July in Nice (Table 3b). Conversely, it was not possible to highlight any clear pattern of the site ranking, due to the high temporal variability, although Genoa consistently showed higher planktonic concentrations than Nice and/or Villefranche, except in Summer4.

### 3.3. Ostreopsis *development related to environmental factors*

#### 3.3.1. *Oxygen, pigments and bacteria concentrations*

A decrease in the oxygen saturation at 0.5 and 3 m depths was observed during summer, particularly between the sampling periods Summer2 (pre-bloom period) and Summer3/Summer4 (mean bloom and post-bloom periods respectively) in the eastern sites of Nice, Genoa and mostly Villefranche-sur-Mer (Fig. 2a). The Spearman test showed significant negative relationships between oxygen saturation and *Ostreopsis* epiphytic and planktonic abundances (Table 2).

Marked increases in chlorophyll *a* concentrations occurred during the period Summer3 in Genoa, Villefranche-sur-Mer and Nice, mostly at 0.5 m (Fig. 2a). Chlorophyll *a* positively correlated with all epiphytic and planktonic *Ostreopsis* abundances. Similar results were obtained for pheophytin, except an absence of a correlation with cell abundance on *Dictyota* spp. Chlorophyll *a* and pheophytin concentrations were strongly correlated. In contrast to pheophytin, chlorophyll *a* was positively related to the temperature.

Finally, bacteria concentration did not correlate with *Ostreopsis* abundances, regardless of substrate.

#### 3.3.2. *Temperature*

Over the entire study period and all sites included, the Spearman test showed high positive correlations between temperature and *Ostreopsis* cf. *ovata* epiphytic and planktonic abundances (Table 2). These correlations were not linear (Fig. 5) and we assumed the presence of minimal and maximal temperature thresholds allowing *Ostreopsis* growth. The first epiphytic cells were detected at the temperature of 13.0 °C and high abundances (>10,000 cells g$^{-1}$ FW) began to occur at 18 °C, which could represent a first minimal threshold for a substantial development of *Ostreopsis*. Planktonic cells were recorded in the water column from 18 °C and were high in number (>5000 cells l$^{-1}$) from 23 °C. Therefore, temperatures ranging from 23 °C to 27.5 °C seemed to favor the occurrence of epiphytic and planktonic blooms. Although maximal *Ostreopsis* peaks were observed at 27.5 °C, cell abundances were relatively low at the maximal measured temperature of 28.0 °C.

#### 3.3.3. *Nutrient concentrations*

The Spearman test highlighted significant negative correlations between nitrite and both epiphytic and planktonic *Ostreopsis* abundances, although low Spearman coefficients indicated weak relations (Table 2). Nitrate and silicate concentrations were weakly and negatively correlated with *Ostreopsis* abundances, only on Corallinales and in the water column. In contrary to the other nutrients, phosphate concentrations were positively correlated with *Ostreopsis* epiphytic cells on all substrates and with planktonic cells. Phosphate also positively correlated with nitrate and silicate.

The ratio N:P (with N = sum of nitrate and nitrite) was significantly and negatively related to *Ostreopsis* abundances on all substrates, contrary to the ratio P:S.

Finally, all nutrients were negatively correlated with temperature, except for phosphate.

## 4. Discussion

### 4.1. Ostreopsis *cf.* ovata *nomenclature*

Despite the morphological concordance between the Mediterranean strains of *Ostreopsis ovata* and the first description of the species by Fukuyo (1981), the high diversity in the size and shape of cells, and the lack of genetic analysis on cells from the

**Table 3**
Results of PERMANOVAs (a) and conclusions of pair-wise tests (b) performed on epiphytic and planktonic data collected during summer sampling periods (Summer1 to Summer4) in the eastern sites Nice, Villefranche-sur-Mer and Genoa. Bold *p* values are significant with $p < 0.05$. In the pair-wise tests conclusions, significantly similar factors ($p < 0.05$) are joined by crossed-bar.

a).

| Source of variation | df | Epiphytic data (N = 268) MS | F | p | Planktonic data (N = 108) MS | F | p |
|---|---|---|---|---|---|---|---|
| Sites (Si) | 2 | $1.81 \times 10^{11}$ | 0.81 | 0.4182 | $2.87 \times 10^{8}$ | 10.82 | **0.0001** |
| Time (Ti) | 3 | $3.93 \times 10^{12}$ | 17.60 | **0.0004** | $5.20 \times 10^{8}$ | 19.60 | **0.0001** |
| Substrate (Su) | 2 | $1.27 \times 10^{12}$ | 5.67 | **0.0227** | | | |
| Depth (De) | 2 | $1.04 \times 10^{12}$ | 4.64 | **0.0346** | $5.23 \times 10^{7}$ | 1.97 | 0.1309 |
| SixTi | 6 | $3.94 \times 10^{11}$ | 1.76 | 0.1301 | $1.98 \times 10^{8}$ | 7.45 | **0.0001** |
| SixSu | 4 | $7.60 \times 10^{10}$ | 0.34 | 0.8289 | | | |
| SixDe | 4 | $3.42 \times 10^{11}$ | 1.53 | 0.1692 | $3.87 \times 10^{7}$ | 1.46 | 0.2044 |
| TixSu | 6 | $8.77 \times 10^{11}$ | 3.93 | **0.0313** | | | |
| TixDe | 6 | $8.49 \times 10^{11}$ | 3.80 | **0.0230** | $5.23 \times 10^{7}$ | 1.97 | **0.0331** |
| SuxDe | 4 | $2.94 \times 10^{11}$ | 1.32 | 0.2376 | | | |
| SixTixSu | 12 | $9.83 \times 10^{10}$ | 0.44 | 0.9107 | | | |
| SixTixDe | 12 | $3.59 \times 10^{11}$ | 1.61 | 0.1166 | $2.90 \times 10^{7}$ | 1.09 | 0.3589 |
| SixSuxDe | 8 | $4.52 \times 10^{11}$ | 2.02 | 0.0689 | | | |
| TixSuxDe | 12 | $4.13 \times 10^{11}$ | 1.85 | 0.0818 | | | |
| SixTixSuxDe | 19 | $3.79 \times 10^{11}$ | 1.70 | 0.0858 | | | |
| Residues | 165/72 | $2.23 \times 10^{11}$ | | | $2.65 \times 10^{7}$ | | |

b).

**Pair-wise tests on interactions for Epiphytic data:**

**Time x Substrate**

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| HAL DIC COR | COR = HAL = DIC | HAL = COR = DIC | COR = HAL = DIC |

| Corallinales | *Halopteris scoparia* | *Dictyota* sp. |
|---|---|---|
| Su1 < Su4 < Su2 < Su3 | Su1 < Su4 < Su2 < Su3 | Su1 Su4 Su2 Su3 |

**Time x Depth**

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| 3 m < 1 m 0.5 m | 3 m < 1 m < 0.5 m | 3 m 1 m 0.5 m | 1 m = 0.5 m = 3 m |

| 0.5 m | 1 m | 3 m |
|---|---|---|
| Su1 < Su4 < Su2 < Su3 | Su1 < Su2 < Su4 Su3 | Su1 < Su2 Su4 Su3 |

**Pair-wise tests on interactions for Planktonic data:**

**Sites x Time**

| Nice | Villefranche | Genoa |
|---|---|---|
| Su1 < Su4 < Su2 Su3 | Su1 < Su4 Su2 < Su3 | Su1 Su4 < Su2 < Su3 |

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| NIC VLF < GEN | VLF < GEN NIC | VLF < NIC < GEN | GEN = NIC = VLF |

**Time x Depth**

| Summer 1 | Summer 2 | Summer 3 | Summer 4 |
|---|---|---|---|
| 3 m < 1 m 0.5 m | 3 m = 1 m = 0.5 m | 3 m = 1 m = 0.5 m | 3 m = 1 m = 0.5 m |

| 0.5 m | 1 m | 3 m |
|---|---|---|
| Su1 Su4 < Su2 Su3 | Su1 < Su4 < Su2 < Su3 | Su1 < Su4 < Su2 Su3 |

*Abbreviations:* NIC = Nice; VLF = Villefranche; GEN = Genoa; Su1 = Summer1; Su2 = Summer2; Su3 = Summer3; Su4 = Summer4; HAL = *Halopteris scoparia*; DIC = *Dictyota* spp., COR = Corallinales.

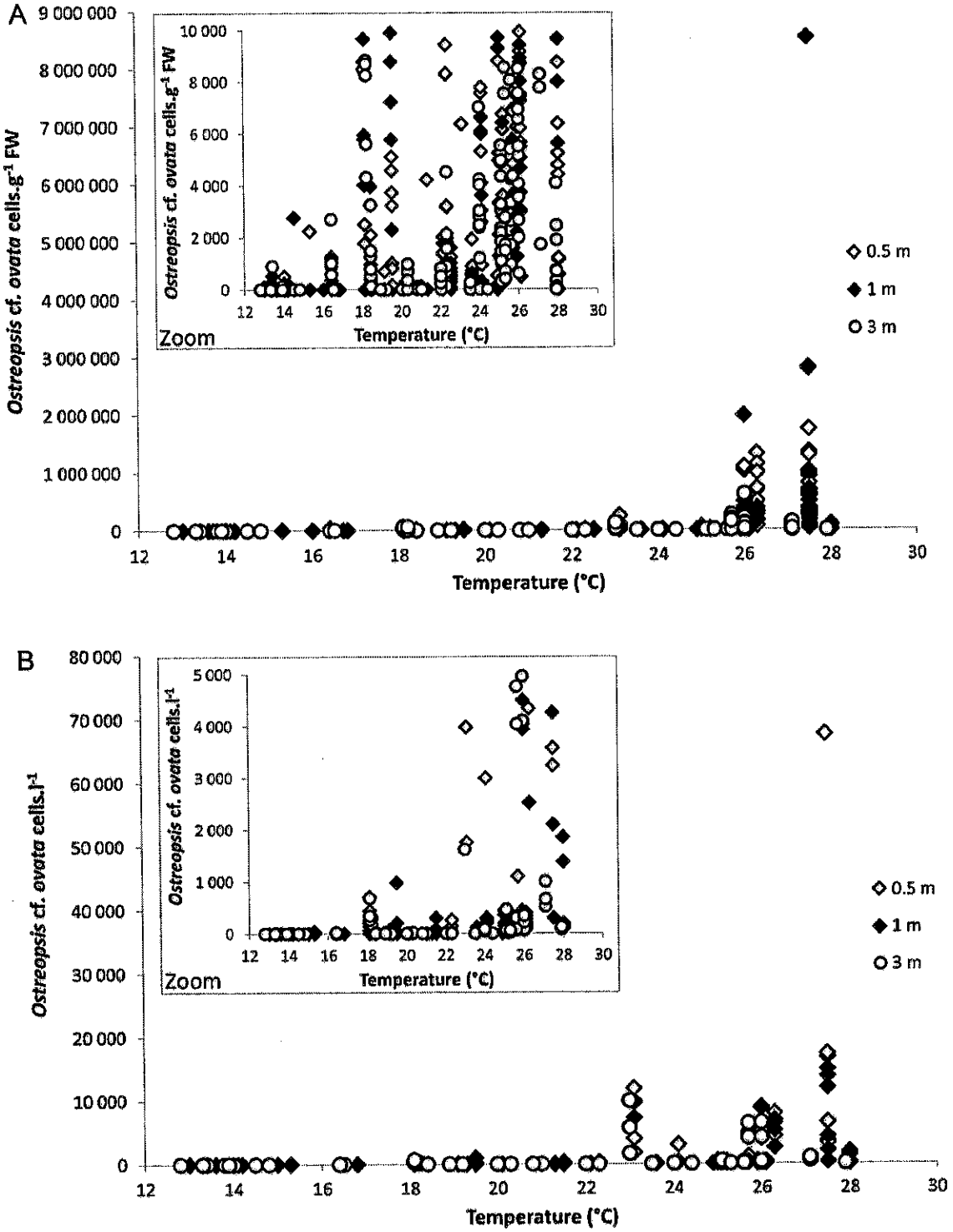



**Fig. 5.** (a) Epiphytic ($N$ = 988) and (b) planktonic ($N$ = 363) *Ostreopsis* abundances depending on the temperature at 0.5, 1 and 3 m depths.

type locality, makes the term "*Ostreopsis* cf. *ovata*" more appropriate for the specimen from the Mediterranean Sea (Penna et al., 2005, 2010). Moreover, genetic analysis carried out on Mediterranean strains by Penna et al. (2005, 2010) showed phylogenetic differences with the first genetic sequences of *O. ovata* registered on GenBank, which belong to a Malaysian strain (Pin et al., 2001).

### 4.2. Temporal and spatial variation in Ostreopsis abundance

*Ostreopsis* spp. proliferation followed a marked seasonal trend, as previously shown in other temperate areas (*e.g.* in the Mediterranean Sea and in New Zealand; Chang et al., 2000; Rhodes et al., 2000; Vila et al., 2001a,b; Simoni et al., 2003, 2004; Turki, 2005; Aligizaki and Nikolaidis, 2006; Shears and Ross, 2009; Ungaro et al., 2010; Mangialajo et al., 2011) and in tropical environments (Ballantine et al., 1988; Morton et al., 1992; Briggs and Leff, 2007; Okolodkov et al., 2007; Parsons and Preskitt, 2007). The present study also highlighted significant variations in *Ostreopsis* cf. *ovata* abundances in the summer period.

We previously reported a high heterogeneity of *Ostreopsis* cf. *ovata* abundances at a small spatial scale (several meters) between the replicates of each site (Cohu et al., 2011; Cohu and Lemée,

2012). The important inter-sites variation, observed on a large spatial scale, from tens to hundreds of kilometers, corresponds to the results reported in temperate and tropical studies (Grzebyk et al., 1994; Chang et al., 2000; Aligizaki and Nikolaidis, 2006; Parsons and Preskitt, 2007). During the summer of 2008, blooms in the eastern sites of Villefranche-sur-Mer, Nice and Genoa appeared in late July, corresponding to the main blooming period reported for the whole Ligurian Sea (Mangialajo et al., 2011). Although Ramatuelle and Saint-Raphaël are also located in the Ligurian Sea, *Ostreopsis* abundances remained low in these sites during the whole sampling year. In Cassis, situated in the Gulf of Lyon, no bloom was recorded, while Mangialajo et al. (2011) noted important developments in nearby sites (Morgiret, Endoume and Méjean) during mid and late August 2008. As the Cassis site was sampled twice in August, the risk of having missed bloom events in this western site seems negligible. Thus, differences in the occurrence of *Ostreopsis* blooms observed in the present study between eastern and western sites were unlikely to be due to a delay in blooms occurring.

The spatial heterogeneity of *Ostreopsis* cf. *ovata* development may therefore reflect a real preference for specific areas. Comparing the characteristics of sampled eastern and western sites, the nature of substrate (natural *vs.* artificial rocks) and hydrodynamic characteristics did not seem to have decisively affected the *Ostreopsis* distribution. However, previous studies suggested a higher *Ostreopsis* spp. growth in calm to slightly turbulent waters (Grzebyk et al., 1994; Chang et al., 2000; Vila et al., 2001b; Simoni et al., 2003; Shears and Ross, 2009).

### 4.3. Anthropogenic pressure

The population density, clearly higher in the three eastern sites of Nice, Genoa and Villefranche-sur-Mer, might be related to substantial *Ostreopsis* cf. *ovata* proliferation. Urbanization, linked to the population density, increases water pollution and modifies the coastal habitats (including macroalgal assemblages, Mangialajo et al., 2008a), which probably impacts the distribution of *O.* cf. *ovata*, as suggested in several studies. In North Line Islands, Briggs and Leff (2007) observed increasing *Ostreopsis* sp. abundances along a gradient of human disturbance, probably linked to the induced change in macroalgae cover. In the Adriatic Sea, Ungano et al. (2010) showed a positive correlation between *O. ovata* abundances and anthropogenic microbiological contamination, related to population density. Similarly, effluents from sewage-treatment plants, generally corresponding to the amount of anthropogenic pressure, seemed to promote the proliferation of some toxic dinoflagellates (Doig and Martin, 1974; Mallin et al., 2005). Harmful algal blooms are also known to be stimulated in eutrophic and confined waters, such as harbors and recreational/industrial coastal areas (Vila et al., 2001a; Vila and Maso, 2005; Maso and Garcés, 2006). Finally, other studies have also shown an important effect of urbanization and human disturbance on the abundance and species composition of dinoflagellates cysts (Saetre et al., 1997; Dale et al., 1999; Pospelova et al., 2002).

### 4.4. Depth preference

*Ostreopsis* cf. *ovata* epiphytic abundances generally decreased from 0.5 to 3 m depth. The occurrence of dense populations of *Ostreopsis* at the sub-surface indicates a resistance to high light intensities. This observation is in agreement with the results of Totti et al. (2010) in the Adriatic Sea, which showed high abundances at 1 and 2 m depths with a decrease in abundance below a 3 m depth, probably related to the decrease of light intensity. The distribution of *Ostreopsis* and the possible daily vertical migration of cells merit further investigations as it could represent the existence of vertical ecological niches.

### 4.5. Substrate preference

The maximal planktonic cell concentration measured in the present study ($68 \times 10^3$ cells $l^{-1}$) is slightly lower than the previous concentrations of *Ostreopsis* spp. reported in Monaco ($213 \times 10^3$ cells $l^{-1}$; Cohu et al., 2011) and in the Villefranche-sur-Mer Bay ($104 \times 10^3$ cells $l^{-1}$; Mangialajo et al., 2011), yet is similar to those found on the Genoa coast ($87 \times 10^3$ cells $l^{-1}$; Mangialajo et al., 2008b). In the Adriatic and Aegean seas, maximal *Ostreopsis* spp. concentrations detected in the water column were lower: $25 \times 10^3$ and $16 \times 10^3$ cells $l^{-1}$ respectively (Aligizaki and Nikolaidis, 2006; Totti et al., 2010).

The maximal epiphytic abundance of *Ostreopsis* cf. *ovata* measured in the present study ($8.54 \times 10^6$ cells $g^{-1}$ FW on *Dictyota* spp.) is one of the highest values ever measured in the NW Mediterranean Sea. In previous studies, densities of *Ostreopsis* spp. reached $7.25 \times 10^6$ cells $g^{-1}$ FW in Catalonia (Mangialajo et al., 2011), $2.77 \times 10^6$ cells $g^{-1}$ FW in Monaco (Cohu et al., 2011), $2.54 \times 10^6$ cells $g^{-1}$ FW on the Genoa coast (Mangialajo et al., 2008b) and $1.70 \times 10^6$ cells $g^{-1}$ FW in the Adriatic Sea (Totti et al., 2010). To the best of our knowledge, the maximal epiphytic abundance in the eastern Mediterranean Sea (Greece) reached $0.41 \times 10^6$ cells $g^{-1}$ FW (Aligizaki and Nikolaidis, 2006).

The positive and significant correlation between epiphytic and planktonic *Ostreopsis* cell abundances observed in this study has been repeated previously (Vila et al., 2001b; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008b, 2011; Totti et al., 2010) and seems to confirm the hypothesis of a cells resuspension from the macroalgae surface to the water column.

Our results show an important variability in the abundance of *Ostreopsis* depending on macroalgal host species, without statistically highlighting a clear substrate preference. However, *Dictyota* species seem particularly able to support very high abundances of *Ostreopsis* during blooms.

### 4.6. Concentration of oxygen and pigments

The oxygen saturation decreased between the pre-bloom and the bloom summer periods, mostly in the three eastern sites. While this depletion partly reflects a summer decrease of dissolved oxygen solubility related to the increase in seawater temperature, it might also be linked to the higher consumption of oxygen related to organic matter decomposition during *Ostreopsis* cf. *ovata* blooms, as suggested by the Spearman negative correlations obtained between *Ostreopsis* abundances and oxygen saturation. The effect of *Ostreopsis* spp. development on dissolved oxygen concentration has received little attention. However, dramatic oxygen depletions have been observed during bloom events of other harmful dinoflagellates (Zingone and Enevoldsen, 2000; Al-Ghelani et al., 2005; Ning et al., 2009).

As expected for autotrophic and mixotrophic microalgae, an increase in chlorophyll *a* concentration was related to the *Ostreopsis* blooms in the three eastern sites, corresponding to previous observations (Parsons and Preskitt, 2007; Ungaro et al., 2010).

### 4.7. Impact of temperature

The seasonal trend of *Ostreopsis* development in the Mediterranean Sea can be easily linked to the seasonal variations of seawater temperature. The present study suggests an optimum growth of *Ostreopsis* cells at temperatures between 23 and 27.5 °C. These results are in accordance with several previous studies

performed in temperate areas (Mediterranean Sea and New Zealand), where temperatures ranging from 22 °C to 30 °C seemed to constitute the best conditions for *Ostreopsis* spp. development (Chang et al., 2000; Simoni et al., 2003; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008b, 2011).

In the present work, *Ostreopsis* cells were found at 13 °C, which is one of the lowest worldwide temperatures associated with the presence of *Ostreopsis* spp. Until now, *Ostreopsis* cf. *ovata* and *Ostreopsis siamensis* have been detected at the minimal temperatures of 14 and 14.5 °C in the Mediterranean Sea (Simoni et al., 2003; Aligizaki and Nikolaidis, 2006), yet were also recorded in Peter the Great Bay (Sea of Japan) when water temperature was only 7 °C (Selina and Orlova, 2010). The presence of *O.* cf. *ovata* in this region of the Sea of Japan which is characterized by cold winter temperatures (dropping below 0 °C at the sea surface), reveals a high tolerance to extreme climates (Selina and Orlova, 2010; Selina and Levchenko, 2011).

The high fluctuations in the abundance of *Ostreopsis* cf. *ovata* during summer cannot be related to temperature variations, suggesting the impact of other physical and chemical factors, as proposed by Mangialajo et al. (2011). Moreover, the obvious seasonality of *Ostreopsis* spp. abundances in tropical climates could not entirely result from the low annual amplitude of temperatures (Ballantine et al., 1985, 1988; Morton et al., 1992; Okolodkov et al., 2007). This also suggests an important effect of other environmental parameters, such as wave activity (Okolodkov et al., 2007), nutrient availability, salinity or light intensity and photoperiod (Morton et al., 1992). Some biotic factors could also be involved, such as the availability of some seasonal macroalgal substrates and the competition with other microphytes (Okolodkov et al., 2007).

### 4.8. Impact of nutrient concentrations

During spring and summer, very high concentrations of nitrate and silicate were recorded at a depth of 0.5 m in Cassis. In this site, a fresh water discharge was observed at the subsurface, which probably provided nutrients. Since low *Ostreopsis* cf. *ovata* abundances were measured simultaneously, the increase in nitrate and silicate concentrations did not seem to impact the microalgae development. Moreover, the freshwater source should have a negative impact on *Ostreopsis* cf. *ovata* growth *via* a decrease of salinity, as observed on *Ostreopsis lenticularis* and others dinoflagellates by Delgado et al. (2006).

While nitrate is usually one of the main nitrogen sources used by microalgae, it did not seem to be a limiting factor. *Ostreopsis* spp., suspected to be a mixotroph, could obtain additional organic sources of nitrogen and other nutrients through phagotrophy and therefore be less sensitive to changes in nutrient supply ratios (Faust and Morton, 1995; Faust, 1998; Stoecker et al., 2006; Burkholder et al., 2008; Ignatiades and Gotsis-Skretas, 2010). In further studies, the role of ammonium and urea in *Ostreopsis* spp. growth should be investigated. These available forms of nitrogen are known to be used by some heterotrophic dinoflagellates, such as *Pfiesteria* spp. (Glibert et al., 2006).

Silicate did not have any clear impact on the abundance of *Ostreopsis* cf. *ovata*, as generally expected for dinoflagellates which do not specifically use silicate for their growth, in contrast to diatoms. It is interesting to note that nitrogen and silicate concentrations were negatively correlated with temperature, as expected in temperate waters where the thermocline formation prevents nutrient replenishment in surface water during summer. This decrease in nutrient concentrations during the warm season, corresponding to the *Ostreopsis* bloom period, could partly explain the observed negative or null correlations with the abundance of *Ostreopsis*.

Finally, phosphate seemed to be the only limiting nutrient for the *Ostreopsis* growth. Since phosphate is positively related to temperature, there might be an interacting or a cumulative effect of these two parameters on the *Ostreopsis* development. The slight increase in phosphate concentrations during summer was surprising and might be due to a seasonal anthropogenic pollution.

In the Mediterranean Sea, Vila et al. (2001b) noted that inorganic nitrogen, phosphate and silicate concentrations appeared to be unassociated with *Ostreopsis* sp. distribution. In the Adriatic Sea, Ungaro et al. (2010) only showed a negative link between *O. ovata* and nitrite, as shown in the present study, and a positive correlation between cell abundances and the ratio $NO_3$:$NO_2$. In Hawaii, Parsons and Preskitt (2007) observed opposing results, as nutrient positively correlated with an undetermined *Ostreopsis* species, yet was not significantly correlated with *O. ovata*. Since an increase in nutrient availability might have an indirect impact on *Ostreopsis* spp. development by inducing a shift in macroalgae substrate cover and/or composition (Briggs and Leff, 2007; Burkholder et al., 2008), the direct effect of nutrient on *Ostreopsis* growth is very difficult to assess *in situ*. Moreover, the coastal habitat of *Ostreopsis* spp. is often impacted by anthropogenic activities, especially in the NW Mediterranean Sea. Therefore *in situ* nutrient concentrations can show important spatial and temporal variations at small scales, which lead to an additional difficulty in ecological studies. It would be useful to investigate the impact of long-term changes in nutrient availability and ratios on *Ostreopsis* bloom frequency and amplitude, as suggested in more general studies (Anderson et al., 2002; Li et al., 2009; Ning et al., 2009).

## 5. Conclusion

The present study highlighted a high spatial heterogeneity in the development of *Ostreopsis* cf. *ovata* within an obvious seasonal trend. Overall, temperature was the most important factor, explaining why *Ostreopsis* growth was restricted to the summer period. Concerning the impact of nutrients, *Ostreopsis* abundance was only related to phosphate concentration. The distribution of epiphytic cells was strongly affected by depth and substrates. In conclusion, the temporal and spatial development of *O.* cf. *ovata* depends on multiple factors which are difficult to discriminate.

Further *in situ* and *in vitro* studies are needed to investigate the effects of substrate and depth on the distribution of epiphytic cells, as well as the impact of urbanization on *Ostreopsis* development.

## Acknowledgement

This work was supported by the French national program MediOs 2 (LITEAU-Ministère de l'Ecologie, Conseil Général des Alpes-Maritimes, Agence de l'Eau Rhône Méditerranée Corse)[SS].

## References

Al-Ghelani, H.M., AlKindi, A.Y.A., Amer, S., Al-Akhzami, Y.K., 2005. Harmful algal blooms: physiology, behavior, population dynamics and global impacts – a review. Journal of Science 10, 1–30.

Aligizaki, K., Nikolaidis, G., 2006. The presence of the potentially toxic genera *Ostreopsis aligialigi*and *Coolia* (Dinophyceae) in the North Aegean Sea, Greece. Harmful Algae 5, 717–730.

Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25, 704–726.

Anderson, M.J., 2005. PERMANOVA: A FORTRAN Computer Program for Permutational Multivariate Analysis of Variance. University of Auckland, Otro.

Ashton, M., Rosado, W., Govind, N.S., Tosteson, T.R., 2003. Culturable and non-culturable bacterial symbionts in the toxic benthic dinoflagellate *Ostreopsis lenticularis*. Toxicon 42, 419–424.

Ballantine, D.L., Bardales, A.T., Tosteson, T.R., Durst, H.D., 1985. Seasonal abundance of *Gambierdiscus toxicus* and *Ostreopsis* sp. in coastal waters of southwest Puerto

Rico. In: Gabrie, C., Salvat, B. (Eds.), Proc. 5th Int. Coral Reef Cong., vol. 4, Tahiti, pp. 417–422.
Ballantine, D.L., Tosteson, T.R., Bardales, A.T., 1988. Population dynamics and toxicity of natural populations of benthic dinoflagellates in southwestern Puerto Rico. Journal of Experimental Marine Biology and Ecology 119, 201–212.
Barroso Garcia, P., Rueda de la Puerta, P., Parrón Carreñob, T., Marín Martínezb, P., Guillén Enríquezc, J., 2008. Brote con síntomas respiratorios en la provincia de Almería por una posible exposición a microalgas tóxicas. Gaceta Sanitaria 22, 578–584.
Battocchi, C., Totti, C., Vila, M., Masó, M., Capellacci, S., Accoroni, S., Reñé, A., Scardi, M., Penna, A., 2010. Monitoring toxic microalgae *Ostreopsis* (dinoflagellate) species in coastal waters of the Mediterranean Sea using molecular PCR-based assay combined with light microscopy. Marine Pollution Bulletin 60, 1074–1084.
Brescianini, C., Grillo, C., Melchiorre, N., Bertolotto, R., Ferrari, A., Vivaldi, B., Icardi, G., Gramaccioni, L., Funari, E., Scardala, S., 2006. Ostreopsis ovata algal blooms affecting human health in Genova, Italy, 2005 and 2006. Eurosurveillance 11, 3040.
Briggs, A., Leff, M., 2007. A comparison of toxic dinoflagellate densities along a gradient of human disturbance in the North Line Islands. Final Report, Stanford University.
Burkholder, J.M., Glibert, P.M., Skelton, H.M., 2008. Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. Harmful Algae 8, 77–93.
Carballeira, N.M., Emiliano, A., Sostre, A., Restituyo, J.A., González, I.M., Colón, G.M., Tosteson, C.G., Tosteson, T.R., 1998. Fatty acid composition of bacteria associated with the toxic dinoflagellate *Ostreopsis lenticularis* and with Caribbean *Palythoa* species. Lipids 33, 627–632.
Chang, F.H., Shimizu, Y., Hay, B., Stewart, R., Mackay, G., Tasker, R., 2000. Three recently recorded *Ostreopsis* spp. (Dinophyceae) in New Zealand: temporal and regional distribution in the upper North Island from 1995 to 1997. New Zealand Journal of Marine and Freshwater Research 34, 29–39.
Ciminiello, P., Aversano, C.D., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Grillo, C., Melchiorre, N., 2006. The Genoa 2005 Outbreak. Determination of putative palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method. Analytical Chemistry 78, 6153–6159.
Cohu, S., Thibaut, T., Mangialajo, L., Labat, J.P., Passafiume, O., Blanfuné, A., Simon, N., Cottalorda, J.M., Lemée, R., 2011. Occurrence of the toxic dinoflagellate *Ostreopsis* cf. ovata in relation with environmental factors in Monaco (NW Mediterranean). Marine Pollution Bulletin 62, 2681–2691.
Cohu, S., Lemée, R., 2012. Vertical distribution of the toxic epibenthic dinoflagellates *Ostreopsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis* in the NW Mediterranean Sea. Cahiers de Biologie Marine 53, 373–380.
Dale, B., Thorsen, T.A., Fjellsa, A., 1999. Dinoflagellate cysts as indicators of cultural eutrophication in the Oslofjord, Norway. Estuarine, Coastal and Shelf Science 48, 371–382.
Deeds, J.R., Schwartz, M.D., 2010. Human risk associated with palytoxin exposure. Toxicon 56, 150–162.
Delgado, G., Lechuga-Devéze, C.H., Popowski, G., Troccoli, L., Salinas, C.A., 2006. Epiphytic dinoflagellates associated with ciguatera in the northwestern coast of Cuba. Revista de Biologia Tropical 54, 299–310.
Doig, M.T., Martin, D.F., 1974. The response of *Gymnodinium breve* to municipal waste materials. Marine Biology 24, 223–228.
Faust, M.A., 1995. Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. Journal of Phycology 31, 996–1003.
Faust, M.A., 1998. Mixotrophy in tropical benthic dinoflagellates. In: Harmul Algae Proc. 8th Int. Conf. Harmful Algae, Vigo, Spain, Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO. pp. 390–393.
Faust, M.A., Morton, S.L., 1995. Morphology and ecology of the marine dinoflagellate *Ostreopsis labens* sp. nov. (Dinophyceae). Journal of Phycology 31, 456–463.
Faust, M.A., Morton, S.L., Quod, J.P., 1996. Further SEM study of marine dinoflagellates: the genus *Ostreopsis* (Dinophyceae). Journal of Phycology 32, 1053–1065.
Gasol, M., Del Giorgio, A., 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Scientia Marina 64, 197–224.
Glibert, P.M., Burkholder, J.M., Parrow, M.W., Lewitus, A.J., Gustafson, D.E., 2006. Direct uptake of nitrogen by *Pfiesteria piscicida* and *Pfiesteria shumwayae*, and nitrogen nutritional preferences. Harmful Algae 5, 380–394.
Grzebyk, D., Berland, B., Thomassin, B.A., Bosi, C., Arnoux, A., 1994. Ecology of ciguateric dinoflagellates in the coral reef complex of Mayotte Island (S. W. Indian Ocean). Journal of Experimental Marine Biology and Ecology 178, 51–66.
Ignatiades, L., Gotsis-Skretas, O., 2010. A review on toxic and harmful algae in Greek coastal waters (E. Mediterranean Sea). Toxins 2, 1019–1037.
Ito, E., Ohkusu, M., Yasumoto, T., 1996. Intestinal injuries caused by experimental palytoxicosis in mice. Toxicon 34, 643–652.
Lenoir, S., Ten-Hage, L., Turquet, J., Quod, J.-P., Bernard, C., Hennion, M.-C., 2004. First evidence of palytoxin analogues from an *Ostreopsis mascarenensis* (Dinophyceae) benthic bloom in Southwestern Indian Ocean. Journal of Phycology 40, 1042–1051.
Li, J., Glibert, P.M., Zhou, M., Lu, S., Lu, D., 2009. Relationships between nitrogen and phosphorus forms and ratios and the development of dinoflagellate blooms in the East China Sea. Marine Ecology Progress Series 383, 11–26.
Lorenzen, C.J., Newton Downs, J., 1986. The specific absorption coefficients of chlorophyllide a and pheophorbide a in 90% acetone, and comments on the fluorometric determination of chlorophyll and pheopigments. Limnology and Oceanography 31, 449–452.
Mallin, M.A., McIver, M.R., Wells, H.A., Parsons, D.C., Johnson, V.L., 2005. Reversal of eutrophication following sewage treatment upgrades in the New River Estuary, North Carolina. Estuaries 28, 750–760.
Mangialajo, L., Chiantore, M., Cattaneo-Vietti, R., 2008a. Loss of fucoid algae along a gradient of urbanisation, and structure of benthic assemblages. Marine Ecology Progress Series 358, 63–74.
Mangialajo, L., Bertolotto, R., Cattaneo-Vietti, R., Chiantore, M., Grillo, C., Lemee, R., Melchiorre, N., Moretto, P., Povero, P., Ruggieri, N., 2008b. The toxic benthic dinoflagellate *Ostreopsis ovata*: quantification of proliferation along the coastline of Genoa, Italy. Marine Pollution Bulletin 56, 1209–1214.
Mangialajo, L., Ganzin, N., Accoroni, S., Asnaghi, V., Blanfuné, A., Cabrini, M., Cattaneo-Vietti, R., Chavanon, F., Chiantore, M., Cohu, S., Costa, E., Fornasaro, D., Grossel, H., Marco-Miralles, F., Masó, M., Reñé, A., Rossi, A.M., Sala, M.M., Thibaut, T., Totti, C., Vila, M., Lemée, R., 2011. Trends in *Ostreopsis* proliferation along the Northern Mediterranean coasts. Toxicon 57, 408–420.
Maso, M., Garcés, E., Pagès, F., Camp, J., 2003. Drifting plastic debris as a potential vector for dispersing harmful algal bloom (HAB) species. Scientia Marina 67, 107–111.
Maso, M., Garcés, E., 2006. Harmful microalgae blooms (HAB); problematic and conditions that induce them. Marine Pollution Bulletin 53, 620–630.
Mattos Nascimento, S., Monteiro, P.O., Leite Ferreira, C.E., González-Rodríguez, E., 2008. Ostreopsis ovata blooms on Rio de Janeiro coast. Harmful Algae News 37, 1–4.
Miraglia, M., Marvin, H.J.P., Kleter, G.A., Battilani, P., Brera, C., Coni, E., Cubadda, F., Croci, L., Santis, B.D., Dekkers, S., Filippi, L., Hutjes, R.W.A., Noordam, M.Y., Pisante, M., Piva, G., Prandini, A., Toti, L., van den Born, G.J., Vespermann, A., 2009. Climate change and food safety: an emerging issue with special focus on Europe. Food and Chemical Toxicology 47, 1009–1021.
Monti, M., Minocci, M., Beran, A., Ives, L., 2007. First record of *Ostreopsis* cfr. *ovata* on macroalgae in the Northern Adriatic Sea. Marine Pollution Bulletin 54, 598–601.
Morton, S.L., Norris, D.R., Bomber, J.W., 1992. Effect of temperature, salinity and light intensity on the growth and seasonality of toxic dinoflagellates associated with ciguatera. Journal of Experimental Marine Biology and Ecology 157, 79–90.
Ning, X., Lin, C., Hao, Q., Liu, C., Le, F., Shi, J., 2009. Long term changes in the ecosystem in the northern South China Sea during 1976–2004. Biogeosciences 6, 2227–2243.
Okolodkov, Y.B., Campos-Bautista, G., Gárate-Lizárraga, I., González-González, J.A.G., Hoppenrath, M., Arenas, V., 2007. Seasonal changes of benthic and epiphytic dinoflagellates in the Veracruz reef zone, Gulf of Mexico. Aquatic Microbial Ecology 47, 223–237.
Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., Ratsimaloto, M., Naoki, H., Yasumoto, T., 1999. Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37, 55–65.
Parsons, M.L., Preskitt, L.B., 2007. A survey of epiphytic dinoflagellates from the coastal waters of the island of Hawai'i. Harmful Algae 6, 658–669.
Parsons, M.L., Aligizaki, K., Dechraoui Bottein, M.Y., Santiago, F., Morton, S.L., Penna, A., Rhodes, L., 2012. *Gambierdiscus* and *Ostreopsis*: reassessment of the state of knowledge of their taxonomy, geography, ecophysiology and toxicology. Harmful Algae 14, 107–129.
Pearce, I., Marshall, J.A., Hallegraeff, G.M., 2001. Toxic epiphytic dinoflagellates from east coast Tasmania, Australia. Harmful Algal Blooms 54–57.
Penna, A., Vila, M., Fraga, S., Giacobbe, M.G., Andreoni, F., Riobo, P., 2005. Characterization of *Ostreopsis* and *Coolia* (Dinphyceae) isolates in the Western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. Journal of Phycology 41, 212–225.
Penna, A., Fraga, S., Battocchi, C., Casabianca, S., Giacobbe, M.G., Riobo, P., Vernesi, C., 2010. A phylogeographical study of the toxic benthic dinoflagellate genus *Ostreopsis* Schmidt. Journal of Biogeography 37, 380–841.
Pérez-Guzmán, L., Pérez-Matos, A.E., Rosado, W., Tosteson, T.R., Govind, N.S., 2008. Bacteria associated with toxic clonal cultures of the dinoflagellate *Ostreopsis lenticularis*. Marine Biotechnology 10, 492–496.
Pin, L.C., Teen, L.P., Ahmad, A., Usup, G., 2001. Genetic diversity of *Ostreopsis ovata* (Dinophyceae) from Malaysia. Marine Biotechnology 3, 246–255.
Pospelova, V., Chmura, G.L., Boothman, W.S., Latimer, J.S., 2002. Dinoflagellate cyst records and human disturbance in two neighboring estuaries, New Bedford Harbor and Apponagansett Bay, Massachusetts (USA). Science of the Total Environment 298, 81–102.
Rhodes, L., 2011. World-wide occurrence of the toxic dinoflagellate genus *Ostreopsis* Schmidt. Toxicon 57, 400–407.
Rhodes, L., Adamson, J., Suzuki, T., Briggs, L., Garthwaite, I., 2000. Toxic marine epiphytic dinoflagellates, *Ostreopsis siamensis* and *Coolia monotis* (Dinophyceae), in New Zealand. New Zealand Journal of Marine and Freshwater Research 34, 371–383.
Rhodes, L., Smith, K.F., Munday, R., Selwood, A.I., McNabb, P.S., Holland, P.T., Bottein, M.Y., 2010. Toxic dinoflagellates (Dinophyceae) from Rarotonga, Cook Islands. Toxicon 56, 751–758.
Riobo, P., Paz, B., Franco, J.M., 2006. Analysis of palytoxin-like in *Ostreopsis* cultures by liquid chromatography with precolumn derivatization and fluorescence detection. Analytica Chimica Acta 566, 217–223.
Saetre, M.M.L., Dale, B., Abdullah, M.I., Saetre, G.P., 1997. Dinoflagellate cysts as potential indicators of industrial pollution in a Norwegian Fjord. Marine Environment Research 44, 167–189.
Selina, M.S., Orlova, T.Y., 2010. First occurrence of the genus *Ostreopsis* (Dinophyceae) in the Sea of Japan. Botanica Marina 53, 243–249.

Selina, M.S., Levchenko, E.V., 2011. Species composition and morphology of dinoflagellates (Dinophyta) of epiphytic assemblages of Peter the Great Bay in the Sea of Japan. Russian Journal of Marine Biology 37, 23–32.

Shears, N.T., Ross, P.M., 2009. Blooms of benthic dinoflagellates of the genus *Ostreopsis*; an increasing and ecologically important phenomenon on temperate reefs in New Zealand and worldwide. Harmful Algae 8, 916–925.

Shears, N.T., Ross, P.M., 2010. Toxic cascades: multiple anthropogenic stressors have complex and unanticipated interactive effects on temperate reefs. Ecology Letters 13, 1149–1159.

Simoni, F., Gaddi, A., Paolo, C.D., Lepri, L., 2003. Harmful epiphytic dinoflagellate on Tyrrhenian Sea reefs. Harmful Algae News 24, 13–14.

Simoni, F., Di Paolo, C., Gori, L., Lepri, L., Mancino, A., Falaschi, A., 2004. Further investigation on blooms of *Ostreopsis ovata*, *Coolia monotis*, *Prorocentrum lima* on the macroalgae of artificial and natural reefs in the Northern Tyrrhenian Sea. Harmful Algae News 26, 6–7.

Stoecker, D., Tillmann, U., Granéli, E., 2006. Phagotrophy in harmful algae. Ecological Studies 189, 177–187.

Taniyama, S., Arakawa, O., Terada, M., Nishio, S., Takatani, T., Mahmud, Y., Noguchi, T., 2003. *Ostreopsis* sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. Toxicon 42, 29–33.

Tichadou, L., Glaizal, M., Armengaud, A., Grossel, H., Lemée, R., Kantin, R., Lasalle, J.L., Drouet, G., Rambaud, L., Malfait, P., Haro, L.d., 2010. Health impact of unicellular algae of the *Ostreopsis* genus blooms in the Mediterranean Sea: experience of the French Mediterranean coast surveillance network from 2006 to 2009. Clinical Toxicology 48, 839–844.

Tosteson, T.R., Ballantine, D.L., Tosteson, C.G., Hensley, V., Bardales, A.T., 1989. Associated bacterial flora, growth, and toxicity of cultured benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus*. Applied and Environmental Microbiology 55, 137–141.

Totti, C., Accoroni, S., Cerino, F., Cucchiari, E., Romagnoli, T., 2010. *Ostreopsis ovata* bloom along the Conero Riviera (northern Adriatic Sea): relationships with environmental conditions and substrata. Harmful Algae 9, 233–239.

Tréguer, P., Corre, P.L., 1975. Manuel d'analyse des sels nutritifs dans l'eau de mer. Utilisation de l'autoanalyseur II Technicon, 2ème édition. Université de Bretagne Occidentale Brest 110 pp.

Turki, S., 2005. Distribution of toxic dinoflagellates along the leaves of seagrass *Posidonia oceanica* and *Cymodocea nodosa* from the Gulf of Tunis. Cahiers de Biologie Marine 46, 29–34.

Ungano, N., Assennato, G., Blonda, M., Cudillo, B., Petruzzelli, M.R., Mariani, M., Pastorelli, A.M., Aliquò, M.R., D'angela, A., Aiello, C., Ranieri, S., 2010. Occurrence of the potentially toxic dinoflagellate *Ostreopsis ovata* along the Apulian coastal areas (Southern Italy) and relationship with anthropogenic pollution. Fresenius Environmental Bulletin 19, 1813–1821.

Ungaro, N., Pastorelli, A.M., Di Festa, T., Galise, I., Romano, C., Assennato, G., Blonda, V., Perrino, V., 2010. Annual trend of the dinoflagellate *Ostreopsis ovata* in two sites along the Southern Adriatic coast. Biology Marine Mediterranean 17, 183–184.

Utermöhl, H., 1958. Zur Vervoll Kommung der Quantitativen Phytoplankton-Methodik. Mitteilungen. Internationale Vereinigung fur theoretische und angewandte Limnologie 9, 1–38.

Vila, M., Camp, J., Garcés, E., Maso, M., Delgado, M., 2001a. High resolution spatio-temporal detection of potentially harmful dinoflagellates in confined waters of the NW Mediterranean. Journal of Plankton Research 23, 497–514.

Vila, M., Garcés, E., Masó, M., 2001b. Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. Aquatic Microbial Ecology 26, 51–60.

Vila, M., Maso, M., 2005. Phytoplankton functional groups and harmful algal species in anthropogenically impacted waters of the NW Mediterranean Sea. Scientia Marina 69, 31–45.

Yasumoto, T., 1998. Fish poisoning due to toxins of microalgal origins in the Pacific. Toxicon 36, 1515–1518.

Zingone, A., Enevoldsen, H.O., 2000. The diversity of harmful algal blooms: a challenge for science and management. Ocean and Coastal Management 43, 725–748.

Harmful Algae 28 (2013) 10–22

Contents lists available at SciVerse ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

# Occurrence of palytoxins in marine organisms from different trophic levels of the French Mediterranean coast harvested in 2009

R. Biré [a], S. Trotereau [a,*], R. Lemée [b,c], C. Delpont [a], B. Chabot [a], Y. Aumond [b,c], S. Krys [a]

[a] Anses, Laboratoire de sécurité des aliments de Maisons-Alfort, 23 Avenue du Général de Gaulle, 94706 Maisons-Alfort Cedex, France
[b] Université Pierre et Marie Curie-Paris 6, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer Cedex, France
[c] CNRS, Marine Microbial Ecology and Biogeochemistry, Laboratoire d'Océanographie de Villefranche, BP 28, 06234 Villefranche-sur-Mer Cedex, France

ARTICLE INFO

*Article history:*
Received 8 February 2012
Received in revised form 30 April 2013
Accepted 30 April 2013

*Keywords:*
Haemolytic test
LC–MS/MS
Marine organisms
Mediterranean
*Ostreopsis* cf. *ovata*
Palytoxins

ABSTRACT

Four sites located in Nice and Villefranche-sur-Mer, on the French Mediterranean coast, were monitored during the summer of 2009 for the presence of epiphytic and planktonic *Ostreopsis* cf. *ovata*, and that of palytoxin (PITX) and 2 of its analogues (ovatoxin-a (OVTX-a) and ostreocin-D (OST-D)) in different marine organisms.

Several of the 15 species that were sampled between June and September 2009 were found to be contaminated with OVTX-a as the major toxin (90% of the toxin profile) and PITX; this included fish, echinoderms, gastropods, crustaceans and cephalopods. The contamination levels varied geographically and between species, with the herbivorous species generally having higher toxin levels than carnivorous ones.

The determination of the toxin distribution between the digestive tube (DT) and the remaining tissue (RT) or roe in the case of the sea urchin *Paracentrotus lividus* showed that the toxins were sequestered in the DT. The highest toxin level ever recorded over the course of the study was of 392.2 μg for the sum of OVTX-a and PITX per kg of DT of the flathead mullet *Mugil cephalus*. No quantifiable levels of toxins were found in the roe of the sea urchins or in the RT of the other marine products. However, in several cases, the toxin level in the whole flesh of the analysed organisms was above 30 μg OVTX-a + PITX/kg, when knowing that the European food safety authority's opinion is that an adult should not ingest more than 30 μg PITX + OST-D per kg of shellfish meat to avoid putting the consumer's health at risk. This was observed for the following four species: the sea urchin *P. lividus*, the red-mouthed rock shell *Stramonita haemastoma*, the warty crab *Eriphia verrucosa* and the flathead mullet *M. cephalus*.

The collection of such data is of great importance to refine and complete the risk assessment of PITX and its analogues and has to be encouraged in order to provide reliable information for setting up a regulatory level that would protect the consumers of edible marine organisms



## 1. Introduction

Palytoxin (PITX) is a complex non-protein compound with amphiphilic properties that was first isolated from the zoanthid *Palythoa* sp. after which it was named (Moore and Scheuer, 1971). The production of PITX and PITX-like compounds by dinoflagellates of the genus *Ostreopsis* has been reported in the literature (Onuma et al., 1999). *Ostreopsis* blooms were solely reported in tropical areas until the beginning of the 2000s with the first blooms in temperate waters, especially in the Mediteranean (Ciminiello et al., 2006) and the Aegean sea (Aligizaki et al., 2008; Aligizaki and Nikolaidis, 2006). This highlighted the human risks associated with the exposure via aquatic activities and through the consumption of contaminated marine organisms.

During the summer of 2005, about 200 people sought medical treatment in Genoa, Italy, because of cutaneous and respiratory problems after being exposed to marine aerosols; Ciminiello and co-workers (2006) showed the presence of PITX associated with *Ostreopsis ovata*. In France, the presence of *Ostreopsis* spp. was responsible for similar symptoms between 2006 and 2009 reported by a few people who went swimming and diving on the Mediterranean coast (Tichadou et al., 2010).

PITX and PITX-like compounds have been reported in marine organisms collected in various tropical countries including Colombia, Madagascar, Philippines, Japan, Australia, and Micronesia (Aligizaki et al., 2011; Gleibs and Mebs, 1999; Munday, 2008) and some cases of food poisoning were reported in the literature, some being fatal (Aligizaki et al., 2011; Deeds and Schwartz, 2010;

* Corresponding author. Tel.: +33 10 49772738; fax: +33 10 49772650.
*E-mail address:* sophie.trotereau@anses.fr (S. Trotereau).

1568-9883/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.hal.2013.04.007

Munday, 2008; Tubaro et al., 2011b). Several symptoms were reported by the consumers after eating contaminated marine organisms; these included a metallic taste, gastrointestinal malaise, diarrhoea, nausea, vomiting, ataxia, dizziness, numbness of extremities, myalgia, dyspnoea, convulsions and bradicardia (Tubaro et al., 2011b).

No food poisoning has been associated with *Ostreopsis* blooms in the Mediterranean, even though the presence of PlTX-like compounds was reported in shellfish collected in the Aegean Sea (Greece) in 2003 (Aligizaki et al., 2008), in mussels and sea urchin from the Ligurian coastline (Bellocci et al., 2008b), and from the French Mediterranean coast (Amzil et al., 2009) and during this bloom of *Ostreopsis* in the French Mediterranean coast no seafood poisoning was reported by the epidemiological system.

The data reported in the literature regarding the presence of PlTX and PlTX-like compounds in edible marine organisms were obtained with different methods, including biological (mouse bioassay, haemolytic test) and chemical methods (LC–MS). The toxin levels accounted for in these reports vary, some being expressed as mouse or haemolytic units per gram or as microgram of PlTX per kilogram of specimen; this makes it difficult to compare the different sets of data and thereby does not facilitate the risk assessment. Yet, an important step has been made in that direction, as a scientific panel under the aegis of the European food safety authority (EFSA) assessed the risk associated with the presence of PlTX and its analogue ostreocin-D (OST-D) in shellfish and estimated that the toxin level should not exceed 30 μg for the sum of PlTX and OST-D per kg (μg PlTX + OST-D/kg) of shellfish meat to protect the consumers' health (EFSA, 2009). This risk assessment was performed on the basis of the scarce toxicological, occurrence and consumption data available at the time. It pointed out the need for additional information on the toxicity of the different analogues, as well as on the occurrence of these toxic compounds in marine products, other than shellfish, destined to human consumption, in order to refine and complete the EFSA risk assessment that currently only concerns the shellfish. The provision of such data can come from monitoring programmes as well as from research projects.

The present study aimed at collecting information on the occurrence and the distribution of PlTX and 2 of its analogues (OVTX-a and OST-D) in a wide variety of edible marine organisms harvested in four recreational sites located in Nice and Villefranche-sur-Mer on the French Mediterranean coast. The sampling campaign took place between June and September 2009, in locations where *Ostreopsis* cf. *ovata* had previously been observed during the same periods.

## 2. Materials and methods

### 2.1. Sampling locations

The choice of the first two sites (Fig. 1), site N (43°41′27.19″ N and 7°17′35.09″ E) located east from Nice harbour and site V1 (43°42′6.87″ N and 7°19′14.45″ E), located in Villefranche, south from "Plage de la Réserve", was based on high *Ostreopsis* cf. *ovata* abundances recorded in 2008.

Two other locations were also sampled when blooms of *Ostreopsis* occurred in the area; these are site V2 in Villefrance, which corresponds to "Plage des jeunes" (43°42′10.81″ N and 7°19′13.04″ E) and site V3, also known as "Rochambeau" (43°41′34.83″ N and 7°18′31.66″ E).

### 2.2. Sampling periods

The edible marine organisms targeted in the study as well as the planktonic and epiphytic *Ostreopsis* samples were collected between the 2nd of June (week 23) and the 31st of September 2009 (week 39), with variations depending on the site. During the course of the sampling period, samples were taken on a bimonthly basis in sites N and V1. The sampling frequency was increased to weekly when the toxic bloom was detected.

Additional sampling on a wider range of marine organisms in sites V2 and V3 was carried because *Ostreopsis* cf. *ovata* blooms occurred in these areas.

### 2.3. Sampling of marine organisms

A wide variety of marine organisms available on site were sampled in the four locations and included molluscs (gastropods and cephalopods), echinoderms, fish, and crustaceans (Table 1).

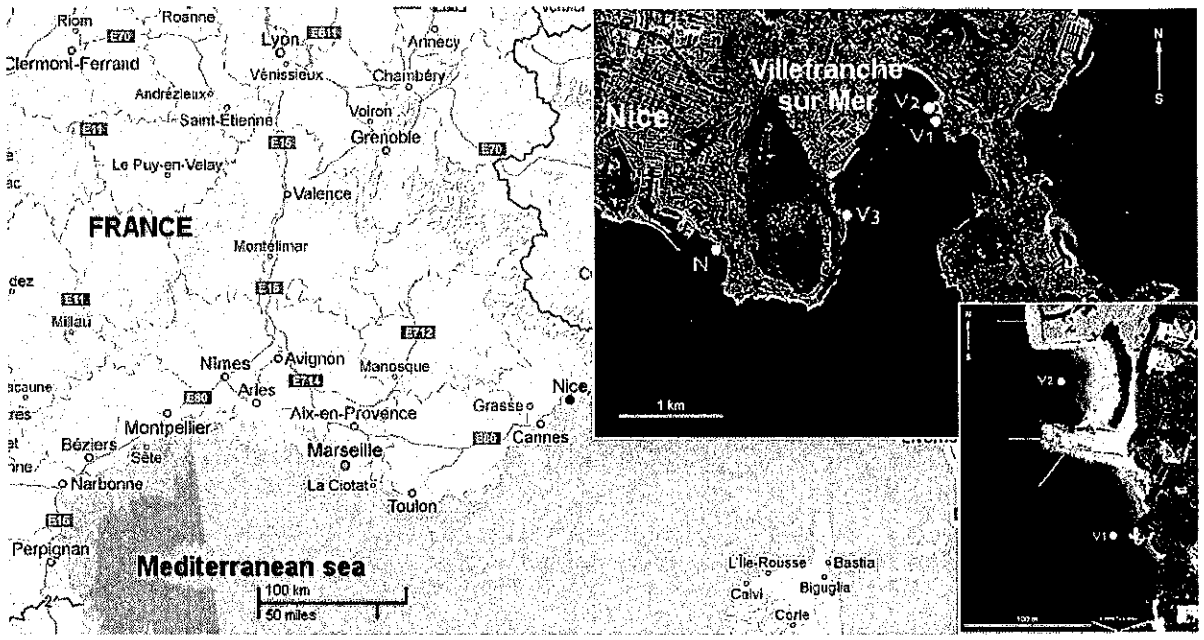



**Fig. 1.** Location of the sampling sites selected for the study. N = Nice, Plage de la Réserve; V1 = Villefranche, beach located below Plage des Jeunes; V2 = Villefranche, Plage des jeunes; V3 = Rochambeau.

**Table 1**
Information regarding the species sampled in the 4 selected sites.

| Group | Species | Authorities | Common name | Diet[1] | | | Sampling sites | | | | Part analysed[2] | Remarks (mainly major diet) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | H | O | C | N | V1 | V2 | V3 | | |
| Gastropods | *Patella* spp. | Linnaeus, 1758 | Limpet | * | | | * | * | * | * | WF | A mix of *Patella coeruela* (Linnaeus, 1758) and *Patella rustica* (Linnaeus, 1758) |
| Gastropods | *Stramonita haemastoma* | Linnaeus, 1767 | Red-mouthed rock shell | | | * | * | * | * | * | WF | Feeding on bivalves and other small sessile animals having a shell |
| Cephalopods | *Octopus vulgaris* | Cuvier, 1797 | Common Octopus | | | * | | | * | * | DT, RT | Feeding on crustaceans and molluscs |
| Crustaceans | *Eriphia verrucosa* | Forskål, 1775 | Warty crab/Yellow crab | | | * | * | * | | * | WF | Opportunistic that feeds on dead or alive prays |
| Crustaceans | *Maja squinado* | Herbst, 1788 | Spinous spider crab | | | * | | | | * | WF | Feeding on molluscs and small crustaceans |
| Echinoderms | *Paracentrotus lividus* | Lamarck, 1816 | Common sea urchin | * | | | * | * | * | * | DT, roe | Feeding on macroalgae |
| Fish | *Diplodus annularis* | Linnaeus, 1758 | Annular seabream | | | * | | | * | | DT, RT | Feeding on crustaceans, molluscs and echinoderms |
| Fish | *Diplodus sargus* | Linnaeus, 1758 | White seabream | | | * | | | | * | DT, RT | Feeding on crustaceans, molluscs and echinoderms |
| Fish | *Mugil cephalus* | Linnaeus, 1758 | Flathead mullet | | * | | | | * | * | DT, RT | Feeding by grazing various soft or rocky substrates and *Posidonia* leaves |
| Fish | *Mullus surmuletus* | Linnaeus, 1758 | Striped red mullet | | | * | * | * | | * | DT, RT | Feeding on worms and crustaceans found while excaving soft substrates |
| Fish | *Muraena helena* | Linnaeus, 1758 | Mediterranean moray | | | * | | | | * | DT, RT | Feeding on fish, crustaceans and cephalopods |
| Fish | *Sarpa salpa* | Linnaeus, 1758 | Seabream | * | | | * | * | * | * | DT, RT | Feeding behaviour depending on age. Adults feed on macroalgae and *Posidonia* leaves |
| Fish | *Scorpaena porcus* | Linnaeus, 1758 | Scorpionfish | | | * | * | * | * | * | DT, RT | Feeding on crabs, shrimps and fish |
| Fish | *Serranus scriba* | Linnaeus, 1758 | Painted comber | | | * | | | * | | DT, RT | Feeding on crustaceans, molluscs and fish |
| Fish | *Symphodus (Crenilabrus) tinca* | Linnaeus, 1758 | East Atlantic Peacock | | | * | | | | * | DT, RT | Feeding on worms and crustaceans |

(1) H = herbivorous; O = omnivorous; C = carnivorous.
(2) DT = digestive tube; RT = remaining tissue; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

Status of scientific names and authorities were checked in WoRMS (World Register of Marine Species; www.marinespecies.org). All these species are edible and can be eaten either directly or as an ingredient of a fish soup for instance.

### 2.4. Sampling techniques and preparation of the samples

All the species were collected by snorkelling or fishing from the shore, from the surface to 3 m depth maximum. Depending on the species, a variable number of specimens were sampled at the time to have enough quantity of matrix for toxin analysis, while making sure that there would be enough resource in the environment for the whole sampling period. Thus a preliminary sampling campaign showed that in order to obtain a minimum of 10 g of each biological matrix (either as whole animal, digestive tube [DT], roe or remaining tissue [RT]) it was necessary to collect 4 specimens of Warty crabs (*Eriphia verrucosa*), 10 sea urchins (*Paracentrotus lividus*), 15 limpets (*Patella* spp.), 8 red-mouthed rock shells (*Stramonita haemastoma*), 4 seabreams (*Sarpa salpa*) and 3 scorpionfish (*Scorpaena porcus*).

The molluscs and crustaceans were analysed as whole flesh (WF), after removing the shell of the molluscs or the exoskeleton of the crustaceans. For all the other marine organisms that were sampled (cephalopods, echinoderms and fish), the DT was separated from the RT or roe in the case of the sea urchins, and each part was analysed separately. The toxin concentration in the whole flesh, composed of the DT and the roe for sea urchins and DT and RT for the other species (except molluscs and crustacean), was then calculated as the sum of the toxin concentration of the different tissues pondered by the weight of the corresponding tissues.

All the samples were stored at −20 °C until being analysed by the haemolytic test and LC–MS/MS.

### 2.5. Ostreopsis *planktonic and epiphytic abundances*

#### 2.5.1. Planktonic cells

To estimate the abundances of *Ostreopsis* cf. *ovata* planktonic cells in the water column of the four selected sites, 250 mL of sea water were collected in a plastic flask at 0.3 m depth and 0.2 m above the macroalgae that were subsequently sampled to estimate the epiphytic abundances of the dinoflagellate. Three different sampling points were selected for each site; and the abundances of the planktonic cells were determined as the average of the 3 counts.

The water samples were fixed in acidic lugol solution (2%; v/v) and stored at 4 °C in the dark. A 50 mL volume of each water sample was settling during 24 h before counting planktonic *Ostreopsis* cells with an inverted microscope (Utermöhl method, 1958). Concentrations were then reported as number of cells per litre (cells $l^{-1}$).

#### 2.5.2. Epiphytic cells

To estimate the abundances of *Ostreopsis* cf. *ovata* epiphytic cells, ca. 10 g fresh weight (FW) of the macroalgae *Halopteris scoparia* (*Linnaeus*) *Sauvageau* (sampling sites V1, V2 and V3) or *Corallina* spp. (site N; *H. scoparia* was not available) were carefully sampled in a 250 mL plastic flask with the surrounding seawater, avoiding as much as possible any loss of microalgae. Macroalgae samples were vigorously shaken and passed through a 500 μm meshed filter to separate the macroalgae from the water containing the microalgae. The macroalgae were rinsed twice

with 100 mL of 0.2 μm filtered seawater to recover a maximum of microalgae, then dried on absorbent paper and weighed (±0.01 g). Epibenthic cell abundances in the filtered water were evaluated with an optical microscope, using calibrated squared chambers (1 mL; Sedgwick Rafter) and then reported as number of cells per gram of fresh weight of macroalgae (cells/g FW).

### 2.6. Determination of the toxin content of the marine products

#### 2.6.1. Reagents used

All the reagents were of analytical grade unless otherwise specified.

PITX was purchased from Wako Chemical GmbH (Neuss, Germany) as a 100 μg lyophilised powder. The toxin quantity was determined gravimetrically. This standard was 90% pure as estimated chromatographically.

De-ionised water (18.2 mΩ) was obtained using a Milli-Q® purification system (Millipore, Molsheim, France).

Methanol (MeOH) and acetonitrile (MeCN) were of HPLC grade and were purchased from Fisher Scientific (Illkirch, France).

Mouse blood containing lithium heparinate as anti-blotting agent was purchased from Charles River Laboratories (L'Arbresle, France).

#### 2.6.2. Haemolytic test

The haemolytic test is based on the capacity of PITX and analogues to convert the $Na^+/K^+$-ATPase pump into a non specific channel, leading to ion imbalance and delayed haemolysis of mammalian erythrocytes (Habermann et al., 1981). This characteristic haemolytic activity was the basis for the development of variants of haemolytic tests to determine the PITX-group toxin content of marine organisms extracts (Bignami, 1993; Riobó et al., 2008; Taniyama et al., 2002).

In this study, the haemolytic test was used as a first level test to screen a large number of samples. Therefore, we considered the sensitivity of the test for the PITX-group toxin as the most important aspect and consequently decided to use mouse red blood cells due to their high sensitivity to PITXs haemolytic activities (Habermann et al., 1981). Furthermore, each positive sample was tested for specificity with ouabain acting as an ATPase blocker and was finally confirmed by an LC–MS/MS analysis.

*2.6.2.1. Extraction procedure.* A 2.5 g portion of marine product homogenate was extracted with 7.5 mL of 50% aqueous MeOH. The mixture was homogenised at 12,000 rpm for 2 min using an Ultra-turrax. After centrifuging the extract at 3700 × *g* for 10 min, the supernatant was transferred to a 25 mL volumetric flask, while the pellet was re-extracted with 7.5 mL of 50% aqueous MeOH. The extract was centrifuged and the supernatant was transferred to the same volumetric flask. The volume was made up to 25 mL using Milli-Q water, bringing the MeOH concentration down to 30% and the final extract was filtered through a 0.45 μm PET filter (Chromafil Xtra PET 45/25 Macherey-Nagel, Hoerd, France).

*2.6.2.2. Haemolytic test.* A series of 11 calibration points was prepared using the PITX standard, with concentrations ranging from 0.0 (blank) to 5.0 ng/mL (1.9 pM). Similarly, the sample extracts had to be diluted several folds to enable the quantification of the toxin content. The dilution factors applied were comprised between 1/2 and 1/256. All dilutions were made in 30% aqueous MeOH.

A 50 μL aliquot of the diluted extract or calibration point was added to 950 μL of phosphate buffer saline (PBS) containing 0.5% (v/v) of mouse erythrocytes obtained from Charles River Laboratories (L'Arbresle, France). The number of erythrocytes in the PBS buffer containing 0.5% of mouse erythrocytes was checked as a quality control; the average count over 131 determinations was $5.7 \times 10^7$ cells (relative standard deviation = 9.7%). The solution was incubated at 37 °C for 4 hours and centrifuged at 1000 × *g* for 10 min. A 200 μL aliquot of each extract or calibration point was put in triplicate in a 96-well plate (Nunc microwell F96 PS NST, Fisher Scientific) and the optical density (OD) was read at 450 nm with a spectrophotometer (MRX 3100, Dynex Technologies). The percentage of lysis is a linear function of the OD (ratio of the difference between OD and the blank OD (OD min) and the difference between the maximum OD and the blank OD). The relationship between the percentage of lysis and the concentration of palytoxin is a sigmoïdal curve that is transformed in a linear curve by using the logit/log model. The percentage of lysis of an extract was determined from the OD obtained for each dilution of this extract. The toxin concentration in the extract was determined using the logit/log model and was calculated using the linear part of the curve Logit (% lysis) = $a \log X + b$, giving the following equations:

$$\text{concentration X (in ng/mL)} = 10^{(\text{Logit}-b)/a} \times \text{dilution factor}$$

with

$$\text{Logit} = \ln \frac{\%\,\text{lysis}}{1 - \%\,\text{lysis}}$$

and

$$\%\,\text{lysis} = \frac{OD - OD_{min}}{OD_{max} - OD_{min}} = \text{slope}_{\text{calibration curve}} \times OD + \text{intercept}_{\text{calibration curve}}$$

As the haemolytic potency was estimated using PITX as a calibrant, the results were expressed in microgram of PITX equivalent per kilogram of the analysed matrix (μg PITX eq./kg).

The limit of quantification (LOQ) of the haemolytic test was determined as 1.2 μg PITX eq./kg.

The specificity of the haemolytic activity of PITX was confirmed on the positive samples by adding 50 μL aliquot of the diluted extract or calibration point to 950 μl phosphate buffer saline containing 0.5% (v/v) of mouse erythrocytes and 500 μM of ouabain pre-incubated at 37 °C for 1 h. In the presence of PITX and ouabain a shift in the response curve compared to the curve without ouabain was observed.

#### 2.6.3. Tandem mass spectrometry (LC–MS/MS) analysis

*2.6.3.1. Extraction procedure.* A 2 g portion of marine product homogenate was extracted with 10 mL of 80% aqueous MeOH. This solvent was shown to be the most efficient by Ciminiello and co-workers in 2011 (2011a).The mixture was homogenised at 10,000 rpm for 2 min using a Polytron and then centrifuged at 4000 × *g* for 8 min. The supernatant was transferred to a 20 mL volumetric flask, while the pellet was re-extracted with 9 mL of 80% aqueous MeOH. After being centrifuged the second extract was transferred to the same 20 mL volumetric flask and the volume was completed to the mark using 80% aqueous MeOH. The extract was then filtered using a 0.45 μm PTFE filter (Chromafil Xtra PTFE 45/25 Macherey-Nagel, Hoerd, France).

*2.6.3.2. Solid-phase extraction (SPE) cleanup.* Prior to LC–MS/MS analysis, the extracts were purified using StrataX (60 mg/3 mL) cartridges (Phenomenex, France).

The cartridges were placed on a Manifold and conditioned with 3 mL of MeOH and 3 mL of 30% aqueous MeOH.

In a 15 mL tube, 4.5 mL of extract were added to 7.5 mL of Milli-Q water to bring the MeOH concentration down to 30%. This solution was then loaded and passed through the cartridge. A

washing step was carried out, using 3 mL of 10% aqueous MeOH and the cartridge was run dry. The elution was performed with 3 mL of 90% aqueous MeOH containing 0.5% of formic acid and the cartridge was run dry to collect the elutant in a 5 mL amber tube.

*2.6.3.3. LC–MS/MS analysis.* The LC–MS/MS analyses were performed on a Dionex Ultimate 3000 (Dionex, France) coupled to an API4000 Qtrap mass spectrometer, a triple quadrupole – linear ion trap hybrid (AB Sciex, Les Ulis, France), equipped with an electrospray ionisation source. The toxins were separated on a Phenomenex Gemini C18 column (150 mm × 2 mm; 3 μm) equipped with a guard column (20 mm × 2 mm; 3 μm) of the same phase. A gradient of mobile phase A (100% $H_2O$ with 30 mM acetic acid) and B (95% aqueous MeCN with 30 mM acetic acid) was run at 200 μL/min starting at 0% B and rising to 100% B in 12 min. The gradient was held at 100% B for 5 min and was decreased to 0% B in 0.1 min. This composition was held for 7.9 min, until the end of the run.

PlTX and palytoxin-like compounds, OVTX-a and OST-D, were analysed by using an ESI triple-quadrupole MS instrument in positive mode with an ionspray voltage of 5.5 kV. MRM experiments were carried out by selecting three transitions (precursor ion → - product ion). For palytoxin, the selected transitions were *m/z* 1340.9 → 327.3 ($[M+2H]^{2+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$), *m/z* 1331.9 → 327.3 ($[M+2H\text{-}H_2O]^{2+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$) and *m/z* 876.2 → 327.3 ($[M+{}^3H\text{-}{}^3H_2O]^{3+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$). The analogues share this same fragmentation behaviour with respectively for OVTX-a *m/z* 1324.9 → 327.3, *m/z* 1315.9 → 327.3 and *m/z* 865.5 → 327.3 and for OST-D *m/z* 1318.9 → 327.3, *m/z* 1309.9 → 327.3 and *m/z* 861.5 → 327.3. The most intense transition was used for quantification purpose ($[M+2H\text{-}H_2O]^{2+} \rightarrow [A\ moiety + H\text{-}H_2O]^+$).

The toxin concentrations in the analysed samples were determined from a PlTX external calibration curve. OVTX-a and OST-D were quantified using PlTX as calibrant, assuming an equimolar response for all these 3 toxins.

The limit of detection (LOD) and LOQ of the LC–MS/MS method were determined as 7.4 and 24.5 μg PlTX/kg, respectively.

## 3. Results

### 3.1. Monitoring of the site N (Nice)

The site N located in Nice was sampled from week 24 to 36 of the year 2009 (Table 2). The epiphytic *Ostreopsis* cf. *ovata* cells started blooming in week 28 and reached a maximum concentration of about 120,000 cells/g FW in 3 weeks (Fig. 2). The toxin levels determined by the haemolytic test in the sampled marine products followed the same trend as the *Ostreopsis* cells with the highest concentrations observed in week 31 for the Warty crab *Eriphia verrucosa* whole flesh (38.4 μg PlTX eq./kg) and the seabream *Sarpa salpa* (29.7 μg/kg). The toxin concentration in the whole flesh of the sea urchin *Paracentrotus lividus* reached a maximum of 61.6 μg/kg in week 32; this organism had the highest toxin concentrations recorded on Nice coast. The red-mouthed rock shell *Stramonita haemastoma* was also found to be contaminated with a maximum level of 34.1 μg/kg in the whole flesh, in week 34. From all the sampled marine products, limpets belonging to the genera *Patella* were the only animals for which the highest toxin concentration (7.5 μg/kg) was recorded in week 30 that is before the maximum of the *O.* cf. *ovata* epibenthic abundance. Over the course of the sampling period, the scorpionfish *Scorpaena porcus* and the striped red mullet *Mullus surmuletus* had toxin levels below the limit of quantification (LOQ) of the haemolytic test (1.2 μg PlTX eq./kg).

From the 7 species that were sampled in site N and analysed, only three had toxin levels in the whole flesh above 30 μg PlTX eq./kg; this echoes to the EFSA opinion reporting that the toxin level in the particular case of shellfish should not exceed 30 μg PlTX + OST-D/kg in order to protect the consumers' health. These species are respectively the Warty crab *Eriphia verrucosa* (week 31), the sea urchin *Paracentrotus lividus* (weeks 32 and 34) and the red-mouthed rock shell *Stramonita haemastoma* (week 34). The toxin concentration in the seabream *Sarpa salpa* in week 31 was equal to 29.7 μg PlTX eq./kg of whole flesh.

The analysis of the different tissues (DT, roe, RT) of the sampled marine products showed that the highest toxin concentration was found in the seabream DT (230 μg/kg) in week 31 (Fig. 3); this toxin level in the DT brought the toxin level in the whole flesh to 29.7 PlTX eq./kg. On two other occasions (weeks 30 and 34), this species (*Sarpa salpa*) had toxin levels in its DT of 66.5 and 99.0 μg/kg respectively. In weeks 31, 32 and 34, the toxin levels found in the sea urchin DT were respectively 41.9, 97.6 and 50.7 μg PlTX eq./kg. However, it is important to notice that despite high toxin levels in the DT of some of the sampled species, the roe of the sea urchins or RT of the other animals were not contaminated (levels below the LOQ). Trace amounts of PlTX-like compounds were found in the DT of the scorpionfish in week 26 (3.6 μg PlTX eq./kg) and the striped red mullet DT in week 34 (3.3 μg PlTX eq./kg).

**Table 2**
Palytoxins concentration (μg/kg) determined by the haemolytic test in different parts of fishery products harvested in Nice (site N) and *Ostreopsis* cf. *ovata* abundances from week 24 to week 36.

| Common name | Latin name | wk 24 | wk 26 | wk 28 | wk 30 | wk 31 | wk 32 | wk 34 | | wk 36 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Nice (N)* | | | | | | | | | | |
| **Patella WF** | *Patella spp* | <LOQ | <LOQ | <LOQ | **7.5** | **1.2** | **2.6** | **2.6** | – | <LOQ |
| **Red-mouthed rock shell WF** | *Stramonita heamastoma* | <LOQ | <LOQ | <LOQ | **2.9** | **6.1** | **13.0** | **34.1** | – | **5.1** |
| **Sea urchin roe** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | 1.4 | <LOQ | – | <LOQ |
| **Sea urchin DT** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **24.4** | **41.9** | **97.6** | **50.7** | – | **5.8** |
| **Sea urchin WF** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **14.6** | **26.6** | **61.6** | **34.7** | – | 3.0 |
| **Warty crab WF** | *Eriphia verrucosa* | <LOQ | <LOQ | <LOQ | **1.1** | **38.4** | **3.2** | **9.5** | – | <LOQ |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | – | <LOQ |
| **Scorpionfish DT** | *Scorpaena porcus* | <LOQ | **3.6** | <LOQ | **1.4** | **2.2** | **1.2** | **1.9** | – | <LOQ |
| Scorpionfish WF | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | – | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| **Seabream DT** | *Sarpa salpa* | <LOQ | <LOQ | **3.9** | **66.5** | **230.0** | **26.2** | **15.6** | **99.0** | **1.2** |
| Seabream WF | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | **12.1** | **29.7** | **5.5** | **2.9** | **21.2** | <LOQ |
| Red mullet RT | *Mullus surmuletus* | – | – | – | – | – | – | <LOQ | – | – |
| **Red mullet DT** | *Mullus surmuletus* | – | – | – | – | – | – | **3.3** | – | – |
| Red mullet WF | *Mullus surmuletus* | – | – | – | – | – | – | <LOQ | – | – |
| *Ostreopsis* cf. *ovata* | **(cells/g fresh weight)** | **189** | **166** | **4749** | **20,786** | **120,736** | **18,054** | **9325** | | **4004** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

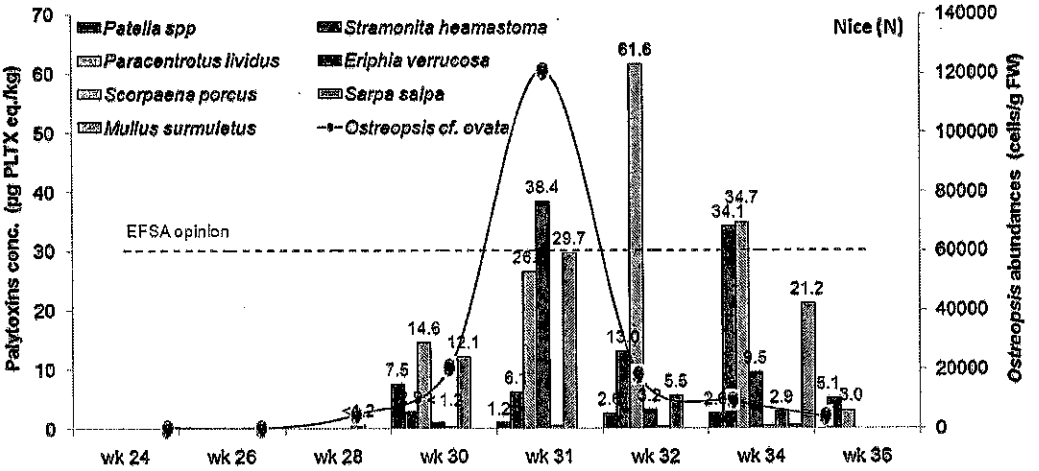



**Fig. 2.** Palytoxins concentration determined by the haemolytic test in the whole flesh of the fishery products harvested in Nice (site N) from week 24 to week 36. The dashed line represents the threshold of 30 µg PITX + OST-D/kg proposed by the EFSA and specifically applicable to shellfish. The blue curve represents the *Ostreopsis* abundances on the same period. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Similarly to the other species, no toxin was detected in the RT of both fish species (*Scorpaena porcus*, *Mullus surmuletus*).

### 3.2. Monitoring of the site V1 (Villefranche)

The site V1 located in Villefranche was sampled from week 23 to week 39 of the year 2009 (Table 3). Similar to what was observed in Nice, the highest *Ostreopsis* cell abundances were recorded in week 31 but with somewhat lower concentrations (ca. 39,000 Cells/g FW). The highest toxin concentration was detected in the whole flesh of the seabream *Sarpa salpa* in week 31 with 16.4 µg PITX eq./kg. Inversely to what was observed in Nice, the highest toxin concentrations in limpets of the genus *Patella* (5.4 µg/kg) coincided with the highest cell counts in week 31. The concentrations of equivalent PITX in the whole flesh of the sea urchin *Paracentrotus lividus* ranged from 1.4 to 5.5 µg/kg in weeks 29 and 30, respectively. The toxin levels measured in the red-mouthed rock shell *Stramonita haemastoma* were comprised between 1.2 and 3.4 µg PITX eq./kg. All the samples of the Warty crab *Eriphia verrucosa*, the scorpionfish *Scorpaena porcus* and the red mullet *Mullus surmeletus* had PITX levels below the LOQ of the haemolytic test.

The analysis of the different tissues of the species showed that the toxins were sequestered in the DT of the sea urchins (7.4 µg PITX eq./kg) and the seabream *Sarpa salpa* (79.4 µg PITX eq./kg), while the corresponding roe or RT did not contain quantifiable PITX levels.

### 3.3. Monitoring of the site V2 (Villefranche – Plage des jeunes)

The results of the monitoring carried out on the Plage des Jeunes (site V2), in Villefranche, are presented in Table 4. A wider panel of species was sampled from week 30 to week 35 of the year 2009, with notably more fish samples. The cell abundances of the epiphytic *Ostreopsis* cf. *ovata* showed that the dinoflagellate reached a maximum concentration in week 31 with ca. 336,000 cells/g FW. This is by far the highest cell abundances recorded in the four sites sampled during this study.

In week 31 and 33, the whole flesh of the sea urchin *Paracentrotus lividus* had equivalent PITX concentrations of 67.5 and 33.7 µg/kg, respectively (Table 4). All the other species had much lower PITX concentrations, the red-mouthed rock shell *Stramonita haemastoma* being the second highest concentrated sample with 10.8 µg PITX eq./kg (week 33), followed by the spinous spider crab *Maja squinado* (8.3 µg PITX eq./kg in week 33), by the seabream *Sarpa salpa* (6.3 µg PITX eq./kg in week 33), by the limpets *Patella* spp. (4.8 µg PITX eq./kg in week 33) and by *Octopus vulgaris* (2.8 µg PITX eq./kg in week 35). All the other fish species

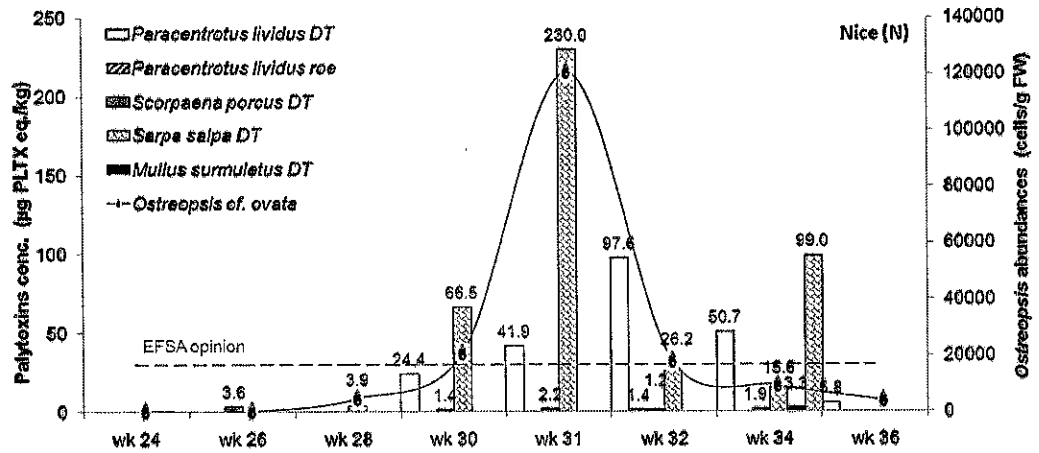



**Fig. 3.** Palytoxins concentration determined by the haemolytic test in the different parts of the fishery products harvested in Nice (site N) from week 24 to week 36. DT = digestive tube; RT = remaining tissue. The dashed line represents the threshold of 30 µg PITX + OST-D/kg proposed by the EFSA and specifically applicable to shellfish. The blue curve represents the *Ostreopsis* abundances on the same period.

**Table 3**
Palytoxins concentration (μg/kg) determined by the haemolytic test in different parts of fishery products harvested in Villefranche (site V1) and *Ostreopsis* cf. *ovata* abundances from week 23 to week 39.

| Common name | *Latin name* | wk 23 | wk 25 | wk 27 | wk 29 | wk 30 | wk 31 | wk 33 | wk 35 | wk 37 | wk 39 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Villefranche (V1)* | | | | | | | | | | | |
| **Patella WF** | *Patella spp* | <LOQ | <LOQ | <LOQ | **1.2** | **1.8** | **5.4** | **4.9** | <LOQ | <LOQ | <LOQ |
| **Red-mouthed rock shell WF** | *Stramonita heamastoma* | <LOQ | <LOQ | <LOQ | <LOQ | **2.6** | **1.2** | **3.4** | <LOQ | <LOQ | <LOQ |
| Sea urchin roe | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| **Sea urchin DT** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **2.0** | **7.4** | **2.1** | <LOQ | <LOQ | <LOQ | <LOQ |
| **Sea urchin WF** | *Paracentrotus lividus* | <LOQ | <LOQ | <LOQ | **1.4** | **5.5** | **1.6** | <LOQ | <LOQ | <LOQ | <LOQ |
| Warty crab WF | *Eriphia verrucosa* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Scorpionfish DT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Scorpionfish WF | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ | <LOQ |
| Seabream DT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | **8.7** | **79.4** | **4.1** | <LOQ | **1.2** | <LOQ |
| Seabream WF | *Sarpa salpa* | <LOQ | <LOQ | <LOQ | <LOQ | **1.9** | **16.4** | <LOQ | <LOQ | <LOQ | <LOQ |
| Red mullet RT | *Mullus surmuletus* | – | – | – | – | – | – | – | <LOQ | – | – |
| Red mullet DT | *Mullus surmuletus* | – | – | – | – | – | – | – | <LOQ | – | – |
| Red mullet WF | *Mullus surmuletus* | – | – | – | – | – | – | – | <LOQ | – | – |
| *Ostreopsis* cf. *ovata* | **(Cells/g fresh weight)** | **167** | **60** | **3580** | **5813** | **14,836** | **38,922** | **4577** | **2054** | **1919** | **1913** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

(*Scorpaena porcus*, *Mullus surmuletus*, *Serranus scriba*, *Mugil cephalus* and *Diplodus annularis*) had toxin levels below the LOQ of the haemolytic test (1.2 μg PITX eq./kg).

The results regarding the distribution of the toxins between tissues of the species are presented in Table 4. The DT is again the most contaminated part of the sea urchin (*Paracentrotus lividus*) and the octopus (*Octopus vulgaris*), with respectively 110.7 and 6.4 μg PITX eq./kg; however, for both species lower toxin levels were detected in the roe or the RT with respectively 6.8 and 2.5 μg PITX eq./kg. As for the fish samples of scorpionfish (*Scorpaena porcus*), seabream (*Sarpa salpa*), and annular seabream (*Diplodus annularis*), toxins were detected in the DT with values ranging from 3.2 (*S. porcus*) to 25.6 μg PITX eq./kg (*S. salpa*), while the RT were not contaminated.

### 3.4. Monitoring of the site V3 (Rochambeau)

The site of Rochambeau (V3) was monitored bimonthly from week 32 to 36 of the year 2009; the maximum *Ostreopsis* cf. *ovata* abundances were recorded in week 32 with ca. 52,700 cells/g FW (Table 5) and rapidly declined in the following weeks reaching roughly 8800 and 4300 cells/g FW in week 34 and 36 respectively. The maximum toxin concentrations in the whole flesh were

**Table 4**
Palytoxins concentration (μg/kg) determined by the haemolytic test in different parts of fishery products harvested in Villefranche Plage des jeunes (site V2) and *Ostreopsis* cf. *ovata* abundances from week 30 to week 35.

| Common name | Latin name | wk 30 | wk 31 | wk 33 | wk 35 |
|---|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Villefranche plage des jeunes (V2)* | | | | | |
| **Spinous spider crab WF** | *Maja squinado* | – | – | **8.3** | – |
| **Red-mouthed rock shell WF** | *Stramonita heama stoma* | – | – | **10.8** | **2.0** |
| **Patella WF** | *Patella spp* | – | **4.5** | **4.8** | **1.2** |
| Flathead mullet RT | *Mugil cephalus* | – | – | <LOQ | – |
| Flathead mullet DT | *Mugil cephalus* | – | – | <LOQ | – |
| Flathead mullet WF | *Mugil cephalus* | – | – | <LOQ | – |
| Sea urchin roe | *Paracentrotus lividus* | – | **6.8** | <LOQ | <LOQ |
| Sea urchin DT | *Paracentrotus lividus* | – | **110.7** | **53.0** | **4.5** |
| Sea urchin WF | *Paracentrotus lividus* | – | **67.5** | **33.7** | **2.6** |
| **Octopus RT** | *Octopus vulgaris* | – | – | – | **2.5** |
| **Octopus DT** | *Octopus vulgaris* | – | – | – | **6.4** |
| **Octopus WF** | *Octopus vulgaris* | – | – | – | **2.8** |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ |
| **Scorpionfish DT** | *Scorpaena porcus* | **3.7** | <LOQ | **3.2** | <LOQ |
| Scorpionfish WF | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ | <LOQ |
| Red mullet RT | *Mullus surmuletus* | – | – | – | <LOQ |
| Red mullet DT | *Mullus surmuletus* | – | – | – | <LOQ |
| Red mullet WF | *Mullus surmuletus* | – | – | – | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | – | <LOQ | <LOQ |
| **Seabream DT** | *Sarpa salpa* | **7.6** | – | **25.6** | <LOQ |
| **Seabream WF** | *Sarpa salpa* | < LOQ | – | 6.3 | <LOQ |
| Painted comber RT | *Serranus scriba* | – | – | <LOQ | – |
| Painted comber DT | *Serranus scriba* | – | – | <LOQ | – |
| Painted comber WF | *Serranus scriba* | – | – | <LOQ | – |
| Annular seabream RT | *Diplodus annularis* | – | – | <LOQ | – |
| **Annular seabream DT** | *Diplodus annularis* | – | – | 10.6 | – |
| Annular seabream WF | *Diplodus annularis* | – | – | <LOQ | – |
| **Ostreopsis cf. ovata** | **(cells/g fresh weight)** | **100,615** | **336,124** | **23,131** | **18,487** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

**Table 5**
Palytoxins concentration (μg/kg) determined by the haemolytic test in different parts of fishery products harvested in Rochambeau (site V3) and *Ostreopsis* cf. *ovata* abundances from week 32 to week 36.

| Common name | Latin name | wk 32 | wk 34 | wk 36 |
|---|---|---|---|---|
| *Haemolytic test results in μg/kg – Rochambeau (V3)* | | | | |
| **Patella WF** | *Patella* spp | **12.7** | **3.8** | **1.2** |
| **Red-mouthed rock shell WF** | *Stramonita heamastoma* | **13.8** | **4.3** | **3.4** |
| **Warty crab WF** | *Eriphia verrucosa* | **4.9** | <LOQ | <LOQ |
| **spinous spider crab WF** | *Maja squinado* | – | **2.0** | – |
| Sea urchin roe | *Paracentrotus lividus roe* | <LOQ | <LOQ | <LOQ |
| **Sea urchin DT** | *Paracentrotus lividus* | **256.6** | **4.5** | **5.2** |
| **Sea urchin WF** | *Paracentrotus lividus* | **179.6** | **2.6** | **3.2** |
| Scorpionfish RT | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ |
| **Scorpionfish DT** | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ |
| **Scorpionfish WF** | *Scorpaena porcus* | <LOQ | <LOQ | <LOQ |
| Seabream RT | *Sarpa salpa* | <LOQ | <LOQ | <LOQ |
| Seabream DT | *Sarpa salpa* | **25.1** | **8.4** | **1.2** |
| Seabream WF | *Sarpa salpa* | **4.8** | **1.3** | <LOQ |
| East Atlantic peacock wrasse RT | *Symphodus tinca* | <LOQ | <LOQ | – |
| East Atlantic peacock wrasse DT | *Symphodus tinca* | **1.9** | **1.8** | – |
| East Atlantic peacock wrasse WF | *Symphodus tinca* | <LOQ | <LOQ | – |
| Flathead mullet RT | *Mugil cephalus* | <LOQ | <LOQ | – |
| **Flathead mullet DT** | *Mugil cephalus* | **392.2** | **72.8** | – |
| **Flathead mullet WF** | *Mugil cephalus* | **53.5** | **13.2** | – |
| Mediterranean moray RT | *Muraena helena* | – | <LOQ | – |
| Mediterranean moray DT | *Muraena helena* | – | <LOQ | – |
| Mediterranean moray WF | *Muraena helena* | – | <LOQ | – |
| **Octopus RT** | *Octopus vulgaris* | – | **18.5** | – |
| **Octopus DT** | *Octopus vulgaris* | – | **1.3** | – |
| **Octopus WF** | *Octopus vulgaris* | – | **16.9** | – |
| **White seabream RT** | *Diplodus sargus* | <LOQ | **1.2** | – |
| **White seabream DT** | *Diplodus sargus* | **5.1** | **1.4** | – |
| **White seabream WF** | *Diplodus sargus* | <LOQ | **1.2** | – |
| **Ostreopsis cf. ovata** | **(cells/g fresh weight)** | **52,734** | **8771** | **4345** |

DT = digestive tube; RT = remaining tissues; WF = whole flesh. In the case of the organisms analysed as different parts (DT, roe, RT), the toxin concentration in the WF was estimated using the toxin concentration and the weight of the different parts.

obtained in week 32: the sea urchin *Paracentrotus lividus* was by far the most contaminated species with 179.6 μg PITX eq./kg, followed by the flathead mullet *Mugil cephalus* (53.5 μg PITX eq./kg), the red-mouthed rock shell *Stramonita haemastoma* (13.8 μg PITX eq./kg) the limpet *Patella* spp. (12.7 μg PITX eq./kg), the Warty crab *Eriphia verrucosa* (4.9 μg PITX eq./kg) and the seabream *Sarpa salpa* (4.8 μg PITX eq./kg). In the following weeks, the toxin levels decreased but some species showed contamination with a maximum of 16.9 μg PITX eq./kg (*Octopus vulgaris*). During the sampling period, some species never showed contamination in the whole flesh: this includes the scorpionfish (*Scorpaena porcus*), the East Atlantic peacock (*Symphodus tinca*), the Mediterranean moray (*Muraena helena*) and the white seabream (*Diplodus sargus*).

The results regarding the distribution of the toxins between the different tissues of the marine organisms are presented in Table 5. Similar to what was observed on the other sites, the DT was the most contaminated tissue with the highest contamination levels being observed for the flathead mullet *Mugil cephalus* and the sea urchin *Paracentrotus lividus* (392.2 and 256.6 μg PITX eq./kg, respectively). *Octopus vulgaris* was the only species having a toxin concentration higher in the RT (18.5 μg PITX eq./kg) than in the DT (1.3 μg PITX eq./kg), contrary to what was observed for the same species in Villefranche – Plage des jeunes (site V2).

### 3.5. Comparison of the toxin levels determined by haemolytic test and LC–MS/MS

All the samples analysed by the haemolytic test and that gave a result above the LC–MS/MS LOQ (24.5 μg PITX/kg) were analysed using the latter technique to determine the toxin profile.

As shown in Fig. 4, the LC–MS/MS analyses showed that the toxin profile of the samples was composed of OVTX-a as the major

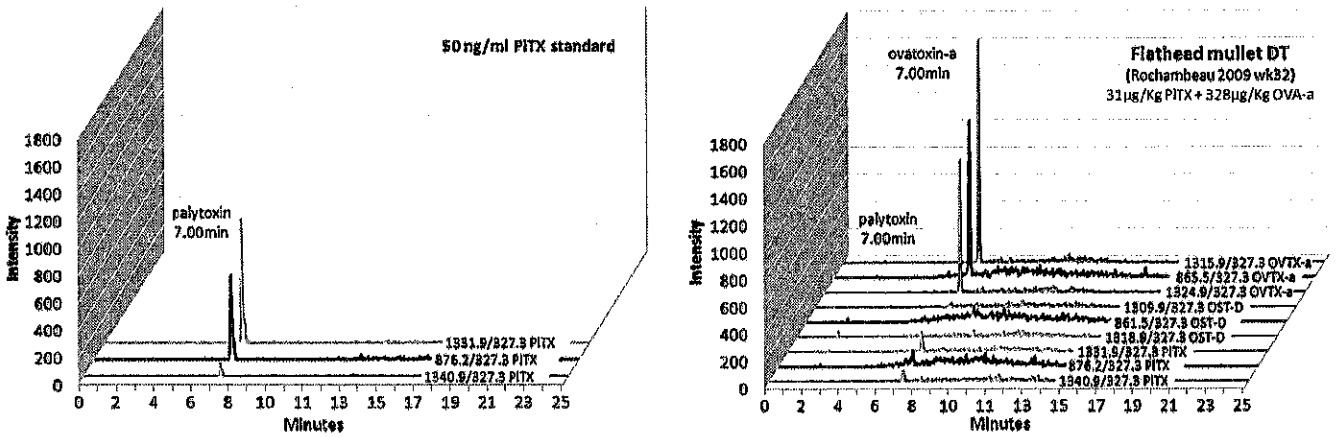



Fig. 4. LC–MS/MS chromatograms of palytoxin standard solution and flathead mullet contaminated sample by OVTX-a and PITX.

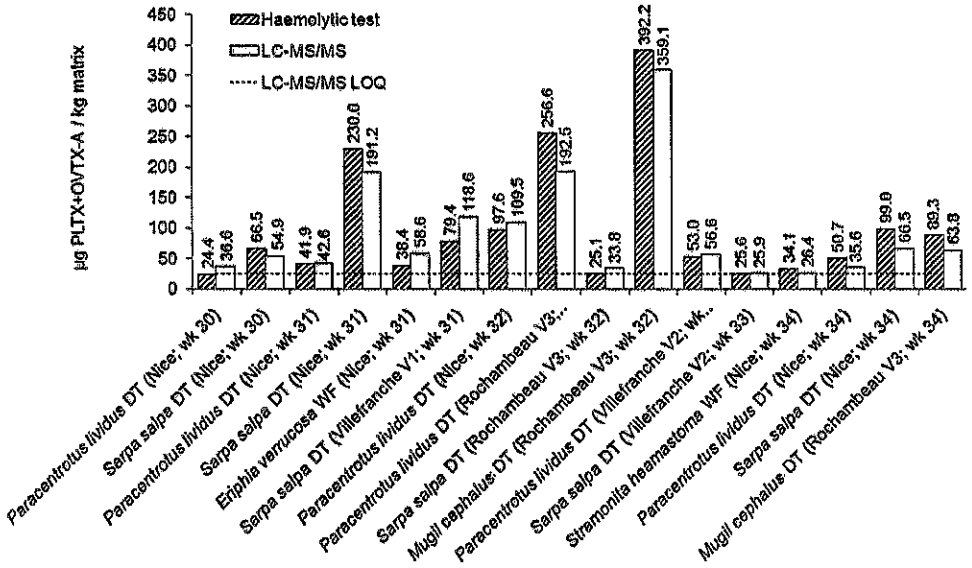



**Fig. 5.** Comparison of the Palytoxins levels determined by the haemolytic test and by LC–MS/MS analysis in the marine products harvested in the four different sampling sites. WF = whole flesh; DT = digestive tube.

compound (90% of the toxin profile) while PlTX only represented 10%. No trace of OST-D was detected in any of the samples.

The results obtained using both methods (haemolytic test and LC–MS/MS) are presented in Fig. 5. As shown in Fig. 6, there is a good agreement between both sets of results whatever the species and the nature of the sample (whole flesh or digestive tube), with a $R^2$ of 0.9533.

The ratio calculated by normalising the LC–MS/MS result with that of the haemolytic test ranges from 0.7 to 1.5 with the median value being comprised between 0.9 and 1.0.

## 4. Discussion

### 4.1. Ostreopsis *abundances and monitoring*

To evaluate the cell abundances in the four sites sampled as part of the present study, it was decided to rely on the epiphytic cells rather than the planktonic ones. This choice was based on the fact that benthic cells are considered to be more conservative as being less susceptible to hydrodynamics disturbances (Mangialajo et al., 2011). The planktonic cell abundances were yet determined for

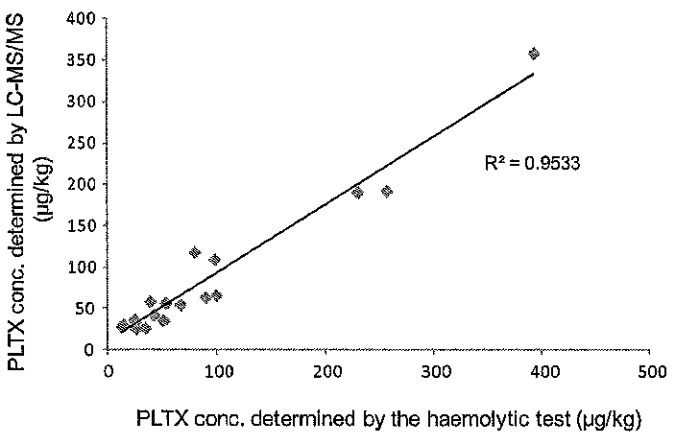



**Fig. 6.** Relationship between the PlTX concentrations determined using the LC–MS/MS method and the haemolytic test. For the LC–MS/MS method, the toxin concentration was determined as the sum of OVTX-a and PlTX, both detected in the samples.

informative purposes in Nice (N), Villefranche (Sites V1 and V2) and Rochambeau (V3) and the maximum values coincided temporally with the corresponding benthic cell abundances (data not shown). This correlation between epiphytic and planktonic cell abundances has already been reported in the literature (Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008; Vila et al., 2001) and this goes along with the hypothesis of the epiphytic cells being the stock of the available *Ostreopsis* cf. *ovata* biomass (Mangialajo et al., 2011). Some authors reported a delay between the benthic and the planktonic cell abundances as a result of different phenomena such as cell re-suspension due to wave action (Totti et al., 2010), to human activities (boat traffic, human trampling; (Mangialajo et al., 2011)) or to vertical migration on a daily cycle (Vila et al., 2001). Mangialajo and co-workers (2011) hypothesised that there may be a link between vertical migration and a "potential critical density threshold" above which the epiphytic cells would move up and down in the water column, thus turning into a planktonic state.

The *Ostreopsis* cell abundances recorded in the four sites that were sampled in 2009 varied greatly with the highest values being found in Villefranche – Plage des jeunes (site V2) with more than 330,000 cells/g macroalgae FW, followed by Nice (ca. 120,000 cells/g FW). The two remaining sites had much lower cell abundances with ca. 53,000 and 39,000 cells/g FW for Rochambeau (V3) and Villefranche (V1), respectively. In all sites except for Rochambeau, the maximum cell abundances were recorded in week 31; in site V3 the highest abundance was recorded in week 32. However, data collected as part of another research programme, MediOs (Méditerranée *Ostreopsis*), showed that the *Ostreopsis* abundances in Rochambeau ranged from ca. 193,000 to ca. 794,000 cells/g FW between week 29 and week 31 in 2009, with the highest counts being recorded in week 30 (data not shown).

Despite general characteristics of the sites (e.g. rocky type, conformation of the coast, macroalgal communities, wave exposure) that can be considered as comparable, some sites seem to be more prone to *Ostreopsis* development than others (Mangialajo et al., 2011). The influence of environmental factors on *Ostreopsis* growth in comparable sites (e.g. shallow waters and rocky areas) is not clear and is a subject of controversy. It makes it difficult to select a monitoring point that would be representative of the cell abundances in a larger area.

As the epiphytic *Ostreopsis* cells are more conservative and represent the stock of available biomass, Mangialajo and co-workers (2011) recommend to policy makers and managers to primarily monitor benthic abundances and to increase sampling frequency during high season (July in NW Mediterranean). According to the data collected as part of our study, the high season would spread from mid-July to mid-August, as the highest cell abundances occurred during this time frame.

Because PITX and its analogues are not regulated yet, either within the EU or internationally, the determination of *Ostreopsis* cell abundances sometimes relies on research projects that are limited in time when it should be included in perennial monitoring programmes. The latter should specifically be dealing with the risk associated with the direct exposure to *Ostreopsis*, via the water (recreational activities) and/or with the consumption of contaminated food organisms.

In France, shellfish production areas located on the coastlines are monitored as part of the Rephy network of Ifremer, an official network mandated by the Ministry of agriculture aiming at monitoring the phytoplankton and the phycotoxins. In the Mediterranean areas at risk regarding *Ostreopsis*, a preventive system has been put in place, with a threshold of 4000 *Ostreopsis* planktonic cells per litre triggering the flesh testing in shellfish present in the area or close by (Ifremer, 2011), even though there is no shellfish production areas in the sites known to be affected by blooms of *Ostreopsis*. The professional or leisure harvesting of sea urchins on the Mediterranean coast is forbidden by local regulation, every year generally from the 1st of May to the 31st of October; therefore there is no monitoring of this species for the presence of PITX during this timeframe. However, during the winter the monitoring is carried out by Ifremer (French Research Institute for Exploitation of the Sea) on a few selected sites, a month before and during the harvesting season (generally from the 1st of November to the 31st of March). The phytoplankton is not used as a trigger but the analysis of sea urchins is directly performed by LC–MS/MS, on a monthly basis; this frequency can be increased to weekly when the contamination levels in PITX and analogues are above the LC–MS/MS LOQ (ca. 25 μg/kg), and will be brought back to monthly as soon as the toxin levels drop below the LOQ (Ifremer, 2011). Although there is no PITX regulation in France, the competent authorities have already foreseen risks management measures to protect human health. The recommendations to be applied between the 15th of June and the 15th of September associated with the consumption of marine organisms coming from leisure activities and from locations where *Ostreopsis* is present are the following: (i) the fish should be eviscerated prior to consumption, (ii) there is a reminder about the period during which the harvesting of sea urchins is forbidden, (iii) all the other marine organisms should not be eaten (DGS, 2010).

Putting in place an efficient monitoring system is not an easy task as many parameters cannot be controlled. For example the toxicity of *Ostreopsis* has to be taken into account, as the sites having the highest abundances may not relate to the most contaminated marine products. This was observed as part of the present study, where the analysis of the water and macroalgae samples collected in the four sites revealed that the site V2 (Villefranche – Plage des jeunes) had cell abundances ca. six folds higher than those in V3 (Rochambeau), and yet the animal species from the latter site had the highest contamination levels.

### 4.2. PITX levels in the marine products

In the present study, herbivorous, omnivorous and carnivorous species were sampled in the four selected sites. There seems to be a relation between the contamination levels of the different animals and their feeding behaviour, as the highest levels were generally found in the herbivorous or omnivorous species. These species, that is the limpet *Patella* spp., the sea urchin *Paracentrotus lividus*, and the fish *Sarpa salpa* and *Mugil cephalus*, were found to be contaminated as determined by the haemolytic test. This can be explained by the grazing behaviour of the animals that feed directly from substrates covered with epiphytic *Ostreopsis* cells. The case of the seabream *S. salpa* is interesting as this fish adapts its feeding behaviour; juveniles (<5 cm) can feed on planktonic aggregates of *Ostreopsis* cf. *ovata* in the water column while the sub-adults and adults have an herbivorous diet and graze on macroalgae colonised by *Ostreopsis*. Whatever its life stage, in the event of *Ostreopsis* blooms, this fish is likely to be contaminated and would be a good sentinel species.

A wider range of carnivorous marine animals (11 different species) was sampled and not all of them were contaminated. While some species such as the mollusc *Stramonita haemastoma* and the crustacean *Eriphia verrucosa* had the highest toxin levels, sometimes close to those of the herbivorous species, other predators especially fish species were never found to be contaminated: the Mediterranean moray *Muraena helena*, the East Atlantic peacock *Symphodus tinca*, the red mullet *Mullus surmuletus* and the painted comber *Serranus scriba*. This could be explained by the fact that the molluscs and the crustacean mentioned above feed on prays that lay on the bottom and are therefore indirectly exposed to the high abundances of *Ostreopsis* cells whereas the fish feed on prays present in the water column with lower cell counts. Wachi and Hokama (2001) reported that both herbivorous (*Acanthurus olivaceus*, *A. scandvicensis*, *Zanclus cornutus*) and carnivorous Hawaiian reef fish (*Myripristis brendti*, *Khulia scandvicensis*) from Barber's Point, in Oahu, were contaminated with PITX.

Fifteen marine species were sampled as part of the study reported herein, but PITX and analogues have been found in a much wider variety of animals including shellfish (*Mytilus galloprovencialis* (Aligizaki et al., 2008; Bellocci et al., 2008a, 2008b; Espina et al., 2009); *Venus verrucosa* and *Modiolus barbatus* (Aligizaki et al., 2008); *Arca imbricate*, *Artina* sp., *Barbatia candida* and *Chama macerophylla* (Gleibs and Mebs, 1999)), crustaceans (*Demania alcalai* and *Lophozozimus pictor* (Alcala et al., 1988; Yasumoto et al., 1986); *Demania reynaudii* (Alcala et al., 1988); *Platypodiella spectabilis* (Gleibs and Mebs, 1999)), fish (*Melichtys vidua* (Fukui et al., 1987); *Decapterus macrosoma* (Kodama et al., 1989); *Scarus ovifrons* (Taniyama et al., 2003); *Chaetodon* spp. (Gleibs and Mebs, 1999); *Herklotsichthys quadrimaculatus* (Onuma et al., 1999)), cephalopods (octopus (A. Milandri personal communication; cited in Aligizaki et al. (2011)), echinoderms (*Paracentrotus lividus* (Amzil et al., 2009; Bellocci et al., 2008a, 2008b); *Acanthaster planci* (Gleibs & Mebs, 1999)), sponges (*Amphimedon*, *Crella*, *Dactylospongia* spp., *Liosina paradoxa*; (Gleibs and Mebs, 1999)), gorgonians (*Briareum* sp. (Gleibs and Mebs, 1999)) and soft corals (*Sarcophyton*, *Sinularia* spp. (Gleibs and Mebs, 1999)).

In our study, the toxin distribution between the different tissues of the organisms was determined for the echinoderms, fish, crustaceans and cephalopods. These marine organisms were dissected in DT and RT or roe for the sea urchins and each tissue was analysed separately. The toxin contribution in the contaminated samples solely came from the DT while the RT was not contaminated whatever the species considered. There is only one case in which the roe of the sea urchin *Paracentrotus lividus* collected in Villefranche (site V2) showed quantifiable levels of PITX as determined by the haemolytic test. The fact that the DT of the sea urchin sample had a high contamination level might have resulted in a cross contamination of the roe while dissecting the different parts. This hypothesis is strengthened by the fact that the sea urchin harvested in week 32 in Rochambeau (site V3) was even more contaminated (ca. 250 μg PITX eq./kg) and yet the roe had a

contamination level below the LOQ of the haemolytic test. This shows how easy it is to cross-contaminate the roe during the dissection process and addresses the question of the risk associated with this procedure when performed by the consumers themselves and not by professionals. This point can be extended to all the marine organisms for which a dissection process would be involved.

During the course of the study, the toxin concentration in the RT was higher than in the DT in only one occasion; this was for the cephalopod *Octopus vulgaris* harvested in site V3 in week 34. An explanation to this phenomenon might be that after being sequestered in the DT, the toxin was absorbed and incorporated to the RT. Yet, the octopus harvested in site V2 in week 35 did not show the same trend with the DT being the most contaminated part. Another hypothesis might be that there were *Ostreopsis* cf. *ovata* cells on the tentacles of the common octopus when it was sampled and subsequently analysed, explaining why the toxin level in the RT (including the tentacles) was higher than in the DT.

Some papers reported the presence of PITX and its analogues in carnivorous and herbivorous fish (Gleibs and Mebs, 1999; Wachi and Hokama, 2001). These studies had a closer look at the toxin distribution and reported that toxins were sequestered in different tissues including the skin, gills, eggs, muscle, viscera, and intestine, the latter part generally being the most contaminated. In these studies, the toxins are not sequestered in the DT, contrarily to what we observed, but are also found in several other tissues, which is the sign that an absorption process took place leading to the transfer of the toxins. Several hypotheses can be made to explain the differences: the origin of fish, from tropical to temperate areas, could lead to differences in the modalities of contamination; the duration of the blooms or the abundances of *Ostreopsis* could also be involved. It would be interesting to have a closer look at the absorption mechanisms to determine the level of absorption and establish whether the recurring exposure to high abundances of *Ostreopsis* blooms would lead to different sequestration patterns with the toxins being absorbed in the DT and transferred to the other organs.

According to Aligizaki and co-workers (2011) one must differentiate the marine organisms that solely carry the toxin in their DT (possibly in the form of the toxigenic macroalgae) that is not further absorbed, from the species that are capable of absorbing and incorporating the toxin in their tissues. In our study, the hypothesis is mostly in favour of a passive carry of toxins in the DT, for the *Ostreopsis* abundances reported, and the absorption seems to be absent or low.

Although no regulation has been put in place yet for PITX and its analogues, either at the European level or internationally, a significant step has been made in this direction with the EFSA opinion reporting that the toxin level in the shellfish should not exceed 30 μg for the sum of PITX and OST-D per kg (EFSA, 2009). This scientific opinion is based on the data available at the time and notably the toxicological study from Ito and Yasumoto (2009) on PITX and OST-D. In all the sites sampled as part of the present study the toxin levels found in the DT of three species (*Sarpa salpa*, *Paracentrotus lividus*, *Mugil cephalus*) were above 30 μg PITX eq./kg. When reported to the whole flesh, the contamination levels dropped but were still above 30 μg PITX eq./kg except in Villefranche (site V1). Because the ESFA opinion has been established for PITX and OST-D and is based on shellfish, for which the portion size of the consumption might be very different from that of other marine organisms such as echinoderms, gastropods and fish, one cannot strictly drive any conclusion from the sanitary status of the marine products tested in this study. Indeed, in the process of risk assessment, the safety threshold determined for a specific contaminant is calculated using the acute reference dose (ARfD), which varies according to the contaminants considered in the toxicological studies, and using the portion size. If we make the assumption that the sanitary threshold for PITX and OVTX-a, the toxins found in the present study, determined for marine organisms is similar to the threshold recommended by EFSA, the consumption of the three species mentioned above would endanger human health and the preventative measures consisting in eviscerating the fish and not consuming other marine organisms as recommended by the French General Directorate for Health (DGS, 2010) would protect the consumers. However, some meals require the use of the entire fish, as the DT confers a specific taste; similarly, the DT of sea urchins can be eaten along with the roe as some consumers dip pieces of bread in the watery intestinal mixture as a delicacy. In some other cases, it is a matter of consumption habits dictated by practical reasons for instance with the animals being too small to be transformed (e.g. evisceration).

The mollusc *Stramonita haemastoma* and the crustacean *Eriphia verrucosa* collected in Nice were analysed as whole flesh and were also found to have toxin levels above the recommended threshold. In this case, no evisceration process can be proposed as we did not collect information about toxin distribution in the different parts and also because evisceration by consumers is not realistic, furthermore, these species are eaten as WF.

When referring to the EFSA opinion to know whether the marine organisms might be at risk, it is important to recall the limits of this opinion. First of all, the experts that were consulted based their work on the limited data available at the time, as only three countries (Greece, France and Italy) answered to the call for data of occurrence. Secondly, the data were obtained from shellfish sampled in contaminated areas and not intended for human consumption; therefore these data do not represent levels of PITX and analogues in shellfish that currently could reach the market. Thirdly, this opinion is about PITX and its analogue OST-D, but the toxin profiles can be markedly different. Indeed, the OVTX analogues constitute up to 90% of the toxin profile of the marine organisms analysed in our study and they represent up to 99% in *Ostreopsis ovata* cells in Italy (2011b; Ciminiello et al., 2010b, 2011c). Additional information is required regarding the toxic potential of PITX analogues; a further step has been done by Tubaro and co-workers (2011a) as they evaluated the oral toxicity in mice of 42-hydroxy PITX isolated from the cnidarians zoanthid *Palythoa tuberculosa*. Fourthly, the portion size taken into account for shellfish is 400 g of meat. For other marine organisms such as fish, crustaceans, sea urchins and cephalopods, the consumption portion size might be significantly different.

### 4.3. Comparison of the haemolytic test and LC–MS/MS results

Although the haemolytic test and the LC–MS/MS methods do not rely on the same detection principles, the quantitative results found using both methods are in agreement.

Ciminiello and co-workers (2010a,b, 2011b) reported the discovery of OVTX-a analogues called OVTX-b, -c, -d and -e, which account for ca. 45% of the *Ostreopsis ovata* toxin profile. As these new analogues were not searched for in the present study and yet the haemolytic test and LC–MS/MS results are in agreement, this could suggest that (i) these analogues were not present in the marine organisms; although the OVTX-a analogues have been detected in Italy in the *Ostreopsis* cells analysed by Ciminiello and co-workers, the French strain of *Ostreopsis* cf. *ovata*, responsible for the contamination of the marine organisms is likely to have a different toxin composition.; (ii) the analogues were present but they have an haemolytic potential markedly lower than that of PITX and OVTX-a, explaining why the results of the haemolytic test were not higher than those of LC–MS/MS. (iii) they were initially present in the dinoflagellate but were metabolised by the marine organisms and were transformed into other compounds with no or

low haemolytic activity. Beyond the considerations mentioned above, the haemolytic test and LC–MS/MS are good candidates as the methods to be implemented in a monitoring programme for PlTX and its analogues. The haemolytic test is less expensive and more sensitive but takes more time, thus leading to a longer sample turn-around, especially when a confirmatory step using ouabain has to be performed. Ciminiello and co-workers (2011c) reported a LOQ of 168 μg/kg for their LC–MS/MS method but the sensitivity of the method can be improved by including a cleanup step such as solid phase extraction (SPE). This would have the advantage of reducing if not eliminating the matrix effect and it would enable the addition of a concentration step increasing the sensitivity of the method. The LC–MS/MS method used in our study included an SPE cleanup resulting in a lower LOQ of 24.5 μg/kg meat, and yet the sensitivity of this method would be border line if the EFSA recommended threshold of 30 μg PlTX + OST-D/kg meat was adopted for shellfish.

## 5. Conclusions

This study aimed to complete the database of marine products contamination in France (Amzil et al., 2012). We made such an inventory of the contamination levels of *Ostreopsis* toxins found during the summer 2009 in different species covering a wide variety of edible marine organisms, sampled in four different sites located on the French Mediterranean coast.

The toxin levels determined by the haemolytic test in the marine organisms followed the same trend as the *Ostreopsis* cell abundances in the sampled sites. The toxin profile of the marine organisms determined by LC–MS/MS showed the presence of OVTX-a as the major compound (90% of the profile) and PlTX.

The results presented herein showed that herbivorous species are more likely to be contaminated and at higher levels, although some omnivorous and carnivorous species were also found to be contaminated. Independently of the feeding behaviour, the toxins were sequestered in the DT suggesting only a passive carry of the toxins and a low absorption rate, in the conditions of our study.

A few samples were found to have toxin concentrations in the whole flesh above 30 μg PlTX eq./kg, which echoes to the EFSA opinion reporting that the toxin level in shellfish should not exceed 30 μg PlTX + OST-D/kg to protect the consumer.

The process of risk assessment is entirely dependent on the availability of data from different nature and origin. Thus, it is necessary to produce toxin occurrence data and particularly to confirm that the evisceration of fish eliminates the contamination even for longer blooms and/or higher abundances, and for other species not collected in our study. For organisms that are entirely eaten, more occurrence data are needed.

Furthermore, the EFSA opinion is based on a 400 g portion size of shellfish meat, and the question that must be addressed is the determination of the portion size of the species other than shellfish.

The availability of toxicological data is also crucial as for now there is a lack of information on this matter.

The collection of additional data would enable in the end the regulation of PlTX and its analogues in marine products destined to human consumption and thereby protecting the consumers.

## Conflicts of interest

The authors declare that there is no conflict of interest.

## Acknowledgements

This work was funded by the General Directorate for Health (French Ministry of Labour, Employment and Health) as part of the programme "Changement climatique et santé humaine" and was also supported by the French national programme MediOs 2 (LITEAU-Ministère de l'Ecologie, Conseil Général des Alpes-Maritimes, Agence de l'Eau Rhône Méditerranée Corse).

The authors wish to thank Sylviane Dragacci for her help and her valuable input while writing this article.[SS]

## References

Alcala, A., Alcala, L., Garth, J., Yasumura, D., Yasumoto, T., 1988. Human fatality due to ingestion of the crab *Demania reynaudii* that contained a palytoxin-like toxin. Toxicon 26 (1) 105–107.

Aligizaki, K., Katikou, P., Milandri, A., Diogène, J., 2011. Occurrence of palytoxin-group toxins in seafood and future strategies to complement the present state of the art. Toxicon 57 (3) 390–399.

Aligizaki, K., Katikou, P., Nikolaidis, G., Panou, A., 2008. First episode of shellfish contamination by palytoxin-like compounds from Ostreopsis species (Aegean Sea, Greece). Toxicon 51 (3) 418–427.

Aligizaki, K., Nikolaidis, G., 2006. The presence of the potentially toxic genera Ostreopsis and Coolia (Dinophyceae) in the North Aegean Sea, Greece. Harmful Algae 5 (6) 717–730.

Amzil, Z., Sibat, M., Chomerat, N., Grossel, H., Marco-Miralles, F., Lemee, R., Nezan, E., Sechet, V., 2012. Ovatoxin-a and Palytoxin Accumulation in Seafood in Relation to Ostreopsis cf. ovata Blooms on the French Mediterranean Coast. Marine Drugs 10 (2) 477–496.

Amzil, Z., Sibat, M., Séchet, V., Lemée, R., Kantin, R., Grossel, H., 2009. First report on palytoxin-like detection in seafood in relation with *Ostreopsis* spp. bloom on French Mediterranean coasts.In: 7th International Conference on Molluscan Shellfish Safety, Nantes.

Bellocci, M., Ronzitti, G., Milandri, A., Melchiorre, N., Grillo, C., Poletti, R., Yasumoto, T., Rossini, G.P., 2008a. Addendum to A cytolytic assay for the measurement of palytoxin based on a cultured monolayer cell line [Anal. Biochem. 374 (2008) 48–55] Analytical Biochemistry 381 (1) 178.

Bellocci, M., Ronzitti, G., Milandri, A., Melchiorre, N., Grillo, C., Poletti, R., Yasumoto, T., Rossini, G.P., 2008b. A cytolytic assay for the measurement of palytoxin based on a cultured monolayer cell line. Analytical Biochemistry 374 (1) 48–55.

Bignami, G., 1993. A rapid and sensitive hemolysis neutralization assay for palytoxin. Toxicon 31 (6) 817–820.

Ciminiello, P., Dell'Aversano, C., Dello Iacovo, E., Fattorusso, E., Forino, M., Tartaglione, L., Rossi, R., Soprano, V., Capozzo, D., Serpe, L., 2011a. Palytoxin in seafood by liquid chromatography tandem mass spectrometry: Investigation of extraction efficiency and matrix effect. Analytical and Bioanalytical Chemistry 401 (3) 1043–1050.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., 2010a. Palytoxins: a still haunting Hawaiian curse. Phytochemistry Reviews 9 (4) 491–500.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., 2011b. A 4-decade-long (and still ongoing) hunt for palytoxins chemical architecture. Toxicon 57 (3) 362–367.

Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Grillo, C., Melchiorre, N., 2006. The Genoa 2005 outbreak. Determination of putative palytoxin in Mediterranean Ostreopsis ovata by a new liquid chromatography tandem mass spectrometry method. Anal Chem 78 (17) 6153–6159.

Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E., Forino, M., Grauso, L., Tartaglione, L., Guerrini, F., Pistocchi, R., 2010b. Complex palytoxin-like profile of Ostreopsis ovata. identification of four new ovatoxins by high-resolution liquid chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry 24 (18) 2735–2744.

Ciminiello, P., Dell'Aversano, C., Iacovo, E.D., Fattorusso, E., Forino, M., Tartaglione, L., 2011c. LC–MS of palytoxin and its analogues: State of the art and future perspectives. Toxicon 57 (3) 376–389.

Deeds, J.R., Schwartz, M.D., 2010. Human risk associated with palytoxin exposure. Toxicon 56 (2) 150–162.

DGS, 2010. Note de service DGS/EA3/EA4 no 2010-238 du 30 juin 2010 relative à la surveillance sanitaire et environnementale et aux modalités de gestion des risques sanitaires pour la saison balnéaire 2010. liés à la présence de la microalgue toxique Ostreopsis spp. dans les eaux de baignade en méditerranée et à la contamination par ses toxiques des produits de la mer issus de la pêche de loisir. Bulletin Officiel Santé – Protection sociale – Solidarité (2010/8) 261–298.

EFSA, 2009. Scientific opinion on marine biotoxins in shellfish – palytoxin group. EFSA Journal 7 (12) 1393–1430.

Espina, B., Cagide, E., Louzao, M.C., Fernandez, M.M., Vieytes, M.R., Katikou, P., Villar, A., Jaen, D., Maman, L., Botana, L.M., 2009. Specific and dynamic detection of palytoxins by in vitro microplate assay with human neuroblastoma cells. Bioscience Reports 29 (1) 13–23.

Fukui, M., Murata, M., Inoue, A., Gawel, M., Yasumoto, T., 1987. Occurrence of palytoxin in the trigger fish *Melichtys vidua*. Toxicon 25 (10) 1121–1124.

Gleibs, S., Mebs, D., 1999. Distribution and sequestration of palytoxin in coral reef animals. Toxicon 37 (11) 1521–1527.

Habermann, E., Ahnert-Hilger, G., Chhatwal, G.S., Beress, L., 1981. Delayed haemolytic action of palytoxin general characteristics. Biochimica et Biophysica Acta (BBA) – Biomembranes 649 (2) 481–486.

Ifremer, 2011. Cahier de procédures et de programmation REPHY 2011. Ifremer, Nantes.

Ito, E., Yasumoto, T., 2009. Toxicological studies on palytoxin and ostreocin-D administered to mice by three different routes. Toxicon 54 (3) 244–251.
Kodama, A., Hokama, Y., Yasumoto, T., Fukui, M., M. S., N. S., 1989. Clinical and laboratory findings implicating palytoxin as cause of ciguatera poisoning due to *Decapterus macrosoma* (mackerel). Toxicon 27 (9) 1051–1053.
Mangialajo, L., Bertolotto, R., Cattaneo-Vietti, R., Chiantore, M., Grillo, C., Lemee, R., Melchiorre, N., Moretto, P., Povero, P., Ruggieri, N., 2008. The toxic benthic dinoflagellate Ostreopsis ovata: quantification of proliferation along the coastline of Genoa, Italy. Marine Pollution Bulletin 56 (6) 1209–1214.
Mangialajo, L., Ganzin, N., Accoroni, S., Asnaghi, V., Blanfuné, A., Cabrini, M., Cattaneo-Vietti, R., Chavanon, F., Chiantore, M., Cohu, S., Costa, E., Fornasaro, D., Grossel, H., Marco-Miralles, F., Masó, M., René, A., Rossi, A.M., Sala, M.M., Thibaut, T., Totti, C., Vila, M., Lemée, R., 2011. Trends in Ostreopsis proliferation along the Northern Mediterranean coasts. Toxicon 57 (3) 408–420.
Moore, R.E., Scheuer, P.J., 1971. Palytoxin: a new marine toxin from a coelenterate. Science 172, 495–498.
Munday, R., 2008. Occurrence and toxicology of palytoxins. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins – Pharmacology, Physiology, and Detection. second ed. CRC Press, Boca Raton, pp. 693–713.
Onuma, Y., Satake, M., Ukena, T., Roux, J., Chanteau, S., Rasolofonirina, N., Ratsimaloto, M., Naoki, H., Yasumoto, T., 1999. Identification of putative palytoxin as the cause of clupeotoxism. Toxicon 37 (1) 55–65.
Riobó, P., Paz, B., Franco, J.M., Vázquez, J.A., Murado, M.A., 2008. Proposal for a simple and sensitive haemolytic assay for palytoxin: toxicological dynamics, kinetics, ouabain inhibition and thermal stability. Harmful Algae 7 (4) 415–429.
Taniyama, S., Arakawa, O., Terada, M., Nishio, S., Takatani, T., Mahmud, Y., Noguchi, T., 2003. Ostreopsis sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus ovifrons*. Toxicon 42 (1) 29–33.
Taniyama, S., Mahmud, Y., Terada, M., Takatani, T., Arakawa, O., Noguchi, T., 2002. Occurrence of a food poisoning incident by palytoxin from a serranid Epinephelus sp. in Japan. Journal of Natural Toxins 11 (4) 277–282.
Tichadou, L., Glaizal, M., Armengaud, A., Grossel, H., Lemée, R., Kantin, R., Lasalle, J.L., Drouet, G., Rambaud, L., Malfait, P., De Haro, L., 2010. Health impact of unicellular algae of the Ostreopsis genus blooms in the Mediterranean Sea: Experience of the French Mediterranean coast surveillance network from 2006 to 2009. Clinical Toxicology 48 (8) 839–844.
Totti, C., Accoroni, S., Cerino, F., Cucchiari, E., Romagnoli, T., 2010. Ostreopsis ovata bloom along the Conero Riviera (northern Adriatic Sea): relationships with environmental conditions and substrata. Harmful Algae 9 (2) 233–239.
Tubaro, A., Del Favero, G., Beltramo, D., Ardizzone, M., Forino, M., De Bortoli, M., Pelin, M., Poli, M., Bignami, G., Ciminiello, P., Sosa, S., 2011a. Acute oral toxicity in mice of a new palytoxin analogue: 42-hydroxy-palytoxin. Toxicon 57 (5) 755–763.
Tubaro, A., Durando, P., Del Favero, G., Ansaldi, F., Icardi, G., Deeds, J.R., Sosa, S., 2011b. Case definitions for human poisonings postulated to palytoxins exposure. Toxicon 57 (3) 478–495.
Utermöhl, H., 1958. Zur Vervoll Kommung der Quantitativen Phytoplankton-Methodik. Mitteilungen Internationale Vereinigung fur theoretische und angewandte. Limnologie 9, 1–38.
Vila, M., Garcés, E., Masó, M., 2001. Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. Aquatic Microbial Ecology 26, 51–60.
Wachi, K.M., Hokama, Y., 2001. Diversity of marine biotoxins in the near-shore ocean area: presence of a palytoxin-like entity at Barber's Point Harbor, Oahu. Journal of Natural Toxins 10 (4) 317–333.
Yasumoto, T., Yasumura, D., Ohizumi, Y., 1986. Palytoxin in two species of xanthid crab from the Philippines. Agricultural and Biological Chemistry 50 (1) 163–167.