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**ASSESSMENT OF CRYPTIC SPECIES DIVERSITY
WITHIN BLOOMS AND CYST BANK OF THE
ALEXANDRIUM TAMARENSE COMPLEX
(DINOPHYCEAE) IN A MEDITERRANEAN
LAGOON FACILITATED BY SEMI-MULTIPLEX PCR**

Benjamin Genovesi, Mi-Sun Shin-Grzebyk, Daniel Grzebyk, Mohamed Laabir,
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**ASSESSMENT OF CRYPTIC SPECIES DIVERSITY WITHIN
BLOOMS AND CYST BANK OF THE ALEXANDRIUM
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MEDITERRANEAN LAGOON FACILITATED BY SEMI-
MULTIPLEX PCR**

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3 Assessment of cryptic species diversity within blooms and cyst bank of the *Alexandrium*
4 *tamarense* complex (Dinophyceae) in a Mediterranean lagoon facilitated by semi-multiplex
5 PCR
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37 **Running title:** Co-occurrence of *Alexandrium* spp. blooms
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40 **Keywords:** *Alexandrium catenella*, *Alexandrium tamarense*, PSP, ITS, Thau lagoon.
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ABSTRACT

The occurrence of *Alexandrium catenella* related to paralytic shellfish poisoning in the French Mediterranean Thau lagoon has been known since 1998. Blooms are recurrent and usually occur each year in spring and/or autumn. Taxonomic diversity of resting cysts and vegetative cells has been studied through morphological examination and molecular typing of 558 clonal strains sampled in 2004 and 2007. Sequencing the nuclear rRNA fragment including ITS1, the 5.8S rRNA gene, ITS2, and the D1/D2 28S rRNA gene enabled two species to be determined, *A. catenella* and *A. tamarense*, which are difficult to distinguish morphologically (cryptic species). In order to carry out extensive and accurate molecular determinations, an original semi-multiplex PCR method, using new ribotype-specific primers targeting the 18S-28S rRNA ITS region has been developed. The relative abundance of each species was then established in seawater in 2007 and in the sediment collected in 2004. The co-occurrence of *A. catenella* (Group IV), which is known as the main species responsible for toxic PSP events since 1998 and of *A. tamarense* (Group III) (non-toxic), which was not formally recognized by microscopic observation since 1995, was examined for several months.

INTRODUCTION

Since the 1970s, Harmful Algal Blooms (HABs) have been recognised as one of the main ecological problems in coastal regions worldwide (Maso and Garces 2006). Among HAB species, some Dinophyceae are toxic and cause neurological and human gastric disorders after consumption of contaminated filter-feeding shellfish (Zingone and Enevoldsen 2000; Smayda and Reynolds 2003; Glibert *et al.* 2005).

In the Mediterranean Sea many species involved in such outbreaks are listed as exotic or non-indigenous phytoplankton (Streftaris *et al.* 2005, Gomez 2008, Molmar *et al.* 2008) and tagged by the International Union for the Conservation of Nature (IUCN). The Mediterranean sea harbours the highest number of reported invasive toxic and non-toxic *Alexandrium* species (Fraga *et al.* 2004), with the exception of the autochthonous Mediterranean *Alexandrium tamarense* described by John *et al.* (2003a) belonging to the group II (Lilly *et al.* 2007). These species spread in the Western Mediterranean region along French, Spanish, Italian and Maghrebian coasts (Penna *et al.* 2005, 2008, Frehi *et al.* 2007, Turki and Balti 2008). Among them many taxa are linked to the paralytic shellfish-poisoning (PSP) syndrome.

Based on the LSU rRNA gene sequence analysis, Scholin *et al.* (1994) delineated eight distinct phylogeographic clusters or “ribotypes” within the genus *Alexandrium*. Among them, the *Alexandrium tamarense* complex consists of three morphospecies *Alexandrium catenella* (Whedon & Kofoid) Balech, *A. tamarense* (Lebour) Balech and *A. fundyense* (Balech). This species complex is distributed into five geographic ribotypes (Scholin *et al.* 1994). These species have been observed worldwide since the 1970's and include both non-toxic and toxic strains (Cordova *et al.* 2003, Ichimi *et al.* 2001, Haya *et al.* 2003). Representatives of the *Alexandrium tamarense* species complex are often difficult to identify, especially for taxa such as *A. catenella*, *A. tamarense* and *A. fundyense*, which can be considered as cryptic species. Indeed, the main classical features such as chain-forming ability, cell shape, and presence/absence of a ventral pore between plates 1' and 4' (Balech 1995) have been demonstrated to be somewhat inconsistent (Lilly *et al.* 2007, Penna *et al.* 2008, Wang *et al.* 2008).

Regarding the Thau lagoon (France), which is one of the largest Western Mediterranean lagoons, the occurrence of *Alexandrium* species involved in significant algal blooms and PSP has been known since 1995 and 1998, respectively. The first attempts to accurately discriminate species involved in PSP in the Thau lagoon were inconsistent. Thecal morphology and toxin content analyses of fixed cells from seawater sampled in autumn 1998

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3 first suggested that both *A. tamarense* and *A. catenella* were present (Masselin *et al.* 2001).
4
5 Lilly *et al.* (2002) analyzed two clonal strains (ATTL01 and ATTL02) established from
6
7 seawater samples also collected during the autumn of 1998. Strain DNA sequences of the D1–
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9 D2 domains of LSU rDNA revealed the Japanese Temperate Asia (TA) ribotype of the *A.*
10
11 *tamarense* complex, i.e. *tamarense/catenella/fundyense* species (Scholin and Anderson 1996).
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13 This evidence was morphologically supported by the thecal analyses of isolated strains
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15 (Laabir *et al.* 2004). Until now, rRNA gene sequencing of strains (88) isolated from 2001 to
16
17 2004 showed that *A. catenella* was the only taxon blooming in the Thau lagoon (Masseret *et*
18
19 *al.* 2009). However, vegetative cells characterized by unusual size and morphology were
20
21 periodically observed in weekly-collected samples. In addition, high concentrations of
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23 *Alexandrium* cells were frequently unrelated to toxin detection (unpublished data). Thus,
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25 these observations (1) questioned the evidence that the toxic *A. catenella* was the only taxon
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27 involved in the Thau lagoon blooms; and (2) prompted us to follow the evolution of densities
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29 of toxic and non-toxic *Alexandrium* species in order to adapt scientific surveys of toxins in
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31 shellfish.

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33 In the present survey, a large-scale sampling was carried out in the water column from May to
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35 October 2007, in order to assess the taxonomic diversity within and between *Alexandrium*
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37 populations periodically blooming in the Thau lagoon. In addition, germination of sexual
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39 cysts, followed by isolation of sibling strains, was performed from sediment sampled in 2004
40
41 and conserved at 4°C (Genovesi *et al.* 2007) in order to explore taxonomic diversity among
42
43 the *Alexandrium* sp. cyst bank. The main results show the co-occurrence of *A. tamarense* and
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45 *A. catenella* belonging respectively to Group III and Group IV. Consequently, a rapid and
46
47 selective method based on ribotyping was developed to discriminate both species through an
48
49 extensive strain determination. Subsequently, to avoid confusion we use the most recent
50
51 designation to discriminate the geographic ribotypes of both species, i.e Group III and Group
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53 IV (Lilly *et al.* 2007) instead of Temperate Asian and Western European clades (Scholin and
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55 Anderson 1996).
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53 METHOD

54 Field survey, sample collection and cultures

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56 The Thau lagoon is located on the French Mediterranean coast (43°24'N-3°36'E). It covers
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58 75 km², one-fifth of which is occupied by shellfish farming structures. This lagoon is
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60 connected to the Mediterranean Sea by three channels, one of which opens into the Sète
international harbour. Sampling was carried out in the Crique-de-l'Angle, which is a shallow

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3 semi-enclosed area at the north of the lagoon (Fig. 1). For the survey program of *Alexandrium*
4 sp. population dynamics, a sampling grid (100 m) including 27 geo-referenced stations was
5 applied in the Crique-de-l'Angle (Fig. 1). Since 1998, water samples were systematically
6 collected by using a pump system during periods favourable for the development of
7 *Alexandrium* sp. blooms (spring and autumn). Samples were at once fixed in formaldehyde
8 (2% final concentration) for the microscopic enumeration of vegetative cells according to the
9 Utermöhl method. Clonal cultures in enriched natural seawater were based on: (1) single
10 vegetative cells isolated from seawater during the bloom period in 2004, and from May to
11 November 2007; (2) sexual cyst germination isolated from sediment collected in 2004 after a
12 week of incubation at 20°C, under an irradiance of 100 $\mu\text{moles photons m}^{-2} \text{ s}^{-1}$ on a 12:12 h
13 Light/Dark cycle (Table I) (Genovesi *et al.* 2009).
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25 **Morphological analyses**

26 Morphological examination was performed on cells from 30 *A. tamarense* (Group III) and 22
27 *A. catenella* (Group IV) clonal strains established in 2004 and 2007. All the strains observed
28 were genotyped. For examination and for each culture, more than 30 vegetative cells were
29 selected at exponential phase and stained with Calcofluor white (20 $\mu\text{g m L}^{-1}$) according to
30 the method of Fritz and Triemer (1985). Observations were carried out using an epi-
31 fluorescence microscope (Olympus AX70) and photographs were taken with a CCD camera.
32 The morphometric analysis (length and width) of fixed vegetative cells (formalin 0.5% final
33 concentration v/v) was performed using a light microscope (Zeiss Axiovert 25) on at least 30
34 cells of each strain.
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44 **DNA extraction**

45 Genomic DNA was extracted from centrifuged culture strains using a modified guanidine
46 buffer method (Matz 2003). After centrifugation, cells were re-suspended in guanidine buffer
47 (4 M guanidine thiocyanate; 30 mM disodium citrate; 30 mM b-mercaptoethanol; pH 8.0-
48 8.5). In order to improve the extraction efficiency, cells were exposed to ultrasound for 20
49 seconds. Chloroform purification was then performed after an incubation step at 65°C lasting
50 10 min. After ethanol precipitation, the DNA was finally re-suspended in TE buffer (10 mM
51 Tris-HCl; pH8.0; 0.1 mM EDTA).
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61 **PCR amplification of the (ITS1-5.8SrDNA-ITS2) rRNA region**

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3 The ribotype of 308 *Alexandrium* spp. strains originating from the Thau lagoon (Table I) was
4 resolved by sequencing the rRNA fragment spanning the region including ITS1, the 5.8S
5 rRNA gene, ITS2, and the D1/D2 28S rRNA gene (totalising 1,246 bp). PCR amplifications
6 were performed with 5-10 ng of genomic DNA template using the primers 18S-ITS1-Ac-F 5'-
7 CTTAGAGGAAGGAGAAGTCG-3' and 28S-D3B-R 5'-TCGGAGGGAACCAGCTACTA-
8 3'. The PCR conditions consisted of 95°C for 2 min, 40 cycles of 95°C for 40 s, 55°C for 4
9 min, 72°C for 1.4 min and a final extension at 72°C for 5 min. The PCR products were
10 purified using ethanol precipitation and were used as templates for direct sequencing with the
11 forward PCR primers and an internal reverse sequencing primer. Sequencing was performed
12 by the Macrogen Company (Seoul, South Korea) using an ABI 3730XL genetic analyzer
13 (Applied Biosystems).
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25 **DNA sequence analyses**

26 Sequences were assembled using the software package BioEdit (Hall 1999). Nucleotide
27 sequence alignments were performed using ClustalX (Thompson *et al.* 1997) with reference
28 sequence data retrieved from Genbank. The rDNA regions from 308 strains were aligned with
29 51 sequences of various species of *Alexandrium* retrieved from GenBank. The sequences of
30 two isolates belonging to *A. catenella* (Group IV) and *A. tamarense* (Group III) have been
31 deposited under the accession numbers FR686536 and FR686537, respectively.
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39 **Development of an allele specific semi-multiplex PCR for species discrimination**

40 An allele specific semi-multiplex PCR (Gagnaire *et al.* 2007) using new ribotype-specific
41 primers targeting the 18S-28S rRNA ITS region was developed in this study. These new
42 primers were designed from the alignment of the sequences from the 308 strains studied.
43 Forward primers FACAT28 (5'-TGATATTGTGGGCAACTGTAA-3') and FATAM143 (5'-
44 TGGTAATTCTTCATTGATTACAATG-3') were used respectively for *A. catenella* (Group
45 IV) and *A. tamarense* (Group III) whereas a common reverse primer RACATAM269 (5'-
46 AACATCTGTTAGCTCACGGAA-3') was designed in a region conserved for both
47 ribotypes. Because 114 bp separate the sites of FACAT28 and FATAM143 in the 18S-28S
48 rRNA-ITS region, the two specific amplification products should differ in their size (126 bp
49 for *A. tamarense* and 240 bp for *A. catenella*), enabling species determination after agarose
50 gel migration. PCR amplifications were performed in 10 µL reaction mixtures containing 5-
51 10 ng of genomic DNA template, 2.0 mM of MgCl₂, 0.2 mM each deoxynucleoside
52 triphosphates, 1X buffer provided with the polymerase, 0.5 µM of each primer and 1U of *Taq*
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3 polymerase (AmpliTaq, Qiagen). Cycling conditions were as follows: 2 min at 95°C, 35
4 cycles of 40s at 95°C, 1 min and 20s at 54°C and 1 min at 72°C, and 5 min at 72°C.

5
6 In order to check the specificity of the newly designed primers, the PCR products obtained
7 from 35 *Alexandrium* spp. strains were sequenced by GenoScreen (Lille, France) using an
8 ABI 3730XL genetic analyzer (Applied Biosystems).
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11 The ratio between both species was determined for all collected samples, i.e. sediment
12 collected in 2004, seawater collected in autumn 2004, and all dates of sampling from the
13 bloom that occurred from May to October 2007. In total, 568 clonal strains were identified
14 using molecular methods: sequencing (308 strains) and semi-multiplex PCR (260 strains).
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19 20 21 RESULTS

22 23 Seasonality of *Alexandrium* blooms

24 Since 1995, *Alexandrium* blooms have occurred mainly during autumn, except for the years
25 2000, 2007 and 2008 that had significant spring blooms (Fig. 2). Throughout the past 13
26 survey years, autumnal blooms were not observed in 2002 and 2006. Maximal vegetative cell
27 densities reached 4.5×10^6 cells L⁻¹ and 16×10^6 cells L⁻¹ in October 2003 and November
28 2004, and resulted in water discolouration during these periods. Such cell concentrations were
29 usually related to blooms of which *Alexandrium* spp. may represent more than 80-90% of the
30 phytoplankton. Under suitable environmental conditions, i.e. temperature around 20-21°C
31 related to calm waters (Laania *et al.* 2008), *Alexandrium* spp. usually needs 10 to 20 days to
32 reach maximum cell density. However, a relatively low cell concentration (from 1000 to
33 50000 cells L⁻¹) could be observed during several weeks before the growth peak. During the
34 survey program in 2007, *Alexandrium* spp. cells were microscopically observed at 8 sampling
35 stations A00, A3, A5, A9, B3, B5, C3 and C5 from May 10 till November 8 (Fig. 1, Table 2).
36 Cell density varied widely within the sampling period and among sampling stations (Table 2).
37 The highest densities were observed on May 24 in C3, C5 and A3, i.e. nearly 2×10^6 cells L⁻¹.
38 In contrast, during the autumnal bloom, the highest densities were recorded on October 15 at
39 B3, A5 and A3 and were lower than 0.2×10^6 cells L⁻¹.
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54 55 Morphological examination

56 Vegetative cells of *A. tamarensense* strains were solitary or in pairs. In contrast, *A. catenella*
57 showed solitary cells but also chains of 2, 4 and 8 cells, mainly observed during the mid-
58 exponential phase. Concerning cell size, there is no significant difference (t-test, p>0.05)
59 between the two morphospecies: 22.20 µm (5.15 s.d.) (H) × 24.82 µm (6.33 s.d.) (W) for *A.*
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3 *tamarensis*; and 23.09 μm (3.78 s.d.) (H) \times 25.48 μm (4.84 s.d.) (W) for *A. catenella*. All
4
5 strains examined of both species exhibit the typical plate formula of the *A. tamarensis*
6
7 complex P0, 4', 6'', 6C, 8S, 5''', 2'''''. Among several morphological characteristics (Apical
8
9 Pore Complex, Posterior sulcal plate, etc.), the presence of a ventral pore (Balech 1995) on
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11 the first apical plate was considered, here, to be the most reliable character to separate *A.*
12
13 *tamarensis* (Group III) from *A. catenella* (Group IV). Our results show the constant presence
14
15 of a small ventral pore located halfway along the right margin of the first apical plate (1') of
16
17 the *A. tamarensis* vegetative cells.

18 19 **Development and test of the rapid molecular determination assay**

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21 The ribotype-specific semi-multiplex PCR method using the newly designed primers allowed
22
23 the resolution of specific amplicons according to their size: 126 bp for *A. tamarensis* (Group
24
25 III) vs. 240 bp for *A. catenella* (Group IV) (Fig. 3). The reliability of this method was tested
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27 on 96 strains whose ribotype was known by sequencing. The results showed that the accuracy
28
29 of the method reached 100%. The sequencing of amplicons obtained by semi-multiplex PCR
30
31 was performed on 34 *Alexandrium* spp. strains and confirmed the accurate specificity of these
32
33 new primers. 240 bp and 126 bp amplicons were obtained for 22 *A. catenella* and 12 *A.*
34
35 *tamarensis* strains respectively.

36 37 **Diversity of *Alexandrium* spp. clonal cultures**

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39 Analysis of the ITS1-5.8SrDNA-ITS2 rRNA region sequences from 308 monoclonal strains
40
41 revealed the presence of both species: *A. catenella* (Group IV) and *A. tamarensis* (Group III)
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43 according to Scholin *et al.* (1994) and Lily *et al.* (2007). Within each ribotype, the sequences
44
45 of all isolates were totally identical. 23 clonal cultures were obtained from resting cysts
46
47 isolated from sediment sampled in 2004. The results showed that 13 and 10 strains belonged
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49 to *A. catenella* and *A. tamarensis*, respectively. This demonstrated the co-occurrence of both
50
51 species in the sediment sampled in April 2004. On the other hand, the *A. tamarensis* ribotype
52
53 was not detected in 79 clonal cultures established from vegetative swimming cells during the
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55 bloom of autumn 2004. In contrast, both ribotypes were isolated from May to September
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57 2007. In October 2007, mainly isolated *A. catenella* cells were able to generate viable
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59 monoclonal cultures (Fig. 4).

60 **DISCUSSION**

Morphological characteristics and ribotype

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3 The main morphological characteristics such as chain-forming ability, cell shape and ventral
4 pore (Balech 1995) are neither discrete nor stable (Taylor 1975, Lilly *et al.* 2007).

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6 Consequently, the chain-forming ability or absence of the ventral pore are considered
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8 plesiomorphic characters and may not be suitable for use as taxonomic markers (John *et al.*
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10 2003a, Leaw *et al.* 2005). For the first time we performed extensive observations on a large
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12 number of strains targeting the most accurate morphological characteristic distinguishing the
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14 two morphospecies *A. tamarense* and *A. catenella*, i.e. the presence/absence of the ventral
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16 pore (Balech 1995) on the first apical plate. The samples examined consisted of ≥ 30 cells for
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18 31 and 28 strains of *A. tamarense* and *A. catenella*, respectively. As previously recorded in
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20 cell observations on natural samples from Thau, both morphospecies are characterized by
21
22 variations in cell size according to previous reports (Wang *et al.* 2008). For all strains
23
24 examined, we show a correspondence of *A. tamarense* and *A. catenella* ribotypes and
25
26 morphospecies designation (Scholin *et al.* 1994) based on the presence/absence of the ventral
27
28 pore on plate 1' and chain-formation, which is related to exponential growth phase in *A.*
29
30 *catenella* (Toulza *et al.* 2010). However, intraspecific variations have been observed
31
32 worldwide in cells with and without a ventral pore (Cembella and Taylor 1986, Orlova *et al.*
33
34 2007, Gayoso and Fulco 2006, Wang *et al.* 2008). Regarding these contradictory
35
36 observations, several characters (such as morphology, toxicity and genetics) seem to be
37
38 required to accurately discriminate specific levels into cryptic/morphospecies (this study,
39
40 Lilly *et al.* 2007).

41
42 The genetic analyses of *Alexandrium sp.* strains isolated in this study place them within
43
44 Group IV which contains *A. catenella* toxic isolates from Temperate Asia and within Group
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46 III including *A. tamarense* non-toxic isolates from Scottish, Irish and English waters (Scholin
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48 *et al.* 1994 and 1995, Higman *et al.* 2001, John *et al.* 2003b, Lilly *et al.* 2007, Collins *et al.*
49
50 2009).

51
52 The succession and the dominance of one and/or the other species could have a significant
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54 impact on the toxicity level measured, through survey programs, in the shellfish during
55
56 HABs. In Thau lagoon, red tides are not related to toxic events according to the Ifremer
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58 survey programme (unpublished data). The rapid and reliable semi-multiplex method
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60 described in this study should allow the detection of both toxic *A. catenella* (Group IV) and
non-toxic *A. tamarense* (Group III). Moreover, the determination of the ecological conditions
favouring each species is an important field of research to be developed in order to estimate
the risk of development of a toxic or non-toxic event.

Identification of cryptic species with the ribotype-specific-PCR method

In this study, the distinction between *A. tamarense* (Group III) and *A. catenella* (Group IV) could be made using morphological features, such as the presence or absence of a ventral pore on the apical plate. However, such morphological examinations are time-consuming and might be questionable (Cembella and Taylor 1986, Lilly *et al.* 2007, Wang *et al.* 2008). Thus, the sequencing of ITS or LSU seems to be the most accurate method to discriminate individuals at the species level within the *Alexandrium tamarense* species complex. However, sequencing is not adapted for routine species discrimination from established culture strains. The original semi-multiplex PCR method developed in this study promptly and accurately discriminates *A. tamarense* (Group III) and *A. catenella* (Group IV) in the laboratory cultures library. This rapid method which only demands PCR amplification followed by agarose gel electrophoresis is moreover more straightforward compared with previous methods, such as the RFLP method (Adachi *et al.* 1994, Lilly *et al.* 2002) or real-time PCR (Hosoi-Tanabe and Sako 2005).

Cultures reveal co-occurrence of two *Alexandrium* species in sediment and seawater

Sequencing and the new semi-multiplex ribotype-specific PCR revealed the co-occurrence of two ribotypes in the water column in 2007 and in the sediments of the Thau lagoon collected in 2004: *A. catenella* (Group IV), which is known as the main species responsible for toxic PSP events in the Thau lagoon since 1998, and *A. tamarense* (Group III), which was not formally recognized by microscopic observation since 1995.

The ribotyping of cyst-derived strains demonstrates the presence of both species in the sediment sampled in April 2004 and also the ability of the resting cysts to grow after 4 to 5 years of storage at 4°C in darkness with a culturing success of approximately 25%. However, the *A. tamarense* ribotype is not detected within 79 ribotyped vegetative cell strains established during the bloom of November 2004 (Masseret *et al.*, 2009, 2010), which can be due to a reduced proportion of this species in the lagoon, but probably also to the sampling and cultivation bias. On the other hand, in 2007, results reveal the presence of these two species within cultures derived from vegetative cells. A prevalence of *A. tamarense* is observed in the cultures grown from May to September while *A. catenella* is the main species isolated from seawater sampled in October 2007. The co-occurrence of *A. catenella* and *A. tamarense* in the same area has been previously reported in the Ofunato bay in Japan (Hosoi-Tanabe and Sako 2005).

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3 Co-occurrence of both cryptic species and their possible succession during blooms may
4 indicate behaviour linked to their life-history traits (e.g. growth and adaptive strategies) and
5 related to environmental conditions. However, our results depend on the isolation and
6 cultivation of microorganisms that are selective and lead to a bias in the description of the
7 diversity and genetic structure of the bloom. During the vegetative pelagic stage in November
8 2004, genotypes belonging solely to *A. catenella* have been isolated compared to the available
9 bank of genotypes present in the sediment as resting cysts (Fig 4). Environmental conditions
10 seemed to be not suitable for *A. tamarensis* cyst germination and vegetative reproduction.
11 Three main processes may also exert selective pressure in culturing phytoplankton: isolation,
12 establishment and maintenance (Lakeman *et al.* 2009). In our study, the isolation and
13 cultivation success were better in October than from May to September. Some of the isolated
14 cells were not taken into account because they did not grow after the beginning of their
15 isolation in enriched seawater and some of them died after a few divisions. This is also the
16 case for cyst-derived strains that exhibited a low culturing success of 25%. After one month,
17 abortive cultures became scarce and very few losses were recorded. It is evident that selective
18 bottlenecks may occur during these steps. A small proportion of tolerant individuals,
19 benefiting from phenotypic properties with capacity for survival in laboratory culture
20 conditions (in Enriched natural seawater, at 20°C, 100 $\mu\text{moles photons m}^{-2} \text{ s}^{-1}$, 12:12 h
21 Light/Dark cycle), grew successfully and for a long period of time. Moreover, an unforeseen
22 result occurred from ribotype analysis performed on clonal cultures of cells isolated from the
23 same sample. During the isolation period, three operators microscopically isolated single
24 swimming cells from field samples. Operator 1 isolated solely *A. catenella* ribotypes whereas
25 operator 2 isolated mainly *A. tamarensis* ribotypes.

46 **The key role of cyst banks in bloom outbreaks and maintenance**

47 The hypothesis of the former presence of both species in the Thau lagoon is now supported by
48 the significant occurrence of *A. tamarensis* resting cysts in the cyst bank of the sediment
49 sampled in April 2004, i.e. >40% of cyst-derived strains belonged to *A. tamarensis*. The cyst
50 densities recorded in the Crique-de-l'Angle (i.e. 200 cysts cm^{-3} in the C7 station (Fig. 1))
51 (Genovesi *et al.* 2008) underline the arrival and/or production of cysts by *A. tamarensis* before
52 2004. The vertical profiles of cyst distribution in sediments represent a useful proxy to
53 estimate the history of bloom development (Mudie *et al.* 2002). In Sequim Bay (Puget Sound,
54 USA), the occurrence of hundreds of *A. catenella* cysts down to a 40 cm sediment depth
55 demonstrates that the presence of the species goes back 30 years at least (Cox *et al.* 2008). In
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3 the sediment of the Thau lagoon, the cyst profiles showed that resting cysts of *Alexandrium*
4 sp. are mostly concentrated in the upper sediment layers (97% down to 12 cm depth),
5 particularly in the first 3 cm where 30% of the overall cysts were observed (Genovesi *et al.*
6 2007). In addition, the mean density of *Alexandrium* sp. cysts in the Thau lagoon (< 20 cysts
7 g⁻¹ dry sediment) is very low (Genovesi *et al.* 2009) compared to other sites where
8 *Alexandrium* sp. blooms (involved in PSP) are often associated with hundreds to thousands
9 cysts per gram of sediment (Yamaguchi *et al.* 1996, Hallegraeff *et al.* 1998, Gayoso 2001,
10 Anderson *et al.* 2005). Therefore, the vertical profiles and the low cyst densities recorded in
11 the Thau lagoon support the hypothesis of recent proliferation of species belonging to the *A.*
12 *tamarensis* species complex.
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22 CONCLUSION

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24 It is already well known that the current expansion of several *Alexandrium* species along
25 the NW Mediterranean coasts may be due to human-mediated transport. The occurrence of
26 toxic and non-toxic strains of *A. catenella* (Group IV) and *A. tamarensis* (Group III) in the
27 Thau lagoon complicates the HAB monitoring performed by Ifremer, in particular for risk
28 management in the shellfish industry. The newly developed semi-multiplex PCR
29 amplification enables the detection of these species. It will thus ease the characterisation of *in*
30 *situ* genetic diversity of *Alexandrium* blooms and promote the understanding of the specific
31 ecological conditions responsible for species succession. The large-scale strain isolation
32 shows the bias of the diversity description from cultures. Exhaustive and precise population
33 genetic studies of HABs must include many samples that are often overlooked because they
34 fail to grow. In the future, the bias of the diversity description from cultures will be
35 minimized by the development of routine single-cell analyses from natural samples. Finally,
36 the regional understanding of *Alexandrium* expansion will require the resolution of the
37 geographic origins of the two species, which could be certainly achieved by a population
38 genetic approach using microsatellite markers.
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TABLE AND FIGURE LEGENDS

Table I. Origin of the 568 clonal strains of *Alexandrium* spp. isolated from Thau lagoon and that have been ribotyped and amplified by semi-multiplex PCR.

Table II. Minimal and maximal abundances (cells L⁻¹) of *Alexandrium* sp. recorded during the bloom survey in 2007 at eight stations belonging to the Crique-de-l'Angle sampling grid (i.e. A00, A3, A5, A9, B3, B5, C3, C5).

Figure 1. Map of Thau lagoon located 30 km Southwest of the city of Montpellier (Hérault, South France). Shellfish farming sectors (S1, S2 and S3) represent 1/5 of total surface of the lagoon.

Figure 2. Maximum *Alexandrium* spp. cell densities recorded in spring and autumn in the Thau lagoon, especially into the Crique-de-l'Angle, since 1995 (data from 1995, 1998 and 1999 are available online at: http://wwz.ifremer.fr/var/envlit/storage/documents/dossiers/toxines10ans/tableau/tab_alex.htm).

Figure 3. Identification of *Alexandrium tamarense* and *A. catenella* resolved by specific amplicons according to their size using the semi-multiplex PCR assay. (M) 100 bp size marker; (a) 126 bp fragment length for *A. tamarense* (Group III-WE); (b) 240 bp fragment length for *A. catenella* (Group IV-TA).

Figure 4. Variation of species proportion, i.e. *Alexandrium tamarense* (Group III) vs. *Alexandrium catenella* (Group IV), on clonal cultures established along the sampling period during the bloom of 2007 into the Crique-de-l'Angle. The specific diversity was studied in samples taken at twelve dates. The proportion of each species was represented by 'pie charts' and reported to the number of established strains for each sampling date.

Table I.

Strains (n)	Sample origin	Period
23	sediment	winter 2004
30	seawater	autumn 2004
515	seawater	spring and autumn 2007

For Peer Review

Table II.

	11-May	16-May	24-May	31-May	07-Jun	14-Jun	21-Jun	30-Aug.	03-Sept.	06-Sept.
Min (cells L ⁻¹)	1500	< 100	600	270	< 100	< 100	< 100	100	< 100	< 100
Max (cells L ⁻¹)	7500	7200	1960000	30600	115300	15000	990	17200	2500	4000
	13-Sept.	20-Sept.	04-Oct.	11-Oct.	15-Oct.	17-Oct.	18-Oct.	22-Oct.	25-Oct.	08-Nov.
Min (cells L ⁻¹)	< 100	3300	860	410	8700	1950	1500	< 100	< 100	< 100
Max (cells L ⁻¹)	5200	123000	5500	146000	168400	139100	124300	120400	62800	5730

For Peer Review

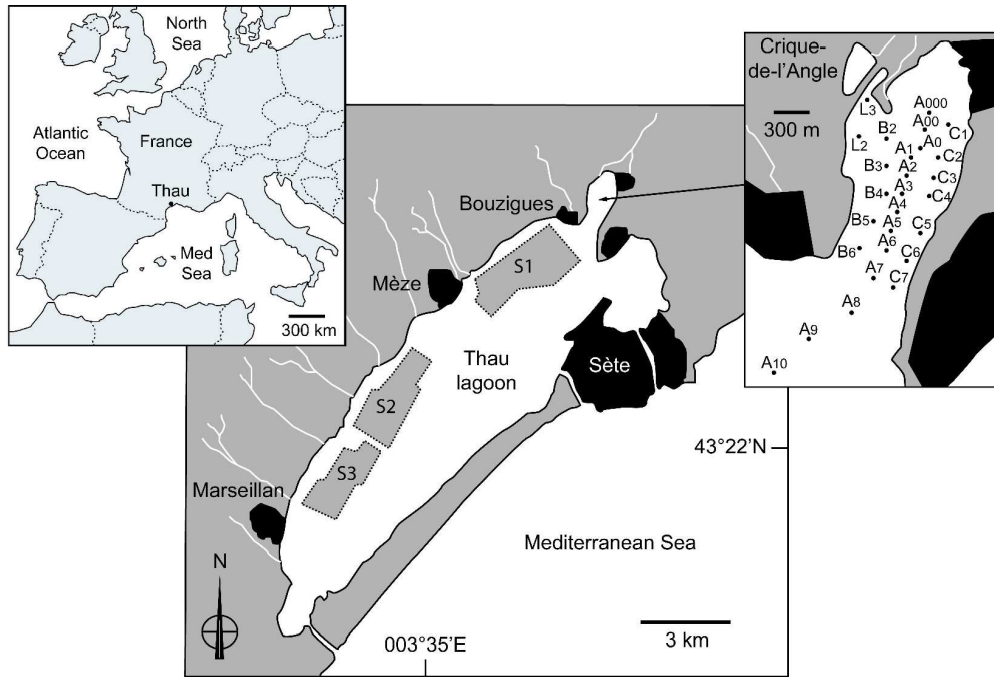


Figure 1
208x139mm (600 x 600 DPI)

Review

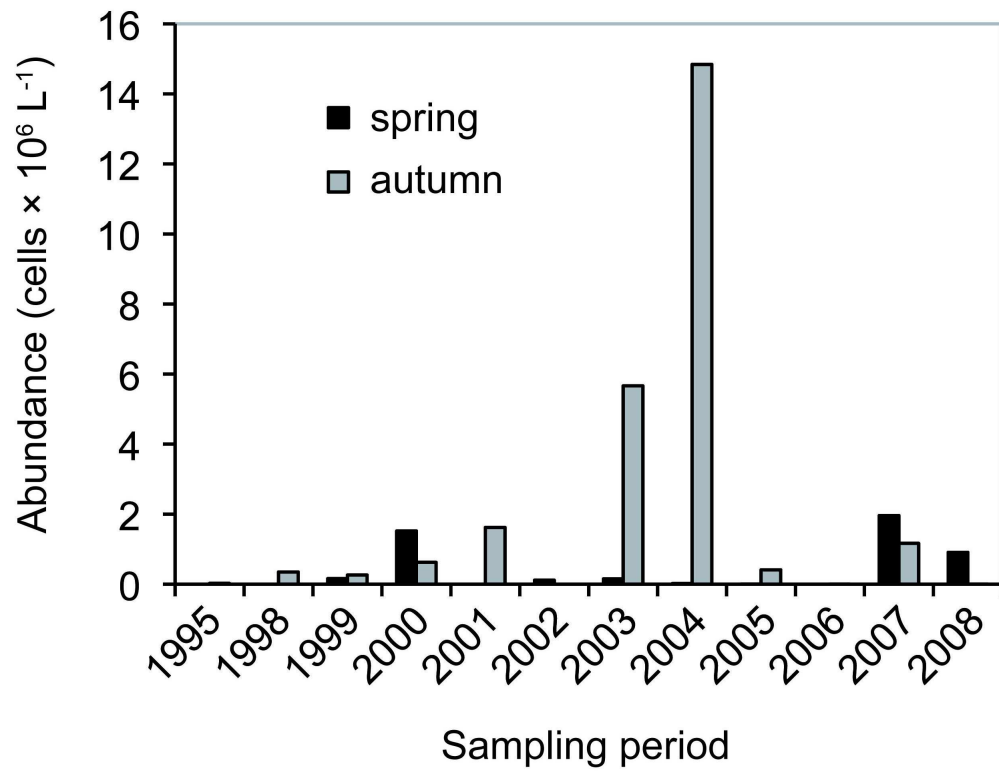


Figure 2
105x80mm (600 x 600 DPI)

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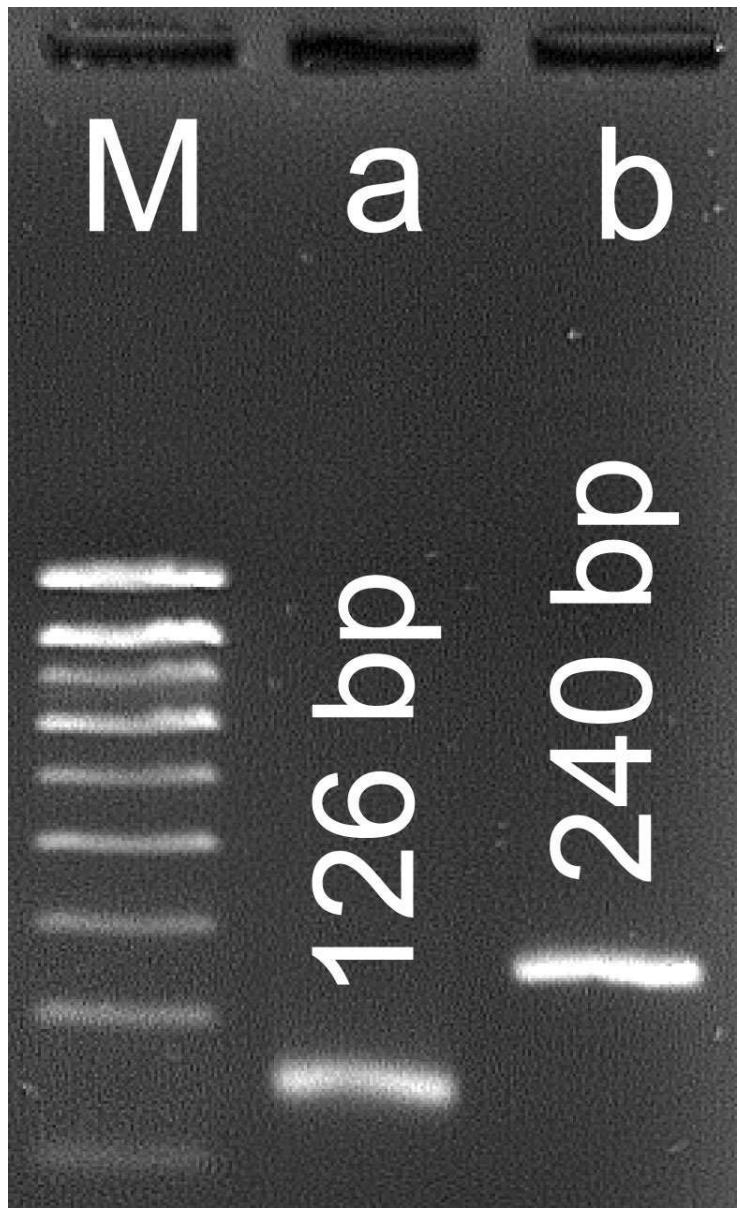


Figure 3
274x452mm (72 x 72 DPI)

