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Title:

IDENTIFICATION OF SEVERAL STRAINS OF THE POTENTIALLY TOXIC GENUS *ALEXANDRIUM* FROM THE BILBAO ESTUARY

Tesi di laurea in Botanica Marina Applicata

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“His eyes guard the beauty of the world that he conquered.

Life goes on...”

Iacopo Monaldi (1992-2017)

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1. INTRODUCTION

1.1. DINOFLAGELLATES

1.1.1. General characters

Fensome et al. (1993) define dinoflagellates as ‘‘eukaryotic, primarily single-celled organisms in which the motile cell possesses two dissimilar flagella: a ribbon-like flagellum with multiple waves which beats to the cell’s left, and a more conventional flagellum with one or a few waves which beats posteriorly’’; this definition is based on flagellar characters.

Dinoflagellates are unicellular eukaryotic microorganisms. They are a highly diverse group of flagellates, most are free-living others, such as the “zooxanthellae” of reef-building corals, are beneficial endosymbionts and still others are parasites of many protists, invertebrate and vertebrate hosts. They are adapted to a variety of habitats: from pelagic to benthic, from tropical to temperate seas and marine, freshwater and estuaries systems.

Dinoflagellates nutritional strategies are three: phototrophy, in presence of plastids, heteroetrophy and mixotrophy where both strategies have been observed, for example among the Gymnodiniales (Diane E. Stoecker, 1999). Typical pigment-pattern of plastid-bearing species include chlorophylls a and c, and peridinin as mayor components. Some species bear chlorophytes, diatoms or haptophytes plastids obtained through secondary or tertiary endosymbiosis, and others bear cryptophytes plastids obtained through kleptoplastidy.

In most dinoflagellates a dinokont arrangement is present and a desmokont arrangement is less common. In the former a pair of flagella is laterally inserted, and in latter they are functionally anterior as we can see in the picture (Fig.1). Desmokonts have two lateral plates: right valve and left valve. In lateral view the right part reveals the flagellar placement in the anterior depression (Fig. 1A). Dynokonts are in general divided in two sections by a groove (cingulum): epitheca, upper part,

hypotheca, lower part (Fig. 1B). Ventral view reveals the flagella arise from cingulum and sulcus (Taylor, 1987).

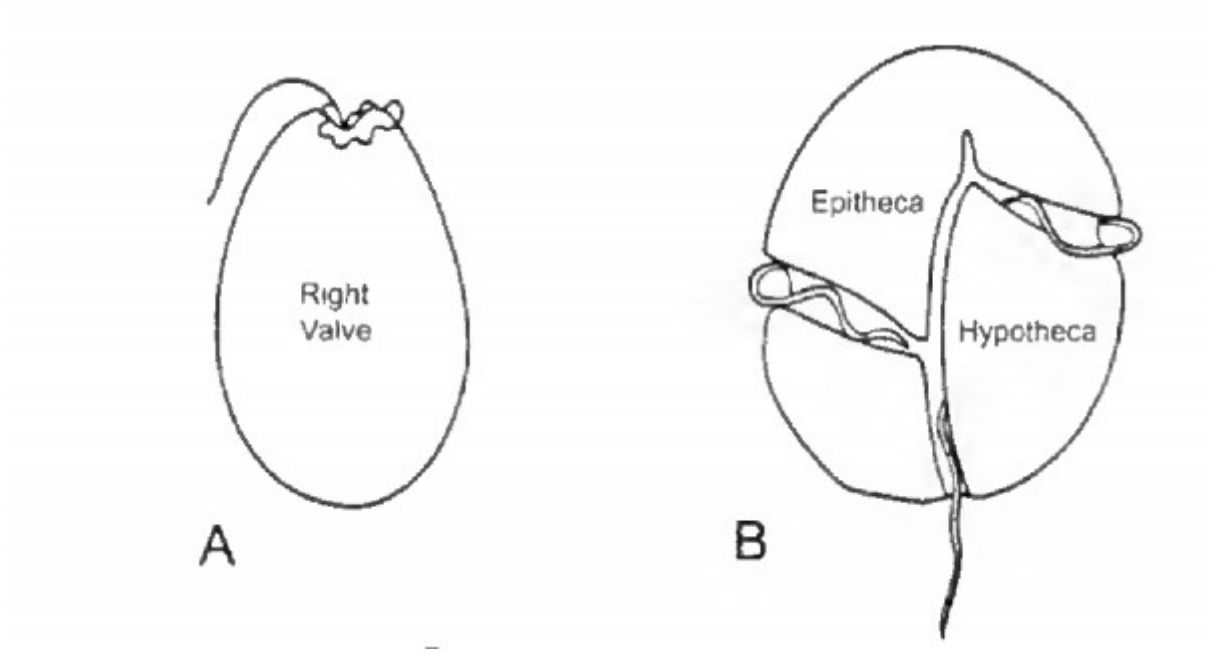
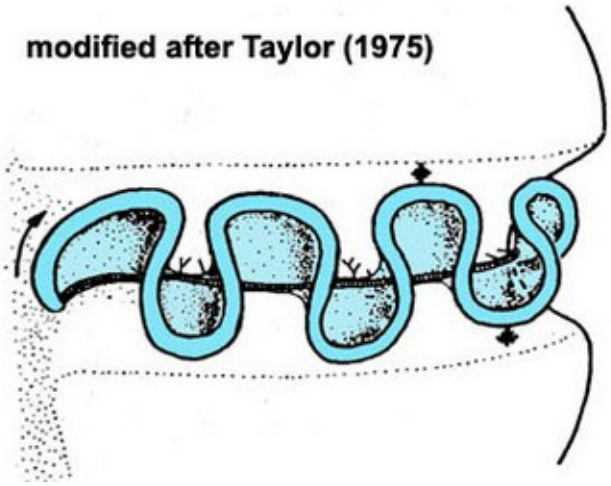


Fig. 1. (A). Desmokont arrangement, lateral view. (B). Dinokont arrangement, ventral view. (redrawn by Faust & Gulledge 2002, from Steidinger & Tangen 1996)

In either cell types a ribbon-like transverse flagellum, which winds around the cell, is present. It has a contractile fibre in the inner edge and the axonema following a spiral path along the outer edge (Fig. 2).



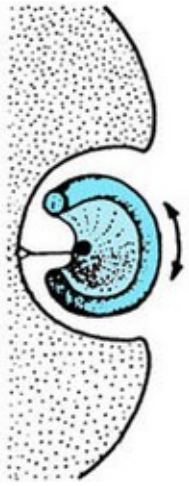


Fig. 2. Left: schematic drawing of the transverse flagellum hosted by the cingulum. Right: schematic draw of the cingulum and transverse flagellum section

Miyasaka et al. (2004) studied the functional role of transverse and longitudinal flagella in the

dinoflagellate *Prorocentrum minimum*. They discovered that *P. minimum* swims in an helical path. Without longitudinal flagellum the cells swim along a straight line. The transverse flagellum enables *P. minimum* cells to achieve a high swimming speed. Gaines & Taylor (1985), noted three important things: flagellar beat always proceeds counterclockwise, seen from apex cell, the cell always rotates in the direction of flagellar beats and the fluid is propelled in the opposite direction.

Dinoflagellates have a cell covering called amphiesma, which is composed of a series of flattened vesicles that can be empty (athecate cell) or can contain cellulose plates (thecate cell). Plates arrangement and morphology are important classification features, that are used as taxonomic traits. For plates' nomenclature the Kofoidian tabulation is generally followed (Fig.3).

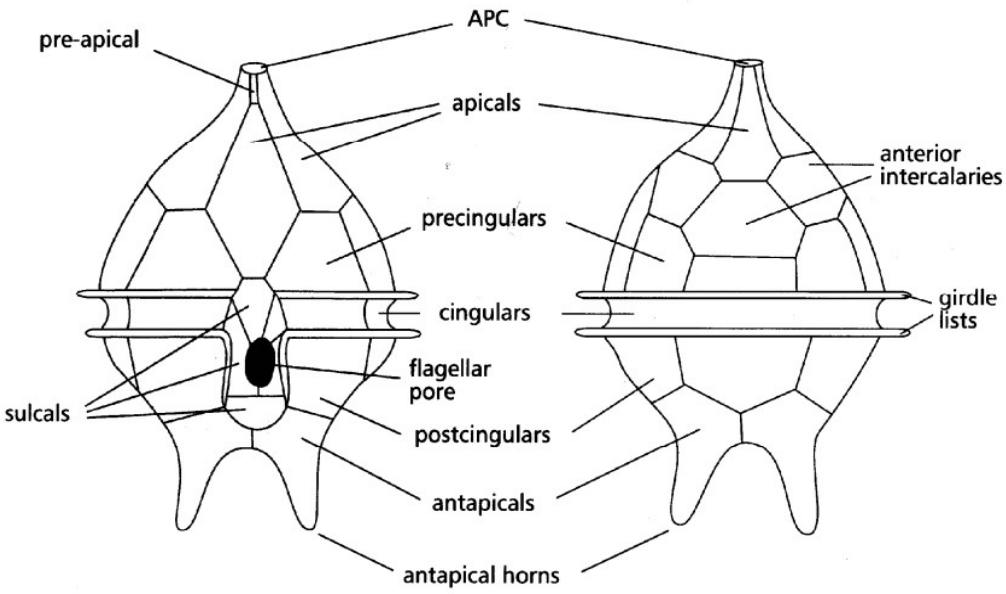


Fig. 3. Kofoidian Tabulation: (left) ventral view; (right) dorsal view. (IOC-UNESCO, modified)

The nucleus, called dinokaryon, is a dinoflagellate peculiar feature. Dinoflagellates contain large amounts of DNA per nucleus and have not been shown to have nucleosome (DNA-histones complex). Their histone-like protein has a low molecular weight, almost 23% lower than histone H4. Fibrillar chromosomes are always condensed because of the presence of Ca^{2+} and Mn^{2+} (Herzog and Soyer, 1983) and divide via closed mitosis with an external spindle.

Most dinoflagellates have an haplontic life cycle. It means that the vegetative stage is haploid.

Asexual reproduction is predominant when there are optimal environmental conditions. Sexual phase allows genetic ricombination, species adaptation and survival. During the sexual reproduction two haploid cells called gametes fuse to form a diploid cell called planozygote (mobile zygote) that will go under meiosis to restore the vegetative form. Part of living dinoflagellates (approximately 13-16%) produce a dormant resting cyst (Head 1996), called hypnozygote. This resting cyst is traditionally associated to sexual dinoflagellates cycle and rapidly sink to the sediment where it can remain viable for up to 100 years (Ribeiro et at., 2011).

The switch from an asexual to a sexual reproduction is currently unknown, it seems that both endogenous and environmental factors are responsible; each species may have its specific condition that driving the switch to sexual reproduction.

1.1.2. Harmful algal blooms

Dinoflagellates greatest diversity is in the marine plankton where they can produce “red tides” and other monospecific blooms (Taylor et al., 2008). The traditional name “red tides” has been partially repleaced by the more general Harmful Algal Bloom (HAB) at the First International Conference on Toxic Dinoflagellate Blooms in 1974 at Boston (LoCicero et al., 1975). Currently, “red tide” is used to specifically refer to HABs causing reddish water discolourations. Gilbert et al., (2005) defined HAB as the phenomena where a elevated uncontrolled production of one, or more, micro or macro algae cause major ecological impacts, for instance the habitat alteration and/or oxygen depletion in the bottom waters, they can also lead to kills of fish and invertebrates like mussels by generating anoxic conditions or by producing exudates or reactive oxygen species (Hallegraeff, 2010). HABs cause important economic damage especially in aquaculture. These problems are caused by toxin production or simply by biomass or also by physical shape.

Many species of dinoflagellates are notorious for producing toxins that can cause human illness through shellfish or fish poisoning (Steidinger, 1993). These toxins were originally designated using

the symptomatology that they cause. PSP as paralytic shellfish poisoning, DSP as diarrheic shellfish poisoning, NSP as neurotoxic shellfish poisoning and ciguatera (Steidinger, 1993).

Two fundamental typologies of algae can create HABs: producers of toxins and non-producers of toxins. Indeed, even non toxic algal blooms can have devastating impact when leads to anoxic conditions that kills fish and invertebrates (like mussels). Other non toxic species can damage the gill tissue of fish by exudates products or reactive oxygen species (Hallegraeff 2010). As stated by Gilbert "the term "HAB" is operational and not technical, but this distinction is irrelevant in terms of human health and economic impacts" (Gilbert et al., 2005).

The most common PSP-producer dinoflagellates belong to the following genera: *Alexandrium*, *Pyrodinium* and *Gymnodinium.* They rely on the germination of benthic resting cysts rather than on a rapid growth strategy (Hallegraeff 1998). For instance, *Alexandrium* produces seasonal bloom events that appear to be restricted in time by cyst production (Anderson 1997).

During the past three decades HABs seem to have become more frequent, more intense and more widespread (Hallegraeff 1995, Van Dolah 2000). The issue of a global increase of HABs has been a recurrent topic of discussions at all major conferences dealing with HABs (Anderson 1989; Hallegraeff 1993; Smayda 1990). The Intergovernmental Panel on Climate Change (Climate Change 2007) is planning to include HAB risk forecasts under a range of climate change scenarios.

Four explanations for increasing of HABs have been proposed: increased scientific awareness; increased utilisation of coastal waters for aquaculture; eutrophication and/or unusual climatologic changes; transporting of resting cysts by ballast water or translocation of shellfish stocks.

1.2. THE GENUS *ALEXANDRIUM*

1.2.1. Introduction

Among the genera responsible of HABs the genus *Alexandrium* is one of the most important in terms of severity, distribution and impacts. Dinoflagellates of the genus *Alexandrium* are the most numerous and widespread saxitoxin producers. They are responsible for Paralytic Shellfish Poisoning (PSP) blooms in subartic, temperate and tropical locations (Hallegraeff et al., 1988; Anderson et al., 1994; Lily et al., 2002) and represent a public health concern and a source of economic loss on aquaculture. Of the more than 30 morphologically defined species of this genus, at least half are known as toxic producers. Three different families of toxins are produced among species within the genus: saxitoxins, spirolides and goniodomins. Furthermore, recently Harju et al. (2016) discovered the production of gymnodimines by *Alexandrium ostenfeldii*.

The most significant of these toxins, in terms of impacts, are the saxitoxins responsible for outbreaks of PSP. These impacts include: human intoxications and death from contaminated shellfish or fish, loss of wild and cultured seafood resources, impairment of tourism and recreational activities, alterations of marine trophic structures, and death of marine mammals, fish and seabirds (Anderson et al 2012).

Alexandrium blooms frequently occur in shallow salt ponds and coastal bays (Su et al 1993, Giacobbe et al. 1996). These blooms can occur either as simply nearshore manifestation or large-scale coastal blooms events, but in many cases are localized as spot events. Another prominent habitat for *Alexandrium* blooms is in open coastal waters or large estuaries (Franks et al., 1992; Anderson et al., 2005; Fauchot et al., 2005; Fauchot et al., 2008).

Blooms observed in bay and salt ponds can last for two or three months (or less) (Anderson et al., 1983), Giacobbe et al. (2006) described an *A. minutum* bloom in a Mediterranean lagoon over a six months period, but only two months (April and May) with a bloom level concentration. The

encystment/excystment cycle which restricts the life of the *Alexandrium* vegetative cell population and even the induction of sexuality precludes the long-term persistence of *Alexandrium* vegetative cells in the plankton.

1.2.2.Life cycle.

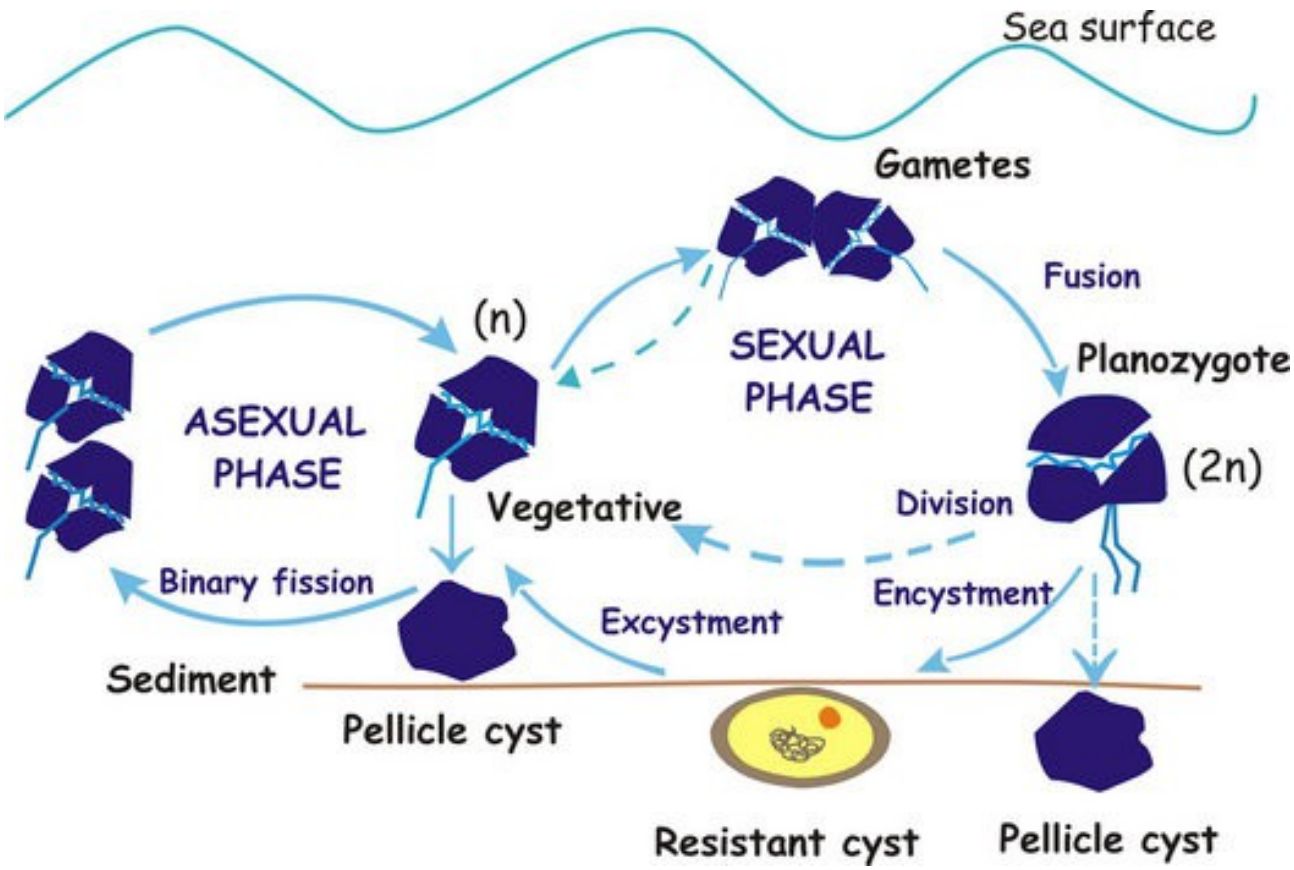


Fig.4. *Alexandrium* life cycle diagram. In this picture a general scheme of the life cycle of *Alexandrium* species is presented. The motile vegetative cells are haploid. Some motile cells can turn into a non-motile pellicle cysts when stress conditions occur. Pellicle cysts can switch back to motile stage when conditions improve. Sexual phase starts with formation of gametes, which fuse and form diploid planozygote. Depending on environmental conditions, the latter can transform into a pellicle cyst or undergo meiosis and produce vegetative cells. Resistant cyst can spend a variable periods of time in the sediments before release a motile cell. (Modified after Walker et al 1984)

Alexandrium life cycle (Fig. 4) consists of an alternation between asexual and sexual reproduction.

A bloom develops with repeated divisions, binary fission, that lead to the proliferation of vegetative cells.

Vegetative cells division usually occurs through desmoschisis (Figueroa et al., 2007), each daughter cell maintains half the thecal plates of mother cells. Their vegetative phase is haploid. This asexual process terminate when a sexual induction occurs and gametes are produced. Gametes are either unrecognizable from vegetative cells or are smaller.

Some species have another resting cyst stage called temporary cyst, or pellicle cysts, it occurs when vegetative cells are exposed to stress conditions (Anderson and Wall, 1978; Bravo et al., 2010). Pellicle cysts are forms of resistance and the vegetative cell can be restored when the stress conditions are over. The production of temporary cysts is an advantageous feature that allows *Alexandrium* populations to persist in short-term environmental fluctuations. In the last decade, several publications reported evidence about resting cysts of *Alexandrium* that the transition between planozygote and hypnozygote is not an obligate one (Figueroa et al., 2008; Figueroa et al., 2007; Figueroa et al., 2006; Figueroa et al., 2005).

1.2.3. Taxonomy

Alexandrium cells were represented for the first time by Kofoid (1911), with excellent drawings that clarified and defined structural features of the species observed, as species of the genus *Gonyaulax*.

In 1925 Marie Lebour described *Gonyaulax tamarensis* distinguished by its simple shape. It lacks an apical horn or conspicuous spines, and showed a thin theca with four apical plates but no intercalary plates.

Whedon and Kofoid (1936) described two species with the same tabular formula: *G. acatenella* and *G. catenella*. The latter has been the most studied because of its capability to produce PSP, that in 1930 caused human illness on humans that consumed bivalve molluscs.

Increased in the years the confusion and hesitation within the genus *Gonyaulax*, other similar

species were grouped with *G. tamarensis*, *G. catenella* and *G. acatenella*.

Eventually, *Alexandrium*, which is the genus that currently includes these species, was formally established with the description of its type species *A. minutum* in 1960 (Halim, 1960) as a "red tide" in the harbour of Alexandria in Egypt.

Halim (1967) established another monospecific genus, *Gessnerium*, when he described a small dinoflagellate found in the Bay of Mochima (eastern Venezuela). Halim assigned a plate tabulation formula to this new species, *Gessnerium mochimaensis*, which was later corrected by Balech (1995). This new corrected formula corresponds to the *Alexandrium* species of the "tamarensis group" and to *Pyrodinium*. Later, it was found that *G. mochimaensis* was a synonym of *Gonyaulax monilata* (Howell 1953). Currently, the genus *Gessnerium* is regarded as a subgenus of *Alexandrium*, and some of the species (for instance, those of the "tamarensis group") described the genus *Gonyaulax*, have been transferred to *Alexandrium*.

The genus *Alexandrium* is subdivided into two subgenera: *Alexandrium* (where the 1' plate is connected to the Apical Pore Complex, APC) and *Gessnerium* (where there is no connection between the 1' plate and the APC) (Balech, 1995). More than 30 species have been reported in the genus *Alexandrium* and new species are continuously being established (Anderson et al., 2012; John et al., 2014).

1.2.4. Biogeography

Alexandrium species are known to be widely distributed across several continental coastal and shelf waters. For a few species, of *Alexandrium*, the observed distributional patterns were dense enough to formulate an evolutionary model. Members of the genus *Alexandrium* are widespread globally, with species present in coastal, shelf and slope waters of subarctic, temperate and tropical regions of the Northern and Southern Hemispheres (Taylor et al., 1995; Lily et al., 2007). The diversity of *Alexandrium* appears to be higher in Mediterranean Sea than elsewhere. This may reflect a high

level of monitoring and laboratory studies more than an actual distribution.

Polymorphic genetic markers such as DNA microsatellites have been developed for some *Alexandrium* species. An example of the application of these molecular tools is in Lily et al. (2005) publication, where a complete biogeography and phylogeny of *Alexadrium mintum* group was presented. It was proposed *A. tamutum* as a valid species, showing morphological and genetic differences from others *A. minutum*. Furthermore, Lily et al. (2005) determined that all of the European *A. minutum* are closely related, and may originate from the same ancestral population. *Alexandrium minutum* were split into two main clades, the larger one containing isolates from locations in Europe and the Southern Pacific, called "Global clade" and the smaller containing only isolates from New Zealand and Taiwan, called "Pacific clade" (Fig. 5). Casabianca et al. (2011) showed the existence of a genetic population substructure for *A. minutum* in the Mediterranean Sea, which was explained by basin-scale transportation patterns through successive generations of vegetative microalga cells.

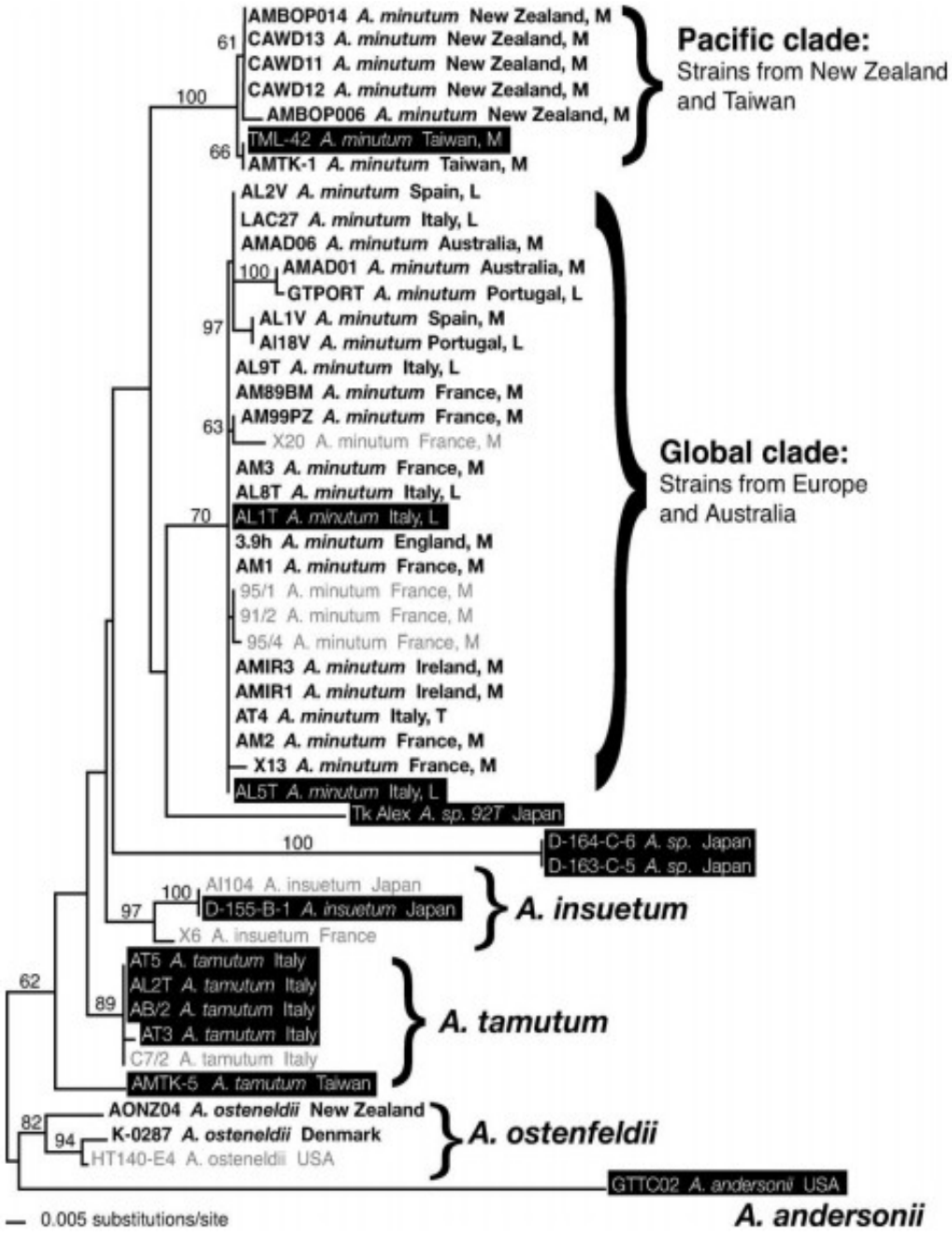


Fig.5. Maximum likelihood tree from Lily et al. (2005). The letter L after a taxon indicates an original morphospecies designation of *A. lusitanicum*. Bootstrap values are shown. Toxic strain are in bold type. Non-toxic are in white type on black. Unknown toxic strain are in grey. (Lily et al., 2005)

Anderson et al. (2012), in a review on the genus *Alexandrium*, pointed out that intraspecific regional genetic patterns might be observed for the majority of *Alexandrium* species, in contrast to expectations of broad genetic uniformity in planktonic marine microbes.

A particular aspect of *Alexandrium* biogeography is the distribution of toxic and non-toxic strains of

the same species, or of closely related species. For instance, the case of *A. minutum* and *A. tamarense* in Ireland, where the peak of concentration of toxic *A. minutum* were found in the North Channel, while the distribution of *A. tamarense* was more homogeneous. Also exceptions were found either in Scotland (Touzet et al., 2010) and in Ireland (Brosnahan et al., 2010).

1.2.5. Morphology

Balech (1995) summoned up several morphological characteristics (Fig. 6) that are keys for the identification of the species of *Alexandrium*, such as the size and shape of cells, the size and shape of the first apical (1') and sixth precingular (6'') plate, thecal ornamentation, presence or absence of a ventral pore and sulcal lists, shape of the pore plate (Po), connection between the Po and 1' plate, the size and shape of sulcal plates and the ability to form chains.

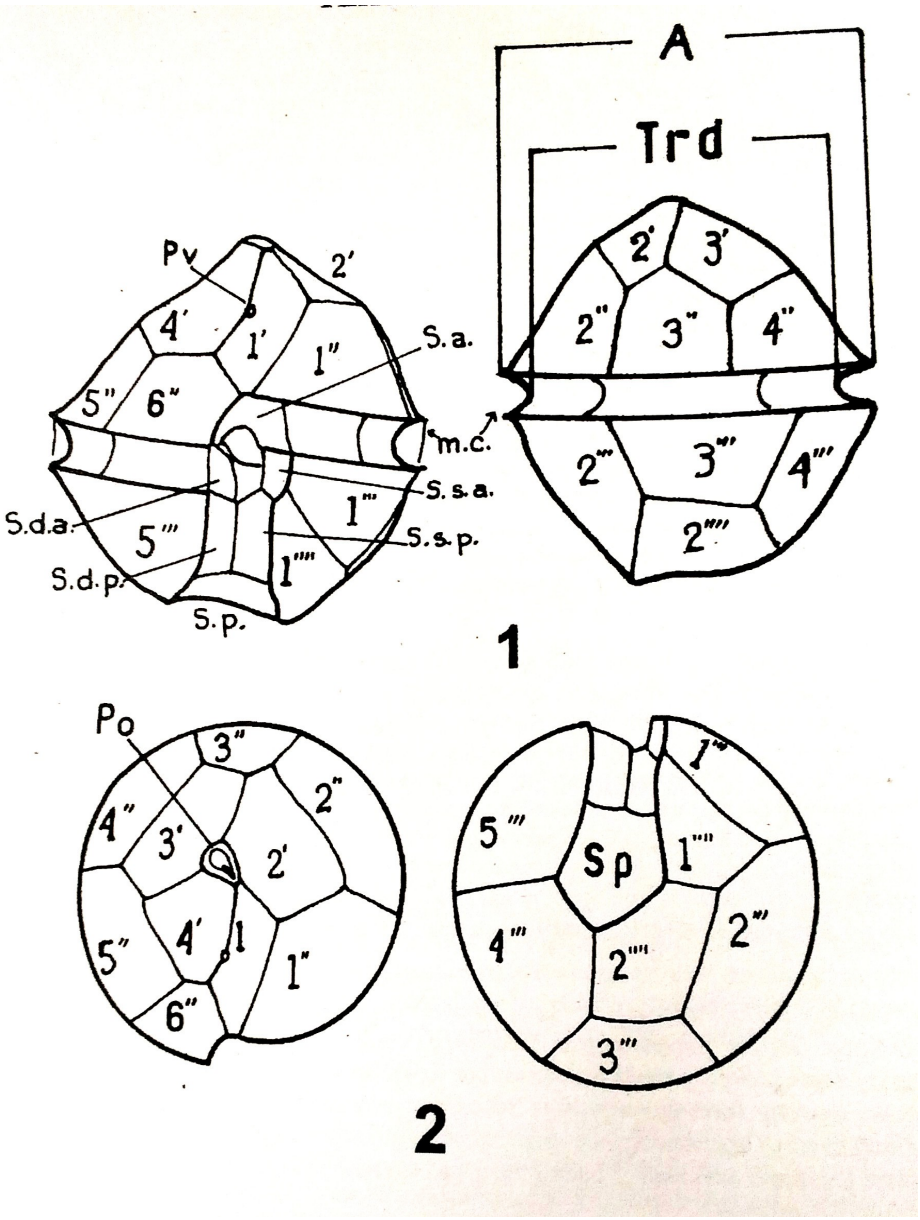


Fig.6. Schematical draws of *Alexandrium* thecal plates. 1) whole theca: ventral view on the left, dorsal view on the right; 2) polar views: apical on the left, antapical on the right. Pv = ventral pore; m.c = curtain fin; A (amplitude) = maximum cingular width; Trd (transdiameter) = minimum cingular width; Po = apical pore; S.p. = sulcal posterior; S.a.= sulcal anterior; S.d.a. = right anterior sulcal ; S.s.a. = left anterior sulcal S.d.p. = right posterior sulcal; S.s.p. = left posterior sulcal. (Balech, 1995)

1.3. *ALEXANDRIUM TAMARENSE* SPECIES COMPLEX

Tamarensis group taxonomy became more and more complicated due to species additions and to the differing opinions. Different names are assigned to one single species, for instance *G. tamarense* and *G. excavata* in Japan, or one single name was assigned to two different species (Balech 1995). Steidinger (1971) was the first to note that these species should be put in another genus. She proposed three new ideas: 1) to separate the whole *tamarensis* group from *Gonyaulax*. 2) the recognition that this "*tamarensis* group" was part of *Alexandrium*. 3) the first formal tabular characterization of the group.

During the seventies, the *tamarensis* group was recognized as a well defined systematic unit that deserved its own designation, and this view was supported by both Steidinger and Balech (Steidinger 1971; Balech 1971).

Nowadays the *A. tamarense* species complex is a recognized group of cryptic species that are essentially unrecognizable from each other without a genetic approach.

All these facts reflect the intricate taxonomic history of these species.

1.3.1. Three morphospecies

Two major species complex produce PSP: *A. tamarense* complex (Atama), refers to three morphospecies which are *A. tamarense*, *A. catenella* and *A. fundyense; Alexandrium minutum* complex that refers to other three morphospecies *A. minutum*, *A. ibericum*, *A. angustitabulatum* (Cembella, 1998). They are grouped into species complexes due to limited morphological differences between the members of each complex (Fig. 7). It was suggested that the three morphospecies from the "Atama complex", one of the most important group within the genus *Alexandrium*, could be differentiated morphologically by cell shape, chain-forming ability, and the presence/absence of ventral pore between the 1' and 4' plates (Balech 1995).

However, latest reviews of the complex tend to consider that they are essentially unrecognizable

from each other (John et al., 2014).

According to extensive study on *A. tamarense* and *A. fundyense* obtained by Balech (1995) these characters were of limited value when identifying species. In support of this sentence, other field and laboratory studies have recorded cells exhibiting morphologies intermediate between those described for the *A. fundyense* and *A. tamarense* morphotypes (Anderson et al., 1994; Destombe et al., 1992; Gayoso and Fulco, 2006; Kim et al., 2002; Orlova et al., 2007; Taylor, 1984)

A. tamarense complex cells had been documented in the Northern and Southern hemispheres, adding South America, South Africa, Australia, the Pacific Islands, India, all of Asia and the Mediterranean to the known range (Hallegraef 1993, Abadie et al., 1999, Vila et al., 2001). Therefore, the original *A. catenella*, *A. fundyense* and *A. tamarense* species descriptions are not useful, because the morphological characters do not allow an unequivocal species circumscription.

Because of the serious human health and economic impacts associated with PSP, the alarming increase of frequency and biogeography expansion of toxic *A. tamarense* complex blooms (Hallegraeff, 1993; Anderson, 1989) has spurred the scientific community to increase the investigation on this species complex in order to improve the environmental controls. However if we are to prevent further spread of *A. tamarense* complex cells, we must understand the causes for their current distribution. This goal requires investigation into taxonomy and DNA phylogeny.

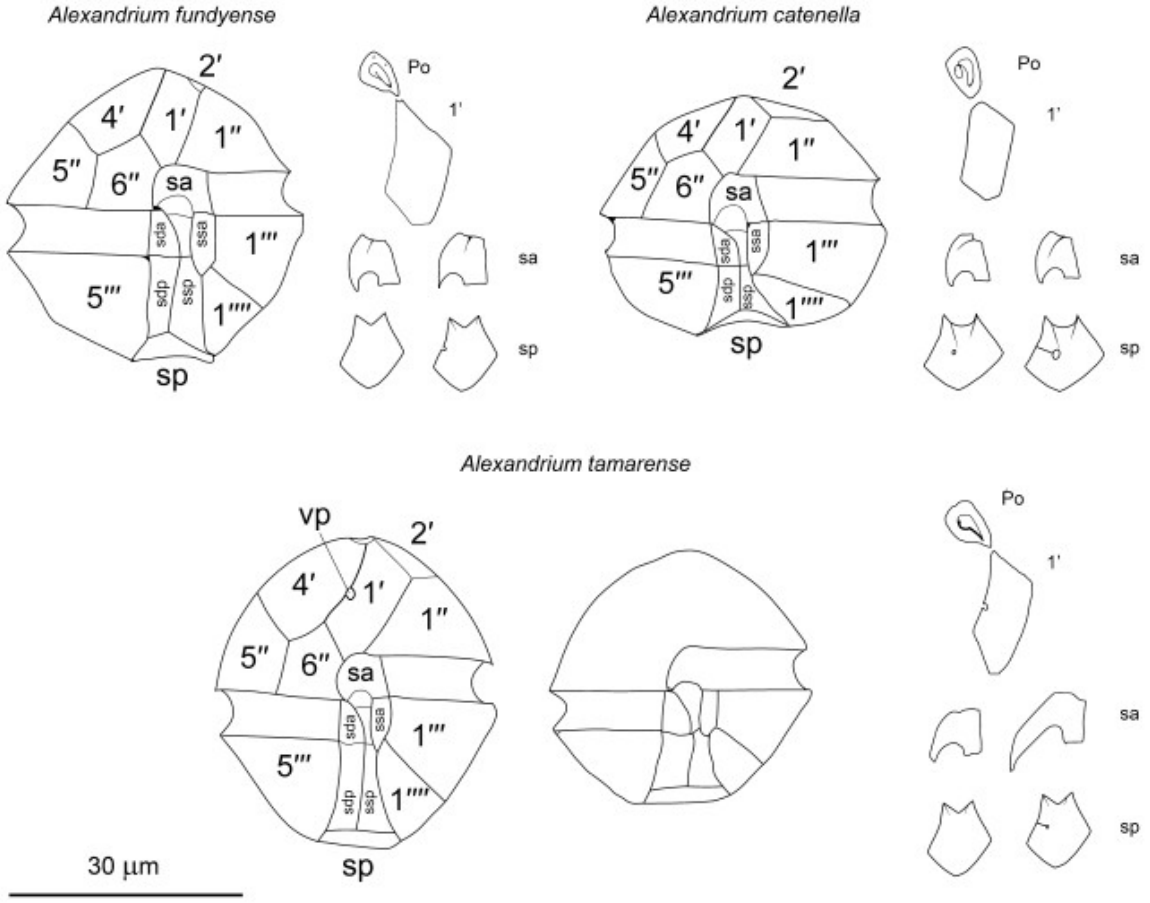


Fig.7. Synthetic representation of the morphological differences among *A. catenella*, *A. fundyense* and *A. tamarense*, as described by Balech (1985; 1990; 1995). The *A. fundyense* and *A. catenella* drawings represent the two extreme morphotypes observed in Atama species complex. Po = apical pore plate; sa = anterior sulcal plate; sda = right anterior sulcal plate; sdp = right posterior lateral sulcal plate; sma = anterior median sulcal plate; smp = posterior median sulcal plate; sp = posterior sulcal plate; ssa = left anterior lateral sulcal plate; ssp = left posterior sulcal plate; vp = ventral pore. (John et al., 2014)

Taxonomy of the *A. tamarense* complex is contentious with some researchers, such as Mayr (1982), believing that the three morphospecies are true biological species. Yet others researchers believe that the morphological variations are not indicative of shared genetic heritage and instead may be variations within single species, as stated by Anderson et al. (1994).

Because of the morphological differences are slight and multiple morphospecies can co-occur (Taylor, 1984; Anderson et al., 1994), researchers have looked for molecular evidence confirming

morphospecies identifications.

1.3.2. *Alexandrium tamarense* (Lebour) Balech

This was one of the better known species of the complex (Fig. 8).

Cells are small to medium size and are somewhat isodiametric (Balech, 1995). Commonly found single or in pairs. The size and shape of this species is highly variable: cells range in size between 22-51 µm in length and 17.5-44 µm in transdiameter width (Balech, 1995). The plate formula is: Po, 4', 6", 6c, 8s, 5"', 2"".

The Po is often very wide and markedly angular, small marginal pores were detected and a comma-shaped foramen is present (John et al., 2014; Tillman et al., 2016; Balech, 1995).

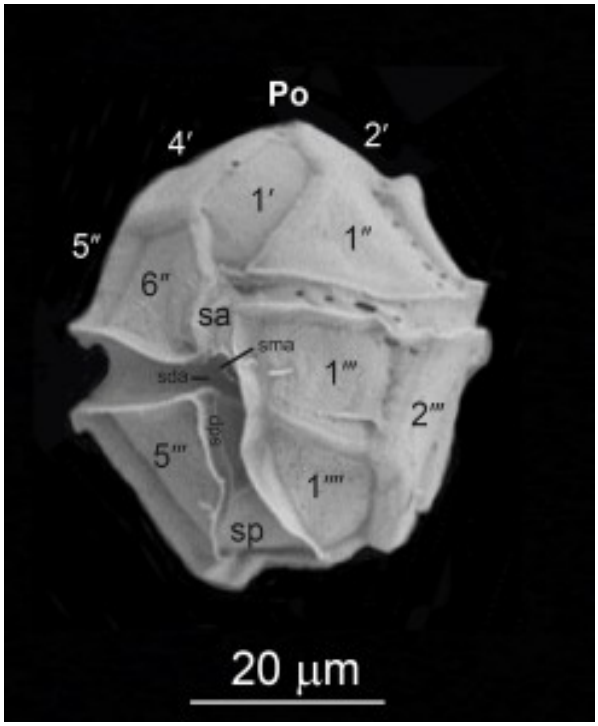


Fig.8. *Alexandrium tamarense.* Calcofluor-stained cell, SEM ventral views. Po = apical pore; sa = sulcal anterior; sma = sulcal median anterior; sda = sulcal right anterior; sp = sulcal plate; sdp = sulcal right posterior

The 1' plate has a relatively viarable width. The ventral pore is small and always exists in the margin with 4'. The sulcal plates (S.p.) may or may no have a connecting pore that is small and displaced to the right and usually connected to the edge by a groove (Balech, 1995).

1.3.3. *Alexandrium fundyense* (Balech)

By its shape and size, it cannot be distinguished from *A. tamarense*; the primary difference from *A. tamarense* is the lack of ventral pore which seemed enough to separate them as species. Cells are as long as wide or slightly longer than wide (Fig. 9).

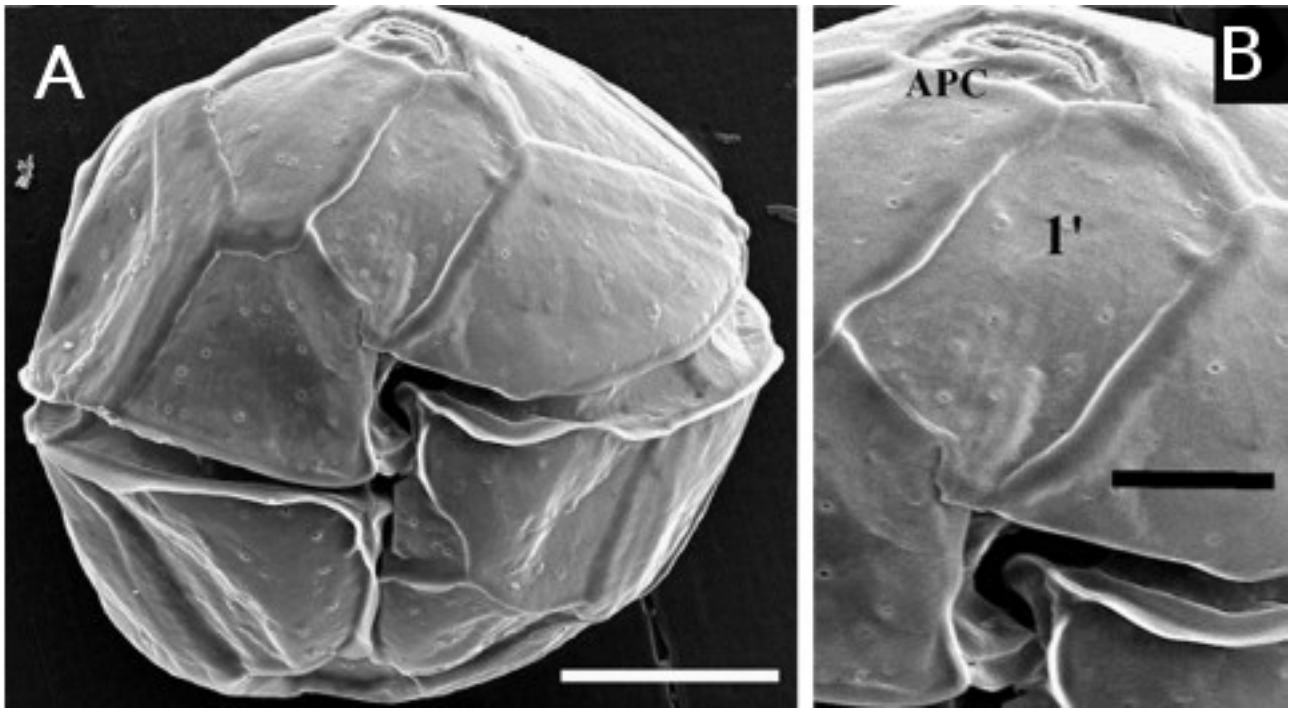


Fig.9. *Alexandrium fundyense* (Balech). A) SEM whole cell. B) SEM image close-up of APC and 1' plate showing the absence of ventral pore. Scale bar = 5 µm. (Modified from Borkman et al., 2014)

Cell surface is usually smooth with many scattered small pores. The plate formula is: Po, 4', 6", 5C, 8-10S, 5'", 2"". Cells are almost always single. A connecting pore on Sp is generally not present.

1.3.4. *Alexandrium catenella* (Whedon & Kofoid) Balech

Alexandrium catenella was first published with detailed description as *Gonyaulax catenella* Whedon & Kofoid (1936) together with *Gonyaulax acatenella* Whedon & Kofoid (Fig. 10). The main difference between them was the ability of *G. catenella* to form chains. At that time the chain formation was a very important taxonomic character while today is considered as a variable character (John et al., 2014; Fraga et al., 2015).

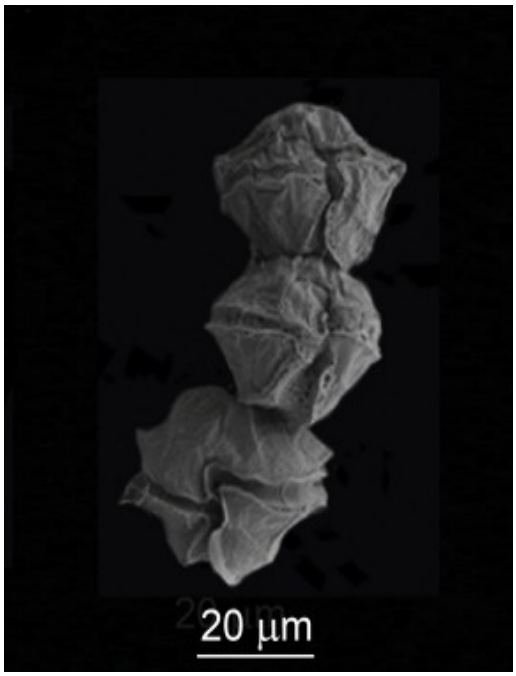


Fig.10.*Alexandrium catenella* (Whedon & Kof.) Balech. SEM image of chain of three cells in ventral views.

Cells are as wide as long or slightly wider than high, with 20-39,5 µm in length. The plate formula is: Po, 4', 6'', 5C, 8-10S, 5''', 2''''.

The ventral pore along margin 1' plate and 4' is generally not present. The absence of a ventral pore distinguishes it well from *A. tamarense*. However, *A. fundyense* lacks this pore but does not form chains and has a different S.p. that is more irregular and elongated (Balech, 1995).

1.4. PHYLOGENETIC RELATIONSHIPS WITHIN ATAMA COMPLEX

1.4.1. Introduction to species identification

The use of DNA sequences as a tool for the identification of species has been widely discussed in the past years (Hebert et al., 2003; Tautz et al., 2003; Hebert et al., 2004; Savolainen et al., 2005, Blaxter, 2004). The mitochondrial DNA, in particular a fragment from the cytochrome oxidase I gene (COI), is a widely used marker for identification of animals species and the base for DNA taxonomy and DNA barcoding (Hebert et al., 2003).

Nevertheless, other markers have also been suggested, for example the nuclear rDNA (Markmann & Tautz, 2005; Scholin et al., 1994). Ribosomal DNA is considered as the best molecular marker for studying the phylogenetic relationships because it is universal and because it has highly conserved portions as well as variable domains (George et al., 1977; Sonnenberg et al., 2007).

The ribosomes consist of a small subunit (SSU) and a large subunit (LSU). Ribosomes contain rRNA and proteins. In most of eukaryotes the LSU contains three rRNA (28s, 18s and 5.8s) while the SSU contains a single rRNA (18s). The LSU is part of the rDNA gene complex which occurs in tandem repeats (Fig. 11), arranged in ribosomal clusters in the nuclear genome (Long & Dawid, 1980).

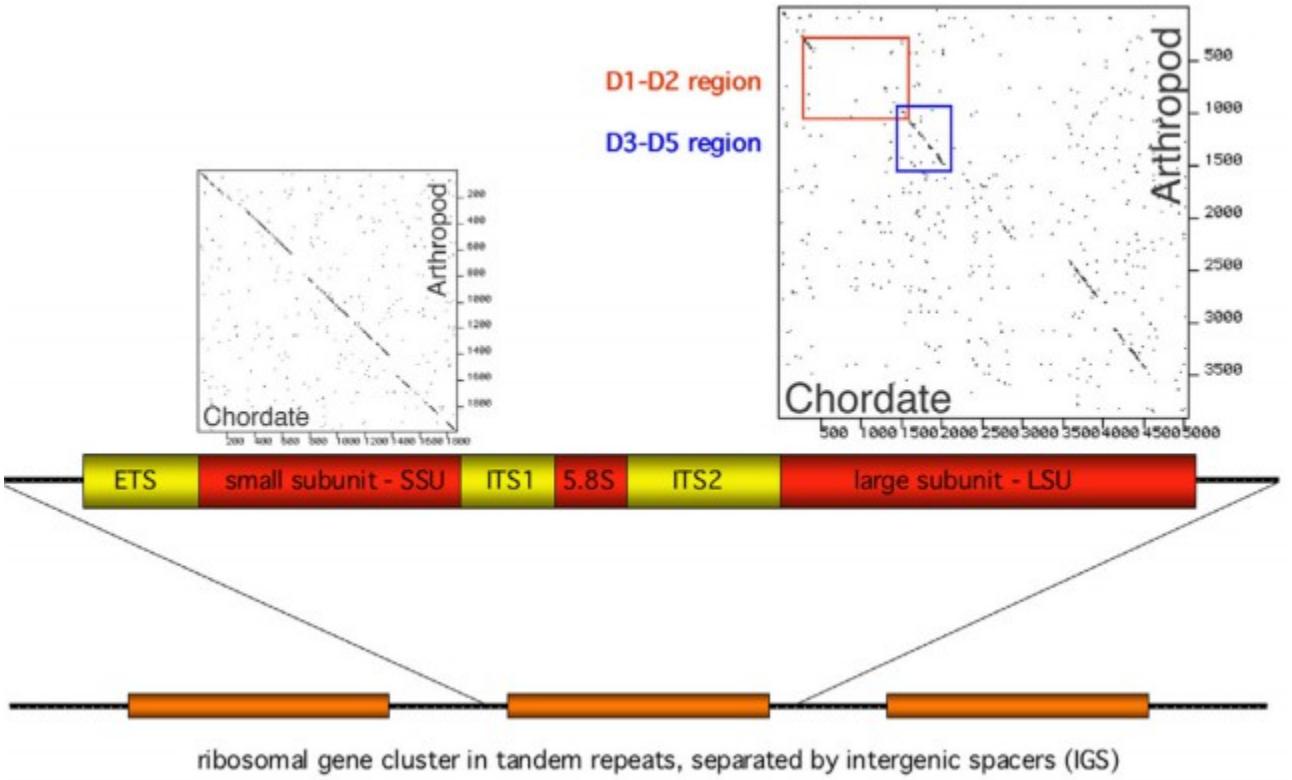


Fig.11. General organization of eukaryotic ribosomal genes. The dot plot comparisons above the SSU and the LSU show the conservation profiles between a chordate and an arthropod sequence. In this presentation, the sequences of two species (mouse and *Drosophila*) are compared and a dot is placed in the diagram at each position where 10 consecutive nucleotides match.(Sonnenbert et al., 2007)

Sonnenberg et al. (2007) evaluated the use of rDNA LSU sequences for the identification of species. Thanks to Sonnenberg et al. (2007) works, it was evident that SSU was more conserved, interrupted by a few less conserved regions; while the LSU showed larger regions of divergence (Fig. 11).

Patwardhan et al. (2014) described the most common molecular markers in a review in order to clarify their use for each circumstance. For example, the 28s rDNA (LSU) is about 811 bp in length and has been widely used for the phylogenetic analyses, especially the D1-D2 region (Patwardhan et al., 2014)

1.4.2. Five cryptic species within Atama complex

Recent field and culture studies with rDNA sequences from the three morphospecies have revealed five phylogenetic groups (Groups I-V) in the *A. tamarense* complex (Miranda et al., 2012). The "Group" naming scheme was proposed as temporary to address the apparent speciation reported in their analysis of LSU sequences from globally dispersed *A. tamarense* species complex isolates (Lily, 2003; Lily et al., 2007).

Scholin et al. (1994) found five ribotypes that named after the geographical origins of the strains: North American Clade (Group I), Mediterranean Clade (Group II), Western European Clade (Group III), Temperate Asian Clade (Group IV), Tasmanian Clade (Group V) (Scholin et al., 1994; Anderson et al., 2012).

Wang et al. (2014) have recently proposed species names for Groups I-V based on ITS rDNA phylogenies. However, they did not deal with the requirements of the Internantional Code of Nomenclature for Algae, Fungi, and Plants (ICN, McNeill et al., 2012) for the valid description of the new species. Their results were in agreement with those obtained previously using SSU and LSU sequences (Miranda et al., 2012; Lily et al ., 2007; Scholin et al., 1995).

John et al. (2014) support the existence of five cryptic species within the *A. tamarense* complex (Fig. 12). Moreover, the genetic distances separating the Groups are as large as those observed between other *Alexandrium* species (Wang et al., 2014; John et al., 2014; Penna et al., 2005, 2008).

John et al. (2014) have found that the greatest divergences (in the ITS/5.8S region) within Group I were due to a deletion in some sequences. Despite this deletion all sequences fell in Group I and not in Groups II-V. This supported the previous analysis which found that highly divergent pseudogenes in Group I never segregate with those found in other groups (Ho et al., 2012; Miranda et al., 2012; Orr et al., 2011).

John et al., (2014) proposed three new species: *A. mediterraneum* (Group II), *A. pacificum* (Group IV), *A. australiense* (Group V). Additionally, they proposed to apply the name *A. fundyense* for

strains from Group I and *A. tamarense* for strains from Group III. They showed that *A. catenella* and *A. fundyense* are synonyms and suggested the rejection of the name *A. catenella* (Whedon & Kof.) Balech, which has priority over *A. fundyense* (John et al., 2014b), a proposal that has not been resolved yet.

Acceptance of proposal 2302 by John et al., (2014b) is still contentious.

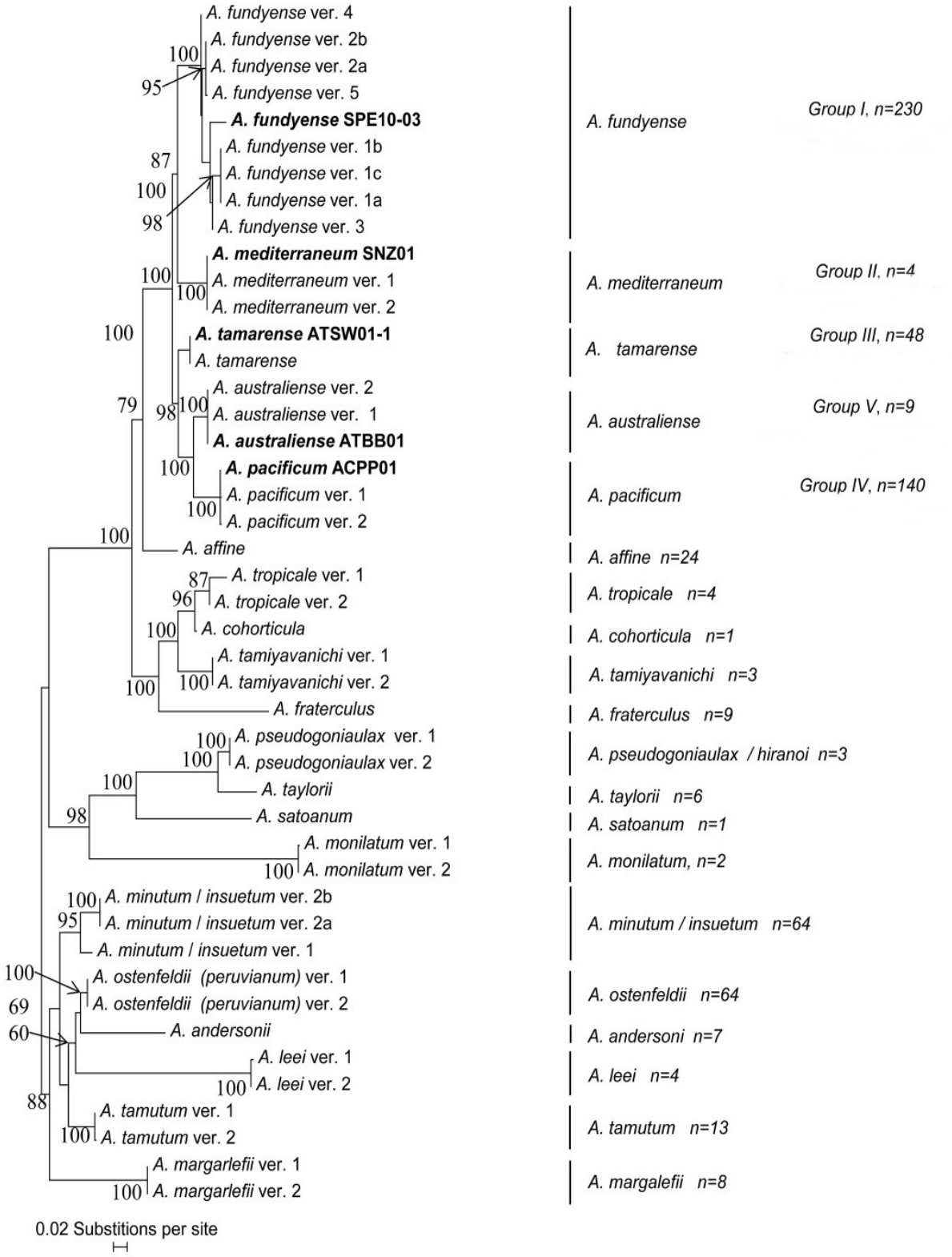


Fig.12. Phylogenetic tree of the D1-D2 LSU rDNA from John et al. (2014). The sequence data were filtered to remove the sequences with differences of ≤ 2 bp. N= number of sequences of each species included in the analysis. (John et al., 2014).

According to John et al. (2014) results, the average genetic distances in the ITS/5.8S region among sequences within groups of the Atama complex remained below p<0.03 substitutions per site (Fig. 13), whereas distances among groups was above 0.152; this supports the *A. tamarense* species complex groups I-V as representing separate species.

	A. fundyense (Group I)	***A. mediteraneum*** (Group II)	***A. tamarense*** (Group III)	***A. pacificum*** (Group IV)	***A. australiense*** (Group V)
A. fundyense (Group I)	Average 0.027 (0.000 - 0.087) n = 3,003	Average 0.192 (0.179 - 0.232) n = 539	Average 0.196 (0.187 - 0.226) n = 1,771	Average 0.218 (0.202 - 0.265) n = 9,779	Average 0.217 (0.206 - 0.253) n = 77
A. mediterraneum (Group II)		Average 0.002 0.000 - 0.004 n = 21	Average 0.152 0.151 - 0.158 n = 161	Average 0.196 (0.188 - 0.217) n = 910	Average 0.187 0.186 - 0.189 n = 7
A. tamarense (Group III)			Average 0.001 (0.000 - 0.008) n = 253	Average 0.177 (0.169 - 0.198) n = 2,921	Average 0.164 (0.164 - 0.166) n = 23
A. pacificum (Group IV)				Average 0.009 (0.000 - 0.038) n = 8,001	Average 0.155 0.147 - 0.176 n = 127
A. australiense (Group V)					0.03 n = 2

Fig.13. Among and within species genetic distances (p, substitutions per site) calculated using aligned ITS1/5.8S/ITS2 rDNA sequences. The n value indicated the total number of pairwise comparisons made for each analysis. The average p value in each, among or within species analysis, is presented. The smallest and largest value observed (in parentheses) is presented (John et al., 2014).

1.5. *ALEXANDRIUM MINUTUM* COMPLEX

As already stated in the chapter 1.3.1, there are two major species complex that are known to produce PSP: *A. tamarense* complex and *Alexandrium minutum* complex.

The species of *Alexandrium minutum* complex share a small size (<30 μm), predominantly oval shape and a posterior sulcal plate that is quite wider than it is long (Balech, 1995). Features used for species separation are based on differences on thecal plates, such as shape and size of the 1', 6", s.a. and s.p. plates, details of the Po plate and the presence or absence of a ventral pore situated between plate 1' and plate 4' (Fig. 14 A).

Balech (1995) assigned four species to the *Alexandrium minutum* group: *A. minutum*, *A. lusitanucum* Balech, *A. angustitabulatum* Taylor and *A. andersonii* Balech.

A. lusitanicum and *A. angustitabulatum* are similar to *A. minutum*, and differ only slightly, with *A. lusitanicum* having an anterior sulcal (s.a.) plate that is wider than it is long and *A. angustitabulatum* having a 1' plate with the two larger margins nearly parallel and displaying no ventral pore between plates 1' and 4' (Fig. 14 C). As showed in Fig. 14 (D) *A. andersonii* differs the most from *A. minutum*. In fact, plate 6" has a characteristc arrow-shaped left margin (Balech, 1995).

The degree of connection between the 1' plate and the Po in *A. minutum* and related species varies from a direct connection to being connected by only a filamentous projection of the plate 1'.

Results of morphological and molecular investigations performed by Montresor et al. (2004) revealed that *A. tamutum* was a new species. It shares several morphological characters with *A. minutum*. It is distinguishable from the latter species by the larger size and width of its plate 6".

Montresor et al. (2004) also showed, performing HPLC analyses, that *A. tamutum* strains do not produce PSP toxins, whereas *A. minutum* is a potential PSP toxins producer. Considering that *A. minutum* and *A. tamutum* are so similar in size and cell outline, they cannot be distinguished during routine cell counts in LM. Their precise identification in monitoring programs becomes a rather important issue.

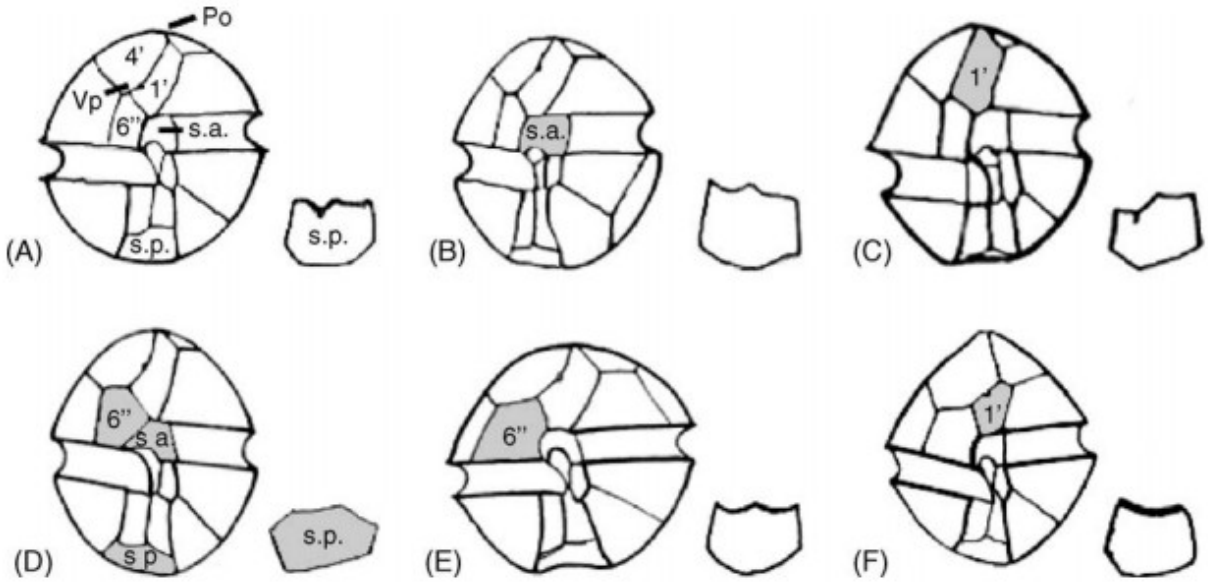


Fig.14. Schematic drawings of ventral view and sulcal plates (s.p.) for the species of the A*lexandrium minutum* complex. Modified from Balech (1995) and Montresor et al. (2004). (A) *A. minutum*, (B) *A. lusitanicum*, (C) *A. angustitabulatum*, (D) *A. andersonii*, (E) *A. tamutum* and (F) *A. insuetum*. In B-F gray highlighting indicates key plates that differ from *A. minutum*. Vp: ventral pore; Po: apical pore; s.a.: sulcal anterior plate. (Lily et al., 2005)

Alexandrium minutum shows a complex population structure and distribution pattern. As stated by Hansen et al. (2003), there is some indication of an *A. minutum* European ribotype and a very different New Zealand ribotype, which perhaps includes western Australian populations. They documented the presence of *A. minutum* cells in Denmark and other European locations that lack a ventral pore, which has been considered a significan species-specific character (Balech, 1995). However, the LSU sequence data did not differentiated between populations with or without ventral pore (Hansen et al., 2003)

Lily et al. (2005) phylogenetic analysis revealed that species of *A. minutum* complex do not appear to be distinctive species. Meanwhile, *A. tamutum* is a valid species, showing consistent morphological and genetic differences from the *A. minutum* isolates.

Furthermore, Lily et al. (2005) proposed *A. insetum* as a valid species that should be considered part of the *A. minutum* complex. They revealed that *A. andersonii* appears to be a separate species and

should not be considered a member of the *A. minutum* complex. They also stated that *A. lusitanicum* and *A. angustitabulatum* are synonyms of *A. minutum*. In the case of *A. lusitanicum*, its status as synonym of A. minutum was already suggested before by other authors (Franco et al., 1995). As already stated in chapter 1.2.4. *A. minutum* was split into two main clades: Global clade and Pacific clade (Lily et al., 2005). Several years later, a study conducted by McCauley et al. (2009), supports the results of Lily et al. (2005) on the existence of these two monophyletic groups using LSU sequences. In particular the Global clade includes both toxic and non-toxic strains. In addition, they conducted a phylogenetic analysis using microsatellite loci, which revealed a geographical structuring of the isolates within the Global Clade. Further, they found that isolates from geographically diverse areas were also genetically distinct from one another. Specifically, strains from Australia, South Africa, France and Portugal formed separate geographic clades; the South African strains contained alleles that were unique to that region. In contrast, isolates from Italy and Spain were genetically diverse.

2. AIM OF THE THESIS

In a routine sampling on 14 September 2015, *Alexandrium* appeared for the first time as the dominant taxon in the microplankton fraction in the estuary of Bilbao, rising the question if this genus posed a risk to the area.

The aim of this work is therefore to study the threat potential of this potentially toxigenic genus in the area. Since this genus had not been addressed before in the area, the first approach to study the issue required the identification of the species present. In order to achieve this, all the *Alexandrium* strains obtained within the monitoring program from year 2004 to 2015 (12 strains collected, 7 of them specifically isolated from the 2015 bloom-event) and never studied, have been characterized. Monitoring programs are mostly based on microscopic analyses and hence, a morphological characterization of the cells is required. Additionally, some of the species form morphologically cryptic complexes, making necessary a genetic identification.

We performed both a morphological characterization, with living cultures and thecal observation of fixed cells stained with Calcofluor M2R white, and a genetic identification using the LSU sequence of the rDNA operon to construct a phylogenetic tree. Finally, the toxicity of the strains was tested using a toxicity assay with *Artemia* sp., which is a branchiopod routinely used as a test organism for screening in ecotoxicological studies (Nunes et al., 2005; Wu Zhenxing et al, 2006).

3. MATHERIALS AND METHODS

3.1. STUDY AREA

The Bilbao estuary (43°23'- 43°14'N, 3°07'-2°55'W), also referred to as the Nervión-Ibaizabal rivers estuary, is the study area for this work. It is a small macro-mesotidal system located in the inner Bay of Biscay on the coast of the Basque Country (Fig. 15). The main characteristics of the estuary are described in Butrón et al. (2009). The climate in the area is temperate, rainy and dominated by NW winds.

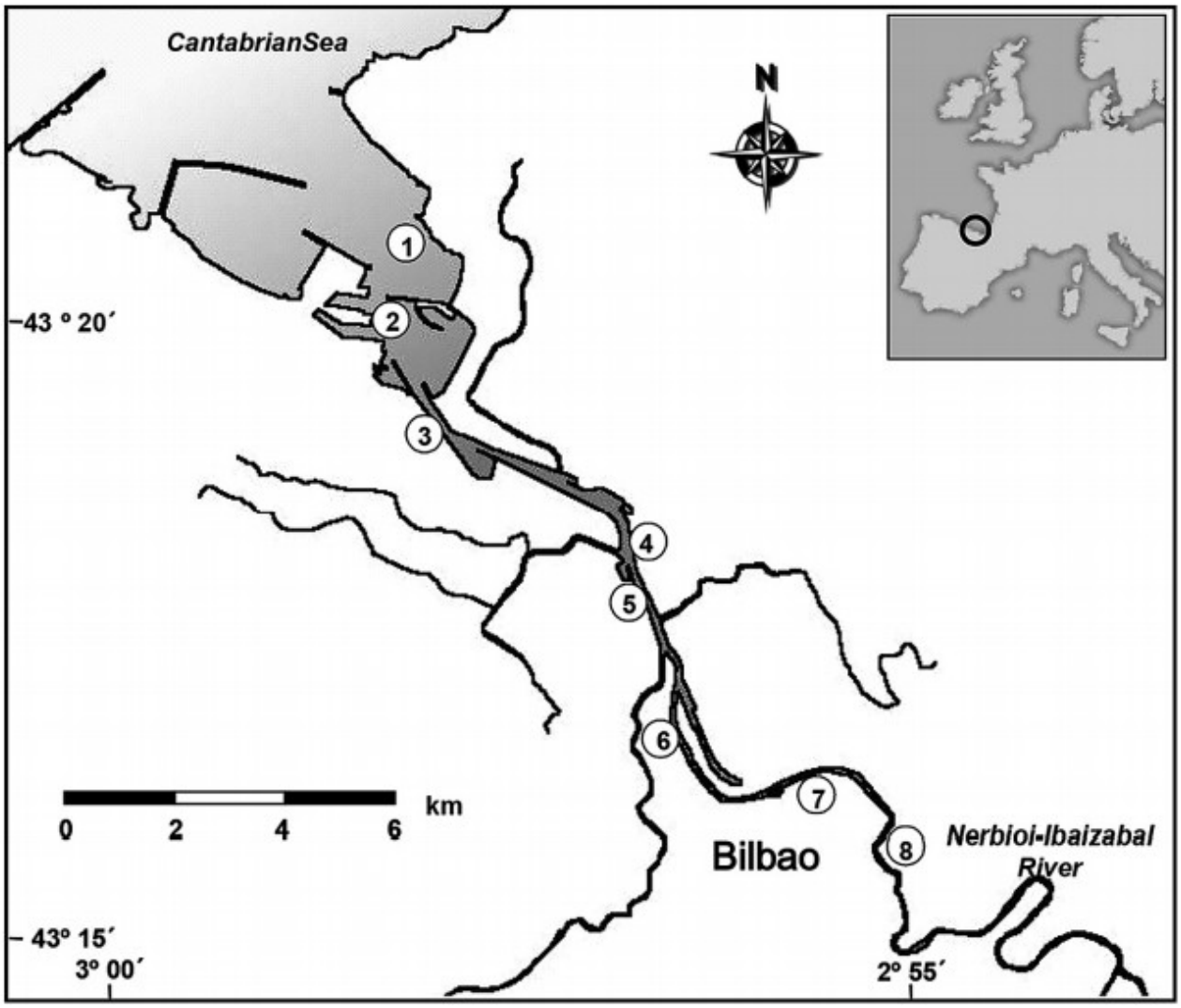


Fig.15. Bilbao estuary. Map showing the location of the estuary and sampling locations: 1, outer Abra; 2, inner Abra; 3, Biscay Bridge (Getxo); 4, Axpe (Erandio); 5, Rontegi (Barakaldo); 6, Zorroza; 7, Deusto; 8, Arriaga

(Abando) (from Cajaraville et al., 2016).

There are two distinct zones: the channel and the bay. The channel si short (15 km long), narrow (50-150 m) and shallow (2-9 m). In the channel a water stratification caused by the salinity occurs. The bay, also called Abra, is a semi-enclosed coastal bay. It is 14-30 m deep, 3.8 km wide and with an area of 30 km². It contains 95% of the total water volume of the estuary.

The estuary of Bilbao has a long history of human activities related to navigation and commerce, also progressive expansion of port, urban and industrial areas. The industrial discharges caused an environmental degradation of estuarine waters, sedimentis and also caused the lost of intertidal areas. Belzunce et al. (2001) showed high levels of pullutants in both water column and sediments. Furthermore, a more recent publication of Cotano and Villate (2006) confirmed the high levels of organic matter and heavy metals in the sediments.

In 2002, a biological treatment came into operation and by that year urban wastes of 80% of the population (800,000 inabitants) were being trated at Galindo WWTP.

The Bilbao estuary (Bay of Biscay, norther Spain) is being monitored for phytoplankton taxonomic composition and abundance since 2000. Although during this period several blooms of harmful and non-harmful species have been observed, no one belonged to the genus *Alexandrium*, which appeared scarcely and occasionally.

3.2. ALGAL CULTURES

Alexandrium strains were previously isolated from samples of the estuary of Bilbao during the years 2004-2015 (Table 1). In total, 12 clonal strains were stablished by picking up single cells with a glass capillary grown in test-tubes and they represented the mother culture. Seven of the strains were isolated from the 2015 bloom event. Stock cultures ere grown in low-light condidions (30 µM photons $m^{-2}s^{-1}$) for maintenance purposes in test-tubes (10 mL of medium) with a light:dark cycle 12:12 at 18° C in a 30 psu salinity f/2 medium (Guillard and Ryther, 1962), modified by adding soil extract and selenium. For the present study, cultures were grown in the same conditions as the stock cultures, except for light, which was rised to standard-intensity conditions (100 µM photons $m^{-2}s^{-1}$). The medium was changed one or two times every month during the study period. For some of the analyses, larger volume cultures were needed and strains were transferred to 75 mL in Erlenmayer flasks (Fig. 16).

Table.1. List of strains used in this work with the sampling date. All strains were isolated from the seaward end of the estuary of Bilbao.

Strain	Date
Alex1	29/09/2015
Alex2	29/09/2015
Alex3	29/09/2015
Alex4	01/09/2014
Alex5	21/10/2014
Alex6	24/08/2015
Alex7	24/08/2015
Alex8	24/08/2015
Alex9	24/08/2015
Alex10	22/05/2004
Alex11	25/06/2004
Alex12	25/08/2009

Fig.16. *Alexandrium* strains cultured in Erlenmayer flasks (100 mL) in the culture-chamber

3.2.1. Preparation of the culture medium

Water at a salinity of 30 psu was prepared from seawater (34 psu) collected from the Bay of Biscay (Bilbao). 874 mL of filtered seawater (34 psu) were brought to a final volume to 1L with distilled water. The resulting water was filtered with a GF/F glass-fibre filter and then pasteurized at 80 °C for 10 minutes. After leaving it to get room temperature, 20 mL of Guillard's (F/2) marine water enrichment solution (Sigma-Aldrich, GmbH), 2 mL of soil exctract and 100 µL of selenium stock solution (0.006 mM Na_2SeO_3, final concentration) were added. Finally, the medium was filtered with a 0.22 µm nitrocellulose (Millipore, Cork) filter and kept in fridge.

3.3. MORPHOLOGICAL CHARACTERIZATION

Morphological observations of live and fixed cells were carried out by examination under a Leica DMRB (Leica Microsystems GmbH, Wetzlar, Germany) direct microscope equipped with epifluorescence. Live and fixed cells were photographed until 100x with Nikon Ds-Fil. For size measurements and thecal plate pattern analysis live cells were harvested from exponentially growing cultures.

3.3.1. Live cells

Cells were harvested directly from cultures and observed under the direct microscope in order to obtain apical and transapical diameter (length and width) measures of 25 cells from each strain in order to perform a significant statistical analysis among strains. Measurements were performed with the program Nis-Elements D 2.30.

3.3.2. Staining with Calcofluor white M2R

20 mL of each strain was fixed with formaldehyde at a final concentration of 5%. Before fixation, the good health status of the cells was checked by light microscopy, since sometimes cells can show aberrant morphologies.

Calcofluor is a specific stain for β-glucans and chitin (Fig. 15). It primarily stains cellulose and requires UV excitation. Fritz and Triemer (1985) found that the fluorochrome Calcofluor White M2R was useful in defining thecal plates of armoured dinoflagellates.(Fig. 17).

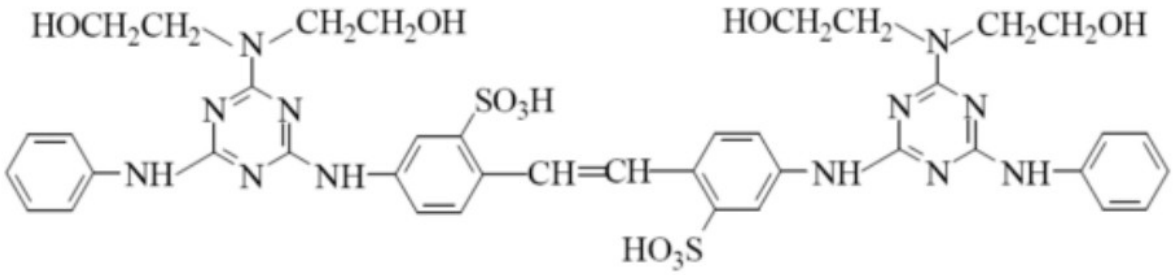


Fig.17. Formula of fluorescent brightener 28 (taken by Rothamasted Bioimaging)

Cells were stained with Calcofluor White M2R (Tinopal, Fluorescent brightener 28, C40 H44 N12 O10 S2) according to the protocol of Fritz and Triemer (1985).

After fixation, the samples were gently centrifugated (500 rpm for 1 minute), the supernatant was removed and the pellet newly suspended in culture medium. The samples were kept in the fridge. At last, 1 mL of each fixed samples was transferred to an eppendorf tube and 100-200 µL of stock solution of Calcofluor White M2R was added.

In the end, thecal plates were visualized at 100x under epifluorescence and then photographed.

3.4. DNA EXTRACTION AND PCR AMPLIFICATION

Approximately 50 mL of an exponentially growing culture were collected through centrifugation (5000 rpm for 5 min). The concentrated culture was moved to 15 mL tubes and centrifuged (5000 rpm for 5 min). The supernatant was removed and the pellet was kept in an eppendorf tube and frozen at -20°C until extraction of total genomic DNA.

DNA was extracted and purified using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

Amplification of the D1-D2 region of the LSU rDNA was performed, with D1C and D2R primers as previously described by Scholin and Anderson (1994). PCR was performed under the following conditions: 95°C for 2 minutes, 50°C for 30 seconds, 72°C for 45 seconds and 35 cycles of 94°C for 30s, 50°C for 90s and a final elongation step at 72°C for 3 minutes.

A Gel electrophoresis procedure was carried out to monitor the success of the PCR and positive results were shipped for sequencing (Fig. 18).

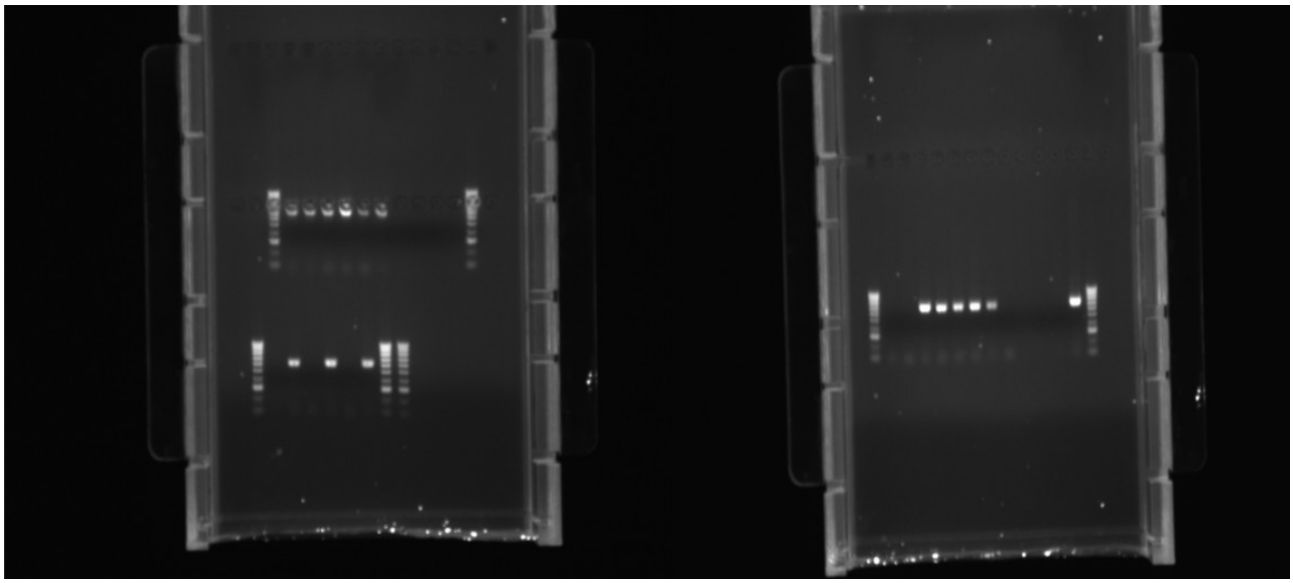


Fig.18. Gel electrophoresis of PCR products using D1C-D2R primers.

3.5. ALIGNMENT AND PHYLOGENETIC ANALYSIS

The partial LSU rDNA sequences (D1-D2 region) of the 12 strains were aligned with 56 other sequences of *Alexandrium* spp. and 3 sequences from other Gonyaulacales retrieved from GenBank (Table 2). The accession numbers are given in the labels of the terminal nodes of the tree. Totally, 19 *Alexandrium* species were represented. Sequences were aligned using CrustalW algorith, with default settings, as implemented in MEGA 6 software (Tamura et al., 2013). Maximum likelihood (ML), maximum parsimony (MP) and neighbour joining (NJ) analyses were conducted using MEGA 6 software. The model TN93+G was selected as the best for the dataset in the phylogenetic model selection for the ML analysis (gamma shape parameter 0.88). MP trees were obtaines with the subtree pruning and regrafting algorithm (search level 1) in which the initial trees were obtaines by the random addition of sequences (10 replicates). Bootstrap values were estimated from 1000.

Table 2. Sequences downloaded from GenBank to be added to the sequences from the present study for the phylogenetic analysis. Here we reported information about: taxon, name of strain, the grographic location, the references and GenBank code. UP: unpublished.

Taxon	Strain	Geographic location	Authors	GenBank accession number
A. affine	-	Vietnam	G. Hansen et al., 2003	AY294612
A. affine	CU1	Thailand, Gulf of Thailand	C.A. Scholin et al., 1994	U44935
A. andersoni	TC02	Thailand, Gulf of Thailand	C.A. Scholin et al., 1994	U44937
A. andersoni	CCMP1718	Massachusetts, USA	R.J. Orr et al., 2011	JF521620
A. australiense	ATBB01/2	Tasmania, Australia	C.A. Scholin et al., 1994	ATU44933
A. australiense	At304A	Japan	S. Hosoi-Tanabe and Y. Sako, 2004 (UP)	AB196481
A. australiense	ATBB01	Tasmania, Australia	C.A. Scholin et al., 1994	KF908810
A. cohorticula	AtMS01	Malaysia	G. Usup et al., 2002	AF174614
A. fraterculus	AF0307MIE07	Japan, Ago-Bay	S. Nagai et al., 2009	AB436943
A. fraterculus	AF0307MIE01	Japan, Ago-Bay	S. Nagai et al., 2009	AB436941
A. fundyense	P2H6	Greenland, Disko Bay	U. Tillman et al., 2016	KP744637
A. fundyense	P2G6	Greenland, Disko Bay	U. Tillman et al., 2016	KP744632
A. fundyense	ACQH01	Washington, USA	U. John et al., 2014	KF908806
A. fundyense	SPE10-3	Massachusetts, USA	U. John et al., 2014	KF908807
A. leei	ASFC01	South China Sea	H. Gu et al., 2013	KF034860
A. leei	AIPA01	-	R. Mohdi Razali 2015 (UP)	KR188516
A. leei	LYD1	Yellow Sea, China	H. Gu et al., 2013	KF034861
A. margalefii	X12	Bay of Concarneau, France	L. Guillou et al., 2002	AF318230
A. margalefii	AGNZ01	Mexico	C.J. Band-Schmidt et al., 2003	AY152707
A. mediterraneum	SZN19	Italy	U. John et al., 2003	AJ535370
A. mediterraneum	SZN01	Italy	U. John et al., 2014	KF908808
A. mediterraneum	SZN21	Italy	U. John et al., 2003	AJ535374
A. minutum	AMIR-3	Ireland	L.A.R. McCauley et al., 2009	EU707474
A. minutum	AL4V	Vigo, Atlantic Spain	L.A.R. McCauley et al., 2009	EU707462
A. minutum	X20	Bretagne, Atlantic France	L. Guillou et al., 2002	AF318232
A. minutum	AMBOPO14	New Zealand	L.A.R. McCauley et al., 2009	EU707469
A. minutum	AMBOPO06	New Zealand	L.A.R. McCauley et al., 2009	EU707468
A. minutum	AMNZ01	New Zealand	L.A.R. McCauley et al., 2009	EU707476
A. minutum	AMNZ02	New Zealand	L.A.R. McCauley et al., 2009	EU707477
A. minutum	SZN30	Italy	U. John et al., 2003	AJ535371
A. ostenfeldii	K0324	Denmark	U. John et al., 2003	AJ535363
A. ostenfeldii	AO1	New River, USA	E.N. Schwarz 2012	JF921171
A. ostenfeldii	AP16	New River, USA	E.N. Schwarz 2012	JF921194
A. ostenfeldii	AOTVA1	Northern Baltic Sea	A. Kremp et al., 2009	FJ011439
A. ostenfeldii	CCNP 4101	Southern Baltic Sea	A. Kremp et al., 2009	KF040968
A. pacificum	ACY12	Ulsan, South Korea	C. Kim et al., 2004	AB088226
A. pacificum	ACPP01	Victoria, Australia	U. John et al., 2014	KF908803
A. pacificum	ATTL01	Thau Lagoon, France	U. John et al., 2014	KF908804
A. pseudogonyaulax	CAWD54	New Zealand	L. MacKenzie et al., 2004	AY338752
A. pseudogonyaulax	APSN	Harimanada, Japan	C. Kim et al., 2004	AB088253
A. satoanum	JHW0007-9	Jinhae Bay, Korea	K.I. Kim et al., 2005	AY438020
A. tamarense	UW42	United Kingdom	W.A. Higman et al., 2001	AJ303428
A. tamarense	UW53	United Kingdom	W.A. Higman et al., 2001	AJ303429
A. tamarense	ATSW01	Gullmar Fjord, Sweden	U. John et al., 2014	KF908805
A. tamiyavanichi	TAMI2207	Harimanada, Japan	C. Kim et al., 2004	AB088267
A. tamiyavanichi	TAMI2201	Harimanada, Japan	C. Kim et al., 2004	AB088264
A. tamutum	S0703601	United Kingdom	L. Brown et al., 2010	GQ120507
A. tamutum	SZN29	Naples, Italy	U. John et al., 2003	AJ535372
A. tamutum	SZN28	Naples, Italy	U. John et al., 2003	AJ535373
A. tamutum	P2E2	Disko Bay, Greenland	U. Tillman et al., 2016	KP744645
A. tamutum	AB/2	Trieste, Italy	E.L. Lily et al., 2005	AY962864
A. tamutum	ALQT01	East China Sea, China	H. Gu et al., 2013	KF034865
A. taylori	Atay99Shio-03	Shioya Bay, Japan	S. Nagai & S. Itakura, 2012	AB607265
A. taylori	Atay99Shio-02	Shioya Bay, Japan	S. Nagai & S. Itakura, 2012	AB607264
A. tropicale	CU-15	-	E.L. Lily et al., UP	AY268607
Gonyaulax elongata	UW388	Orkney, United Kingdom	M. Ellegaard et al., 2003	AY154964
Gonyaulax digitale	UW394	-	M. Ellegaard et al., 2003	AY154963

3.6. TOXICITY BIOASSAY

PSP toxins are tetrahydropurine neurotoxins, which bind to voltage-gate sodium channels of nerves and muscle cells. The saxitoxin is probably the most known representative. A combined approach linked to genomics and proteomics is required to describe toxin biosynthesis and regulation. The early investigations into the biosyntethic pathway of PSP toxins supposed that this pathway was the same in all PSP toxin-producing organisms, and that the corresponding genes was homologous (Shimizu et al., 1984).

Toxicity tests were performed with 10 *Alexandrium* strains (Alex 1 – Alex 8, Alex 11 and Alex 12), selected after the molecular analyses to represent the three identified species. Strain Alex 10 was not tested because it had already been analysed in a previous study (Aylagas et al. 2014). A strain of the non-toxic *Ensiculifera loeblichii* was used as a negative control. The toxicity bioassay was performed in order to test the potential toxicity of the three species founded in this work.

The toxicity of the strains was tested at a cell concentration of 10000 cell/mL. For this, 50 mL of each strain was transferred to Erlenmayer flask (100 mL Kimble) at the same growth conditions of mother culture. The choice of the used strains was based on the greater amount of cells that were visible on the bottom of the flasks.

3.6.1. Monitoring of algal growth

The toxicity tests were performed with cells in the early stationary growth phase. The growth phase of the cultures was monitored by measuring the daily cell density. To achieve this, the *in vivo* fluorescence of cultures was used.

The biophysical properties of phytoplankton pigments and their structuring in functional photosynthetic units determine, at room temperature, emission of red fluorescence from the reaction of photosystem PSII centres, in response to excitation in the visible spectrum. In aquatic ecology this property has been used for decades to detect photoautotrophic organisms (Lorenzen, 1966).

In vivo fluorescence was used to measure *Alexandrium* growth using a Turner Designs 10-100R fluorometer (Fig. 19). Measurements with the fluorometer are made in test-tubes.

Daily, flasks were gently shaken to uniform the cultures and 10 mL aliquots of each strain were transferred from Erlenmayer flasks to the test-tubes for the measurements.

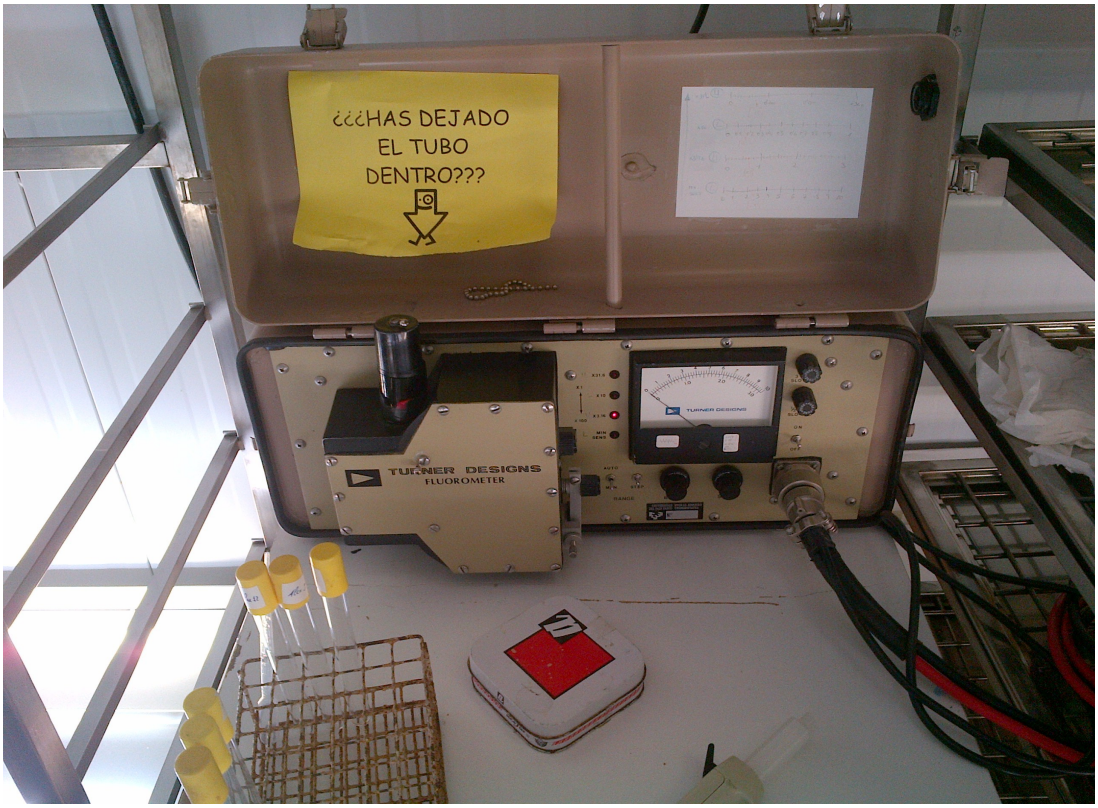


Fig.19. Picture of fluorometer used for measurements of algal growth.

3.6.2. Cell count with Neubauer chamber

The cultures were harvested in the early stationary growth phase. It was necessary to estimate the harvested cell-concentration in order to know the necessary volume to collect 10000cells. The Neubauer chamber, or hemocytometer, is the most common method used for cell counting. It is a thick crystal slide with the size of a glass slide (30 x 70 mm and 4 mm thickness). The central area is where cell counts are performed. The chamber has three parts; the central one is where the counting grid has been set on the glass. Double chambers can be present (Fig. 20).

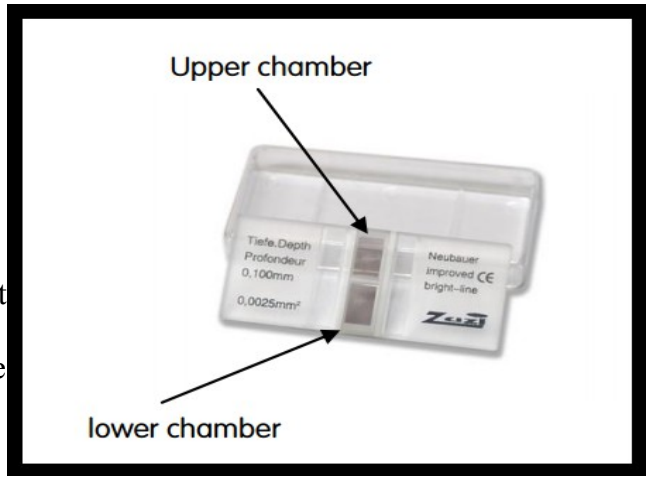


Fig.20. Neubauer commercial chamber (from www.celeromics.com)

After loading the Neubauer chamber, the cell count consisted in counting the number of cells in the four larger squares (Fig. 21 and Fig. 22).

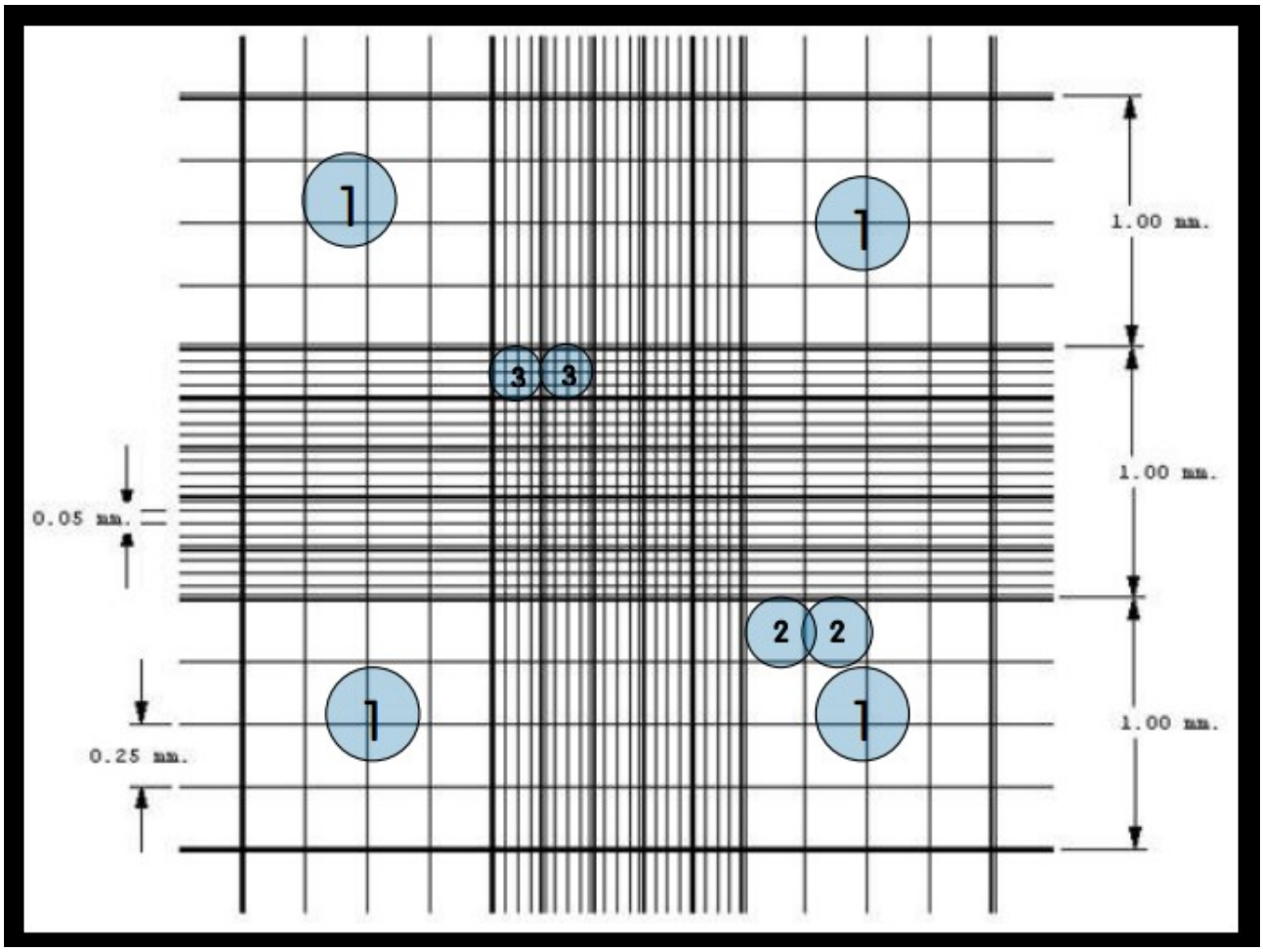


Fig.21. Neubauer-improved chamber grid detail. Squares 2: square where cell count is performed. Squares 1: it represents replicates of cell count, 1 mm width and is characterized by subdivision of 16 squares of 0.25 mm width. (from www.celeromics.com)

Cells clusters in the bottom of the flask (they congregate because of phototrophy) were harvested in

an eppendorf tube (approximately 2 mL). 195 µL were transferred in another eppendorf and fixed with 5 µL lugol; this suspension was used for cell count and the effect of the dilution by fixative addition corrected (÷ 0.975).

$$\text{Concentration} = \frac{\text{Number of Cells x 10.000}}{\text{Number of square x dilution}}$$

Fig.22. Cell concentration formula (from technical note www.celeromics.com)

The appropriate volume to inoculate 10000 cells was derived from the following proportion:

Cell concentration (cell/mL) : 1 mL = 10000 cell/mL : X

3.6.3. *Artemia franciscana*

Artemia franciscana is a Branchiopod crustacean. It is one of the standard experimental organisms used for toxicity bioassay (Sorgeloos et al., 1978). After harvesting and processing, Artemia's cysts are available in cans as storable 'on demand' live feed.

Persoone and Wells (1987) described a test protocol for routine, screening toxicology tests.

Nowadays, after years of scientific works, sustained by debate over *Artemia* characteristics as test organism, this protocol is validated and commercially available (Artoxkit, 1990).

In this work the nauplii stage was used to perform the toxicity bioassay as suggested by Artoxkit.

Artemia franciscana cysts were hatched in 10 mL of standard filtered seawater placed in a 6-well polystyrene multiwell plates, at 20°C in darkness during 30 hours in order to obtain the nauplii stage.

3.6.4. Preparation of the plates and inoculation of strains

A first set of assays was performed with Alex 9 and Alex 12 and second set with Alex 2, Alex 5, Alex 11 and the control *Ensiculifera loeblichii*.

The experiment was carried out in 24-well polystyrene multiwell plates using three replicates for each strain with a final volume of 1 mL each. Three replicates of *Ensiculifera loeblichii* with 1 mL of water culture was used as a negative control. An additional negative control, without any microalga added, was included for each assay round in order to control Artemia's health without algae. Each replicate of toxicity test contained: ten nauplii larvae, microalgae culture with 10000 cells and culture medium added to a final volume of 1mL. Multiwell plates were covered with parafilm and stored at 20°C in darkness. After 24h and 48h dead larvae were counted. When no movement was detected after 10 seconds of observation, larvae were considered dead. When larvae were lying in the bottom of the plate but still showed some appendage movement, they were considered semi-dead.

To consider the test as valid, the mortality in the control must be lower than 10%.

After the assay, dead and alive larvae were observed under the direct microscope to check if microalgae were ingested by larvae.

3.7. STATISTICAL ANALYSES

Normality distribution of cell size parameters (length and width) was tested using the Shapiro-Wilk test (Shapiro & Wilk, 1965). The homogeneity of variances of morphological data was tested using Bartlett's test (Bartlett, 1937). Since the data were not normally distributed and the hypothesis of homoscedasticity was rejected, Kruskal-Wallis test was performed (Kruskal & Wallis, 1952). Post-hoc Dunn's test for multiple comparison was performed (Dunn, 1964), the test has been repeated with Bonferroni correction.

It should be noted that Dunn (1964) originally used a Bonferroni adjustment to account for Type I error inflation. This latter test is more conservative than the first (no adjustment) and could not show significant differences even if they are present. Hence, in this study both adjustments were performed.

The potentially toxic effects of algal strains on Artemia's larvae was tested with a non-parametric procedure for the analysis of longitudinal data in factorial experiments (F1-LD-F1), Wald-type statistics and ANOVA-type statistics were performed. Dunn's multiple comparisons with and without Holm's adjustment (Holm, 1979) were performed on 72h time point.

R-Studio Version 0.99.489 was used to perform the statstical analyses; “PMCMR”, “nparLD” and “agricolae” R-packages were also used (De Mendiburu, 2016; Pohlert, 2014; Noguchi et al., 2012).

The tables and boxplots of data were made with the spreadsheet from an opensource Openoffice 4.1.2 and R-Studio Version 0.99.489.

4. RESULTS

4.1. MORPHOLOGICAL CHARACTERIZATION

4.1.1. Live cells

Cell length ranged from 41,42 µm to 14,66 µm. Bartlett test of homogeneity of variances and Shapiro-Wilk normality test for length data wasn't significant, the output for each test is presented below:

```
> bartlett.test(dataset$Length, dataset$Strains)

      Bartlett test of homogeneity of variances

data:  dataset$Length and dataset$Strains

Bartlett's K-squared = 26.638, df = 11, p-value = 0.00521

> shapiro.test(dataset$Length)

      Shapiro-Wilk normality test

data:  dataset$Length

W = 0.95261, p-value = 2.855e-08
```

Cell width ranged from 40,67 µm to 12,72 µm. Shapiro-Wilk normality test and Bartlett's test of homogeneity of variances for width data wasn't significant. The output for each test is presented below:

```
> shapiro.test(dataset$Width)

      Shapiro-Wilk normality test

data:  dataset$Width

W = 0.96355, p-value = 7.484e-07

> bartlett.test(dataset$Width, dataset$Strains)

      Bartlett test of homogeneity of variances

data:  dataset$Width and dataset$Strains
```

```
Bartlett's K-squared = 20.828, df = 11, p-value = 0.0352
```

Since the data violate the assumptions of normality and homoscedasticity of the ANOVA, a Kruskal-Wallis test was performed on both the length and width data. The p-value of the length data turns out to be nearly to zero (p-value < 2.2e-16). The null hypothesis of same populations was rejected. The output is presented below:

```
Kruskal-Wallis rank sum test

data:  dataset$Length by dataset$Strains

Kruskal-Wallis chi-squared = 188.84, df = 11, p-value < 2.2e-16
```

The p-value of the width data turns out to be nearly to zero (p-value < 2.2e-16). Hence de null hypothesis of same populations was rejected. The output is presented below:

```
        Kruskal-Wallis rank sum test

data:  dataset$Width by dataset$Strains

Kruskal-Wallis chi-squared = 184.32, df = 11, p-value < 2.2e-16
```

Table 3. Size measurements of strains. L = average length; W = average width; L/W = average length/width ratio; n = number of samples.

Strains	L (µm)	W (µm)	L/W ratio	n
Alex 1	29.22±3.69	26.84±3.12	1.09±0.05	25
Alex 2	33.39±3.47	29.33±3.19	1.14±0.08	25
Alex 3	30.2±2.61	26.73±2.47	1.13±0.07	25
Alex 4	31.62±3.09	30.09±3.36	1.05±0.06	25
Alex 5	30.85±3.71	30.34±3.34	1.02±0.09	25
Alex 6	35.7±3.58	30.95±3.90	1.16±0.08	25
Alex 7	29.82±2.47	27.32±2.49	1.09±0.06	25
Alex 8	30.37±2.78	28.16±2.74	1.08±0.05	25
Alex 9	31.42±2.74	30.17±3.10	1.05±0.06	25
Alex 10	18.88±1.80	18.13±1.96	1.04±0.07	25
Alex 11	18.41±2.73	16.33±2.44	1.13±0.09	25
Alex 12	22.09±4.31	20.19±3.88	1.1±0.11	25
Overall mean	28.5±5.59	26.22±5.09	1.09±0.04	12

As we can see on the table above (Table 3) we have a length/width ratio approximately homogeneous, close to 1 and this means that all the strains are as wide as long or slightly longer then wide.

As the Kruskal-Wallis Test statistics of both length and width is highly significant (width: $\chi^2(11) = 184.32$, $p<0.001$; length: $\chi^2(11) = 188.84$, $p<0.001$), the Dunn's post hoc test for multiple comparisons have been applied. Strains from Alex 1 to Alex 9 have approximately the same average length and width, while Alex 10 and Alex 11 are different from all the others and Alex 12 also is significantly different from the other strains. The results of the post hoc Dunn's test are shown in Table 4.

Table 4. Summary table of post hoc Dunn's test. (A) Width and (B) length multiple comparisons, no p-value adjustment. (C) Width and (D) length multiple comparisons, Bonferroni adjustment. The color of each table represents the degree of significance; red: almost zero; blue: 0.001; yellow: 0.01.

(A)

STRAINS	Alex 1	Alex 10	Alex 11	Alex 12	Alex 2	Alex 3	Alex 4	Alex 5	Alex 6	Alex 7	Alex 8
Alex 10	2,00E-005	-									
Alex 11	8,90E-007	0,51538	-								
Alex 12	0,00091	0,34312	0,10993	-							
Alex 2	0,0402	2,70E-010	3,30E-012	8,00E-008	-						
Alex 3	0,96034	2,50E-005	1,10E-006	0,00109	0,0356	-					
Alex 4	0,0091	6,30E-012	5,40E-014	3,10E-009	0,5777	0,00786	-				
Alex 5	0,00465	1,30E-012	9,60E-015	7,90E-010	0,4363	0,00398	0,82453	-			
Alex 6	0,00111	5,30E-014	3,00E-016	4,80E-011	0,22672	0,00093	0,51433	0,66691	-		
Alex 7	0,63579	2,20E-006	7,10E-008	0,00015	0,11454	0,60075	0,03277	0,01844	0,00532	-	
Alex 8	0,18476	2,30E-008	4,40E-010	3,40E-006	0,4816	0,16883	0,19977	0,1326	0,05307	0,39386	-
Alex 9	0,0051	1,60E-012	1,20E-014	9,50E-010	0,45379	0,00436	0,84745	0,97659	0,6457	0,01995	0,14032

(B)

STRAINS	Alex 1	Alex 10	Alex 11	Alex 12	Alex 2	Alex 3	Alex 4	Alex 5	Alex 6	Alex 7	Alex 8
Alex 10	6,40E-006	-									
Alex 11	1,80E-006	0,7917	-								
Alex 12	0,00079	0,2474	0,15538	-							
Alex 2	0,00179	2,20E-014	2,70E-015	9,10E-011	-						
Alex 3	0,40159	8,60E-008	1,90E-008	2,70E-005	0,02232	-					
Alex 4	0,05904	1,50E-010	2,60E-011	1,60E-007	0,21655	0,29414	-				
Alex 5	0,18022	4,80E-009	9,40E-010	2,60E-006	0,0745	0,61615	0,58385	-			
Alex 6	7,80E-006	< 2e-16	< 2e-16	5,00E-015	0,1781	0,00028	0,00981	0,00175	-		
Alex 7	0,6323	6,00E-007	1,50E-007	0,00013	0,00817	0,71863	0,15872	0,38891	6,60E-005	-	
Alex 8	0,34687	4,90E-008	1,10E-008	1,70E-005	0,02904	0,91884	0,34354	0,68958	0,00042	0,64395	-
Alex 9	0,06298	1,80E-010	3,20E-011	1,80E-007	0,20613	0,30746	0,97724	0,60359	0,00903	0,16733	0,35827

(C)

STRAINS	Alex 1	Alex 10	Alex 11	Alex 12	Alex 2	Alex 3	Alex 4	Alex 5	Alex 6	Alex 7	Alex 8
Alex 10	0,00133	-									
Alex 11	5,90E-005	1	-								
Alex 12	0,06027	1	1	-							
Alex 2	1	1,80E-008	2,10E-010	5,30E-006	-						
Alex 3	1	0,00165	7,60E-005	0,07192	1	-					
Alex 4	0,60028	4,20E-010	3,50E-012	2,10E-007	1	0,51852	-				
Alex 5	0,30705	8,60E-011	6,30E-013	5,20E-008	1	0,26255	1	-			
Alex 6	0,07338	3,50E-012	2,00E-014	3,20E-009	1	0,06151	1	1	-		
Alex 7	1	0,00014	4,70E-006	0,00996	1	1	1	1	0,35114	-	
Alex 8	1	1,50E-006	2,90E-008	0,00023	1	1	1	1	1	1	-
Alex 9	0,33641	1,10E-010	8,00E-013	6,30E-008	1	0,28805	1	1	1	1	1

(D)

STRAINS	Alex 1	Alex 10	Alex 11	Alex 12	Alex 2	Alex 3	Alex 4	Alex 5	Alex 6	Alex 7	Alex 8
Alex 10	0,00042	-									
Alex 11	0,00012	1	-								
Alex 12	0,0519	1	1	-							
Alex 2	0,1179	1,50E-012	1,80E-013	6,00E-009	-						
Alex 3	1	5,70E-006	1,30E-006	0,0017	1	-					
Alex 4	1	1,00E-008	1,70E-009	1,00E-005	1	1	-				
Alex 5	1	3,20E-007	6,20E-008	0,00017	1	1	1	-			
Alex 6	0,00052	< 2e-16	< 2e-16	3,30E-013	1	0,0186	0,64762	0,11532	-		
Alex 7	1	3,90E-005	9,70E-006	0,00825	0,53895	1	1	1	0,00433	-	
Alex 8	1	3,20E-006	7,10E-007	0,00114	1	1	1	1	0,02747	1	-
Alex 9	1	1,20E-008	2,10E-009	1,20E-005	1	1	1	1	0,59601	1	1

The post hoc Dunn's test shows that strains Alex 10-Alex 12 show more significant differences to all 12 strains either regarding the length and the width, both Bonferroni and no Bonferroni p-value adjustment (Table 4).

These results show that the twelve strains are subdivided in two groups (Alex 1-9, Alex 10-12) based on size parameters.

From the boxplots, a clear division between the strains from Alex 1 to Alex 9 and the strains from Alex 10 to Alex 12 was visible on length and width (Fig. 23).

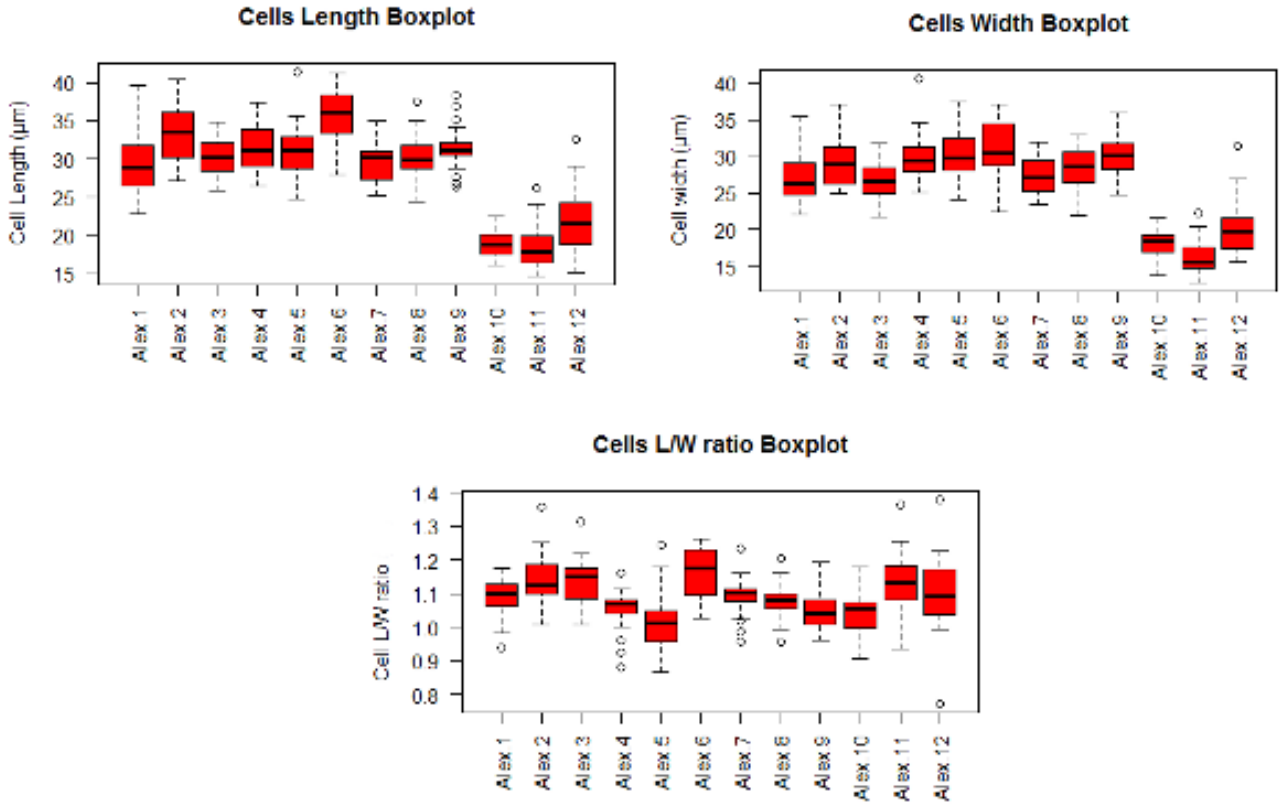


Fig.23. Size parameters boxplots. Length (µm), on the left; width (µm), on the right ; L/W, stands for length and width ratio.

Cells of the strains from Alex 1 to Alex 9 were almost always single-celled and were rarely found in chains of two. The epitheca was helmet-shaped and the hypotheca roughly trapezoidal (Fig. 24). These descriptions are in agreement with the species of the *Alexandrium tamarense* complex from

John et al. (2014).

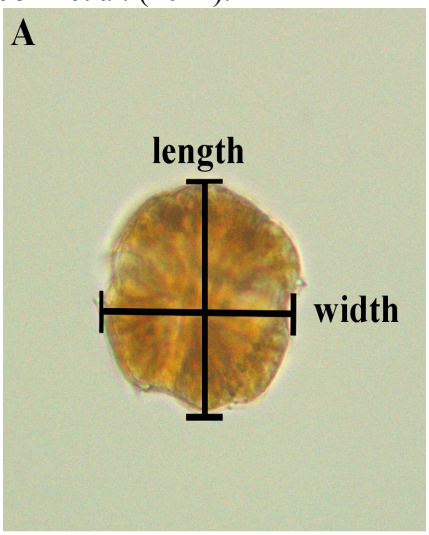


Fig.24. Live cells observations (x40) captured by Leica DMRB. (A) Cell of the strain Alex 4 with lines that marked the length and width measurements. (B) Cell of the strain Alex 1.

Cells of the strains Alex 10 and Alex 11 were always single. Furthermore, cells were small with cells of Alex 11 with an average length of 18.41 ± 2.73 (µm) and an average width of 16.33 ± 2.44 (µm), slightly longer than wide. Cells of Alex 10 had an average length of 18.88 ± 1.80 (µm) and an average width of 18.13 ± 1.96 (µm). Cells were somewhat oval (Fig. 25), the epitheca was hemielliptical and the hypotheca was short and hemielliptical with a convex to flat antapex.

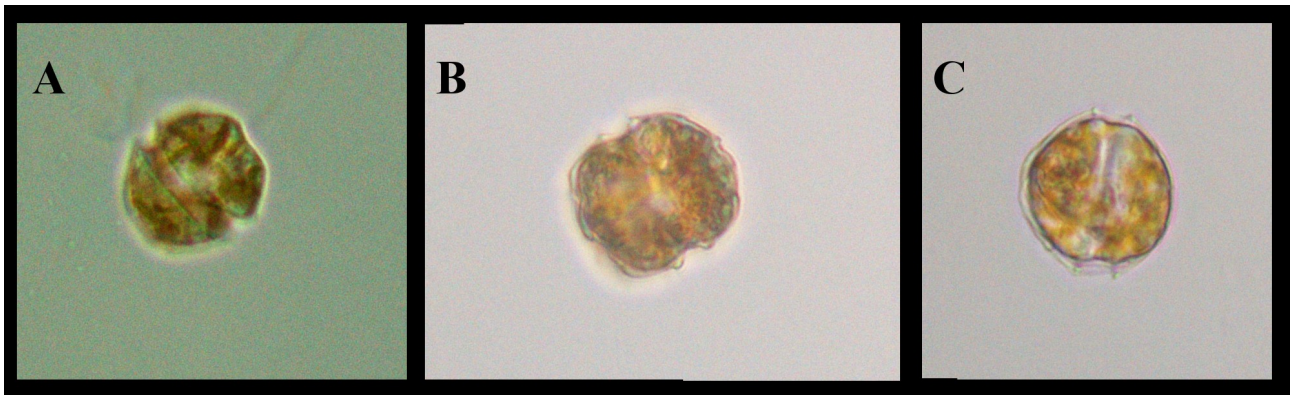


Fig.25. Live cells observations (x40) captured by Leica DMRB. (A) Cell of the strain Alex 11. (B and C) Cell of the strain Alex 10.

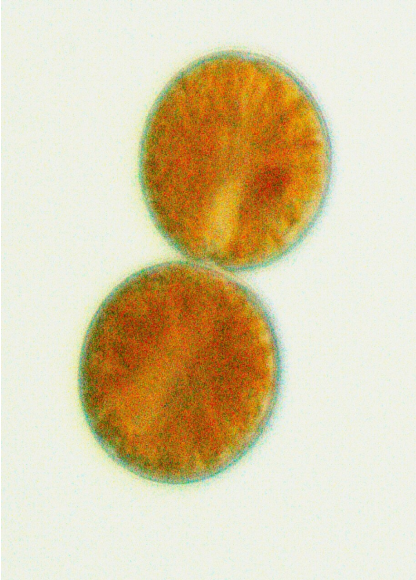


Fig.26. Live cells observations (x40) captured by Leica DMRB of the strain Alex 12.

There were no obvious differences between the cells of strain 12 and the cells of strains 1-9. However, they were more similar in size to strains 10-11. General shape was spherical, more similar to that of Alex 10-11 (Fig. 26). Cells were almost always single but chains of two were also observed.

4.1.2. Calcofluor-stained cells

Strains of the larger cell-size group (Alex 1-9) showed the same plate-pattern, which coincides with that of the *Alexandrium tamarense* complex.

The apical pore (Po) was ornamented by several small pores and it presented a comma-shaped foramen. The presence of a connecting pore in the Po was a variable feature (Fig. 27 C-D): it was present in some strains but not in others. Alex 2, Alex 4, Alex 6, Alex 7 and Alex 9 showed a connecting pore in the Po. In some strains both cells with and without connecting pore were observed (Alex 2, Alex 4, Alex 6 and Alex 9). In all cells observed in Alex 1, Alex 3 and Alex 5 a connecting pore on Po was absent. Plate 1' was irregularly rhomboidal, its apical portion contacted the Po and its antapical portion contacted the sulcal anterior plate (s.a.). The ventral pore (v.p.) along the margin between Plate 1' and 4' was always present. V.p. position and size dimension were variable among the strains (Fig. 27 E-H). In Alex 1-3 the size ranged from medium to small while in the other strains it always ranged from medium to small size. Its position along the right margin was variable with dominance of median and inferior position. Plate 6" is as wide as tall or slightly wider

than tall (Fig. 27 N). Its anterior margins along plates 1' and 4' are generally straight making a roof-ridge shape. The anterior-right margin was observed to be concave while the anterior-left margin was convex to the plate 1'; the left margin was generally concave.

The s.a. was generally A-shaped and longer than wide (Fig. 27 L). This plate always showed a distinct line-form mark in the anterior part. The posterior sulcal plate (s.p.) was pentagonal and almost always horizontal grooves were observed. It was as long as wide or slightly longer than wide (Fig. 27 I, M). The connecting pore on the s.p. was observed in Alex 7 and in few cells of Alex 5.

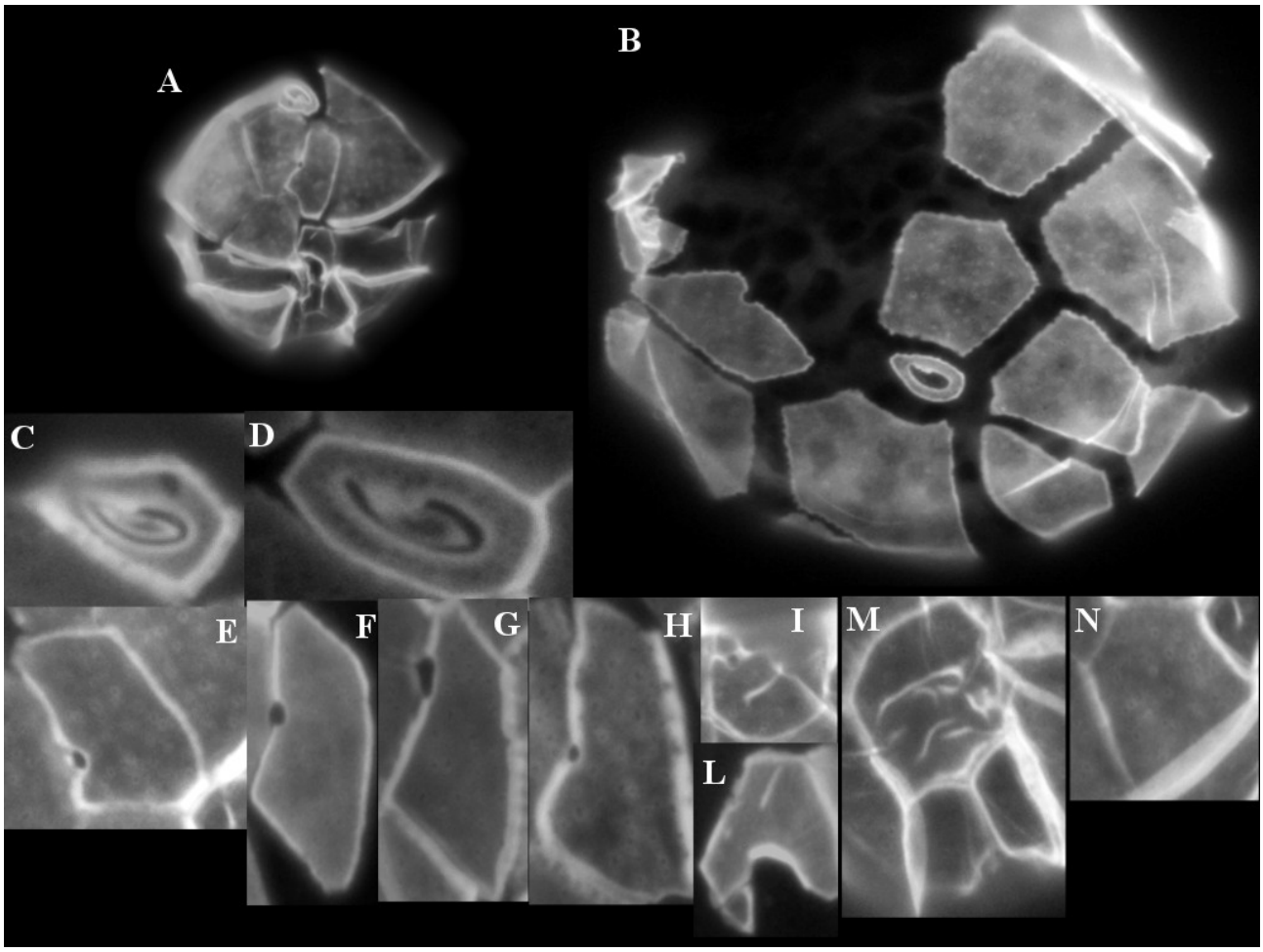


Fig.27. Strains from Alex 1 to Alex 9. Fluorescence images of calcofluor stained cells. (A) Whole cell in ventral view. (B) Apical view showing the series of epithecal plates. (C-D) Detailed view of pore plate with (C) and without (D) connecting pore. (E-H) Detailed view of plate 1' and variability on v.p. position and dimension, (E) inferior and normal size, (F) median and normal size, (G) superior and large size, (H) inferior and small size. (I, M) Detailed view of the posterior sulcal plate (s.p.) with (I) and without (M) connecting pore. (L) Detailed view of anterior sulcal plate (s.a.). (N) Detailed view of the last precingular plate 6". Plate nomenclature according to Kofoidian system.

Among the smaller sized strains, two plates' related morphotypes were distinguished: Alex 10-11 on the one side and Alex 12 on the other.

Morphology of strains Alex 10-11 coincided with that of the *Alexandrium minutum* complex.

The Po plate had a distinct comma and scattered marginal pores (Fig. 28 A, C). Either the strains didn't presented the connecting pore on the Po plate. The first apical plate (1') shape displayed

considerable variation (Fig. 28 A, E, H, I). It was always connected to the Po by a more or less narrow extension (Fig. 28 H, I). In both strains, the anterior tip of the 1' plate rarely appeared divided by a sort of transverse ridge; this feature was already described by Hansen et al. (2003). No v.p. between plate 1' and plate 4'' has been founded in Alex 11 while it has been observed in Alex 10, always in the inferior part of the plate 1', the size ranged from small to normal size.

The plate 6'' was narrow, longer than wide, with a roof-ridge shape of either the edges of plate 1' and plate 4'' (Fig. 28 B). The s.a. usually had a more or less straight anterior margin and a length:width ratio close to one (Fig. 28 D).

The s.p. was short and no grooves were observed, a symmetric roof-shape was sometimes recorded (Fig. 28 G) while others specimens had rounded edges (Fig. 28 F). The connecting pore on the s.p. was a variable feature. It wasn't present in Alex 11 while it was in the strain Alex 10 although not in all the cells.

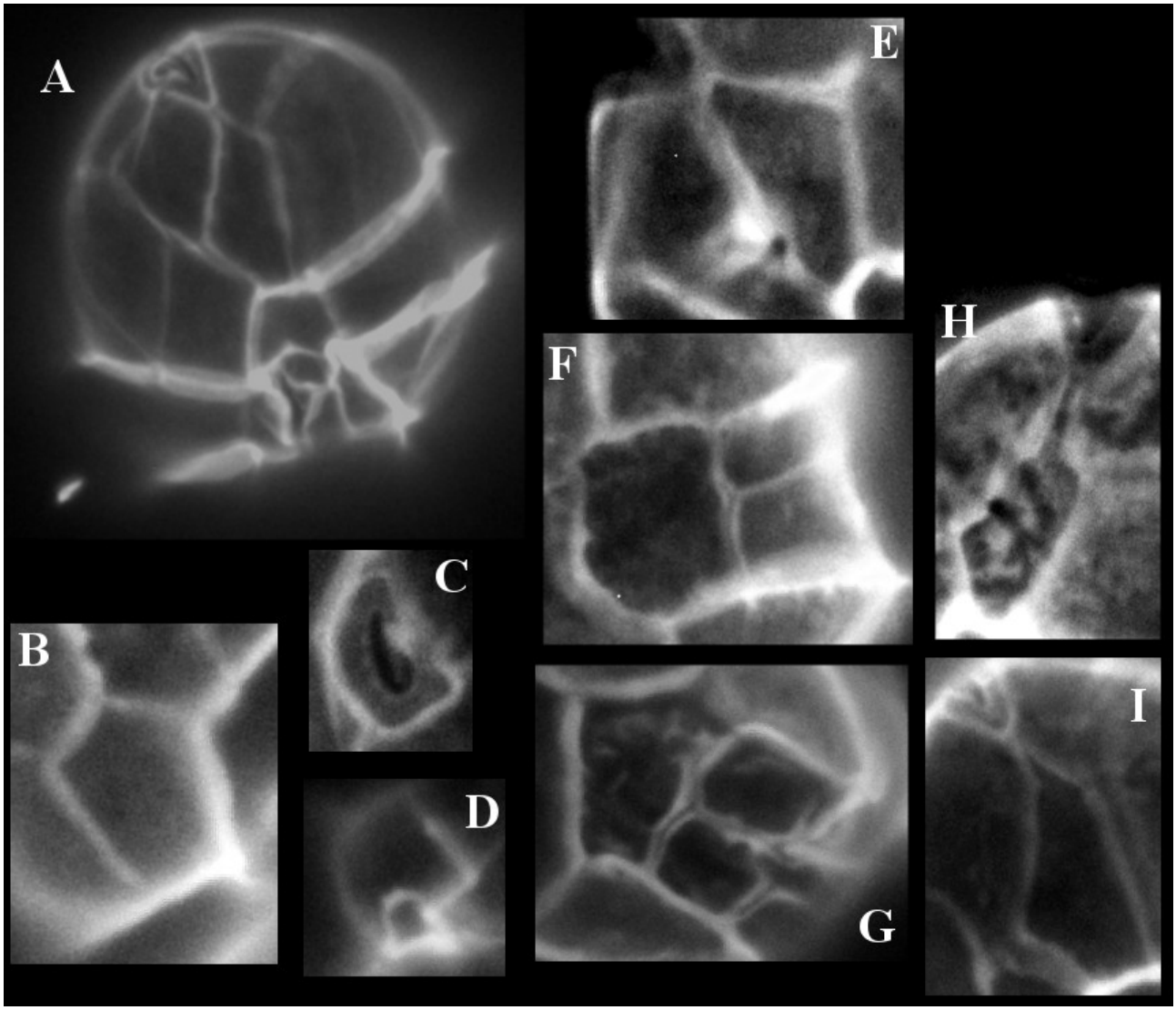


Fig.28. Alex 10 and Alex 11. Fluorescence image of calcofluor stained cells. (A) Whole cell in ventral view. (B) Detailed view of the last precingular plate 6". (C) Detailed view of pore plate (Po). (D) Detailed view of anterior sulcal plate (s.a.). (E, H, I) Detailed view of plate 1' and variability of connection to Po and presence/absence of ventral pore. (F, G) Detailed view of posterior sulcal plate (s.p) with rounded edges (F) and with roof-shape edges (G). Plates' nomenclature according to Kofoidian system.

Plate morphology of Alex 12 coincided with *Alexandrium tamutum.* The first apical plate, plate 1', contacted Po directly and was irregularly rhomboidal. A small ventral pore was always present on

the middle right margin (Fig. 29 C-E).

The pore plate (Po) was comma-shaped bordered with marginal pores.

The sixth precingular plate (6") was as wide as long with some specimens wider than longer. The margins with plate 1' and plate 4" converged giving a shape-roof (Fig. 29 B).

The anterior sulcal plate (s.a.) was A-shaped, as wide as long slightly taller than wider (Fig. 29 F, G). The right margin was oblique and in contact with plate 6".

The posterior sulcal plate (s.p.) was almost asymmetric and rectangular, as wide as long slightly longer than wider (Fig. 29 H, I). The margin that faced with cingulum was concave with double humps caused by cingulum plates (s.d.p. and s.s.p.). The opposit margin was rounded. It is smooth , no grooves were observed, no connecting pore.

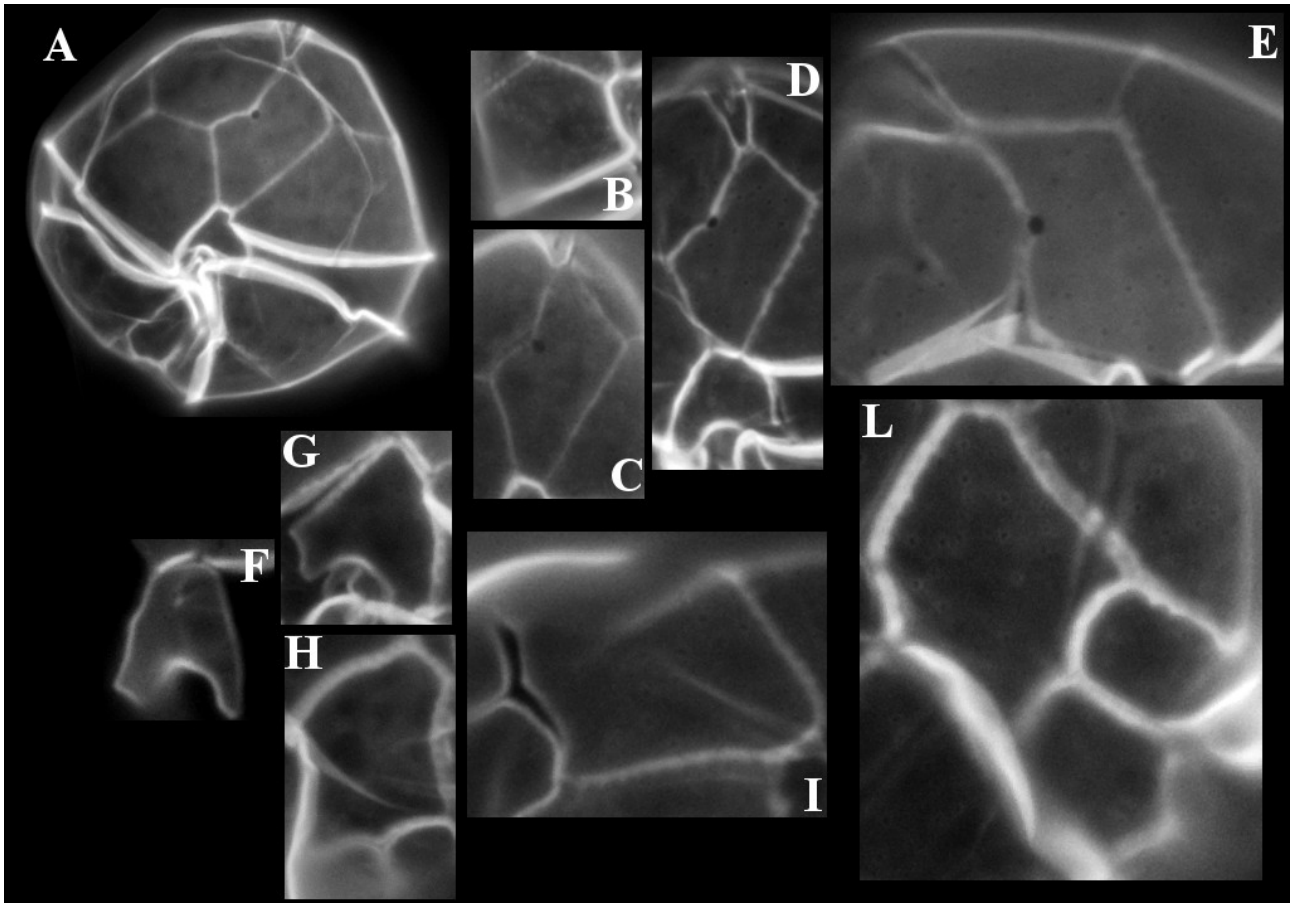


Fig.29. Strain Alex 12. Fluorescence image of calcofluor stained cells. (A) Whole cell in ventral view. (B) Detailed view of the last precingular plate 6". (C-E) Detailed view of plate 1' and his connection with s.a. (D). (F, G) Detailed view of anterior sulcal plate (s.a) with rounded apical margins (F) and roof-shape (G). (H, I) Detailed view of the posterior sulcal plate (s.p). Plates' nomenclature according to Kofoidian system

4.2. MOLECULAR ANALYSES

The Maximum likelihood analysis of LSU rDNA revealed that sequences of the *Alexandrium* isolates from the Bilbao estuary clustered in three different clades. All larger-sized strains (Alex1 to Alex9), including the 7 strains from the 2105 bloom event and the two isolated in 2014, clustered with *A. mediterraneum* (Group II of *A. tamarense* species complex) sequences. The 2 strains isolated in 2004 (Alex 10 and Alex 11) clustered with *Alexandrium minutum* sequences, while the last strain (Alex 12) isolated in 2009 clustered with *Alexandrium tamutum* sequences.

Alexandrium minutum sequences were divided in two clades. The local strains clustered in the clade containing other European strains (Fig. 30).

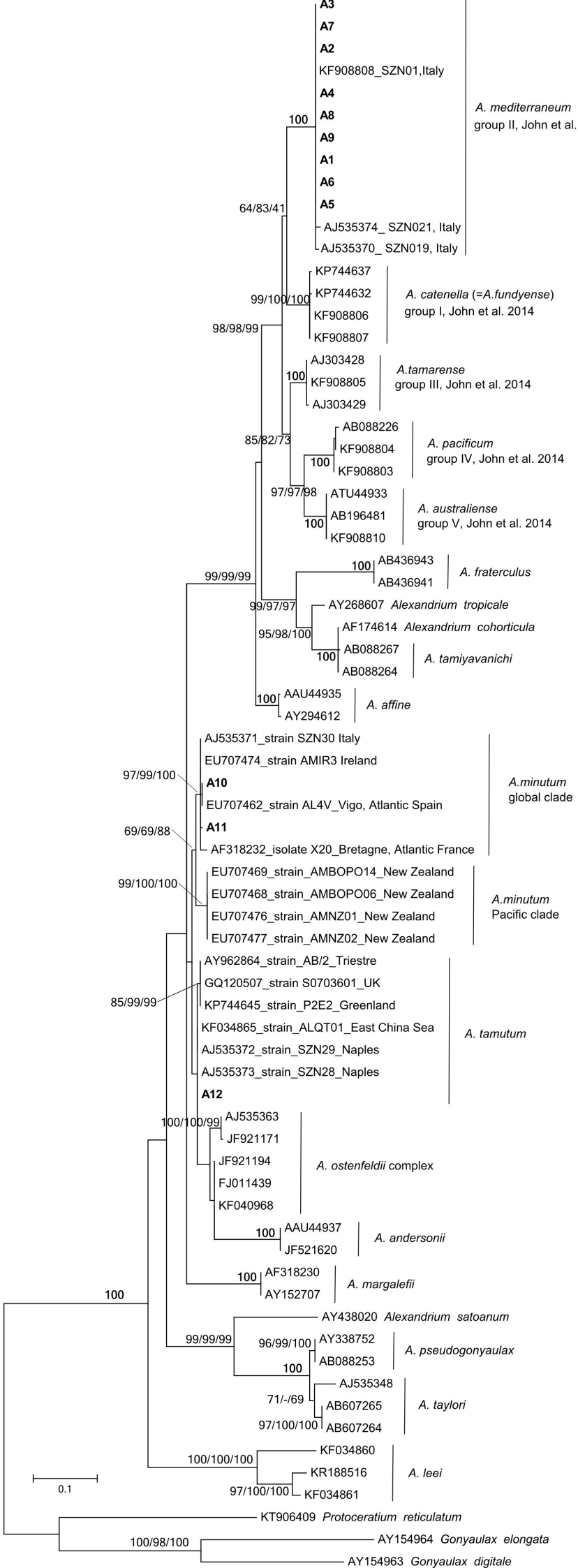

A3
A7
A2
KF908808_SZN01,Italy
A4
A8
A9
A1
A6
A5
AJ535374_ SZN021, Italy
AJ535370_ SZN019, Italy
A. mediterraneum
group II, John et al. 2014
100
64/83/41
KP744637
KP744632
KF908806
KF908807
A. catenella (=A.fundyense)
group I, John et al. 2014
99/100/100
98/98/99
AJ303428
KF908805
AJ303429
A.tamarense
group III, John et al. 2014
100
AB088226
KF908804
KF908803
A. pacificum
group IV, John et al. 2014
85/82/73
100
ATU44933
AB196481
KF908810
A. australiense
group V, John et al. 2014
97/97/98
100
AB436943
AB436941
A. fraterculus
100
99/99/99
AY268607 Alexandrium tropicale
99/97/97
AF174614 Alexandrium cohorticula
95/98/100
AB088267
AB088264
A. tamiyavanichi
100
AAU44935
AY294612
A. affine
100
AJ535371_strain SZN30 Italy
EU707474_strain AMIR3 Ireland
A10
EU707462_strain AL4V_Vigo, Atlantic Spain
A11
AF318232_isolate X20_Bretagne, Atlantic France
A.minutum
global clade
97/99/100
69/69/88
EU707469_strain_AMBOPO14_New Zealand
EU707468_strain_AMBOPO06_New Zealand
EU707476_strain_AMNZ01_New Zealand
EU707477_strain_AMNZ02_New Zealand
A.minutum
Pacific clade
99/100/100
AY962864_strain_AB/2_Triestre
GQ120507_strain S0703601_UK
KP744645_strain_P2E2_Greenland
KF034865_strain_ALQT01_East China Sea
AJ535372_strain_SZN29_Naples
AJ535373_strain_SZN28_Naples
A12
A. tamutum
85/99/99
AJ535363
JF921171
JF921194
FJ011439
KF040968
A. ostenfeldii complex
100/100/99
AAU44937
JF521620
A. andersonii
100
AF318230
AY152707
A. margalefii
100
100
AY438020 Alexandrium satoanum
99/99/99
96/99/100
AY338752
AB088253
A. pseudogonyaulax
100
AJ535348
AB607265
AB607264
A. taylori
71/-/69
97/100/100
KF034860
KR188516
KF034861
A. leei
100/100/100
97/100/100
0.1
KT906409 Protoceratium reticulatum
AY154964 Gonyaulax elongata
100/98/100
AY154963 Gonyaulax digitale

Fig.30. Maximum likelihood tree inferred from *Alexandrium* LSU rDNA (D1-D2 region) sequences in addition to three Gonyaulacales sequences used as outgroup. Labels from the terminal nodes refer to GenBank accession numbers. In clades containing sequences from this study, strain name and origin are also given. Species names are on the right. Species from the *A. tamarense* complex groups according to John et al. (2014) are also included. Strains from this study are written with bold type character. Values in the nodes indicate support in ML/MP/NJ analyses (bootstrap values estimated from 1,000 replicates; 100=100/100/100).

4.3. TOXICITY BIOASSAY

All the nauplii larvae from both negative control assays (without any algae added and with *Ensiculifera loeblichii*) survived till 72 hours.

Growth curves of several strains used for the toxicity bioassay are reported in Fig. 31. Some differences can be observed even within the same species. For example, in *A. mediterraneum*, Alex 2 had a short exponential phase until the fifth day followed by a long stationary phase characterized by a very slow growth. Alex 9 had a long exponential phase from the third up to tenth day while Alex 5 had an exponential phase from the third up to eighth day, with a slow growth till the tenth day. Except for Alex 9, which had low cell density, all the *Alexandrium mediterraneum* strains have been used for the toxicity bioassay.

Alexandrium tamutum (Alex 12) had a slow and long exponential phase till the thirteenth day. *Alexandrium minutum* (Alex 11) had a long lag phase and a late exponential phase that lasted from the sixth day up to eighth.

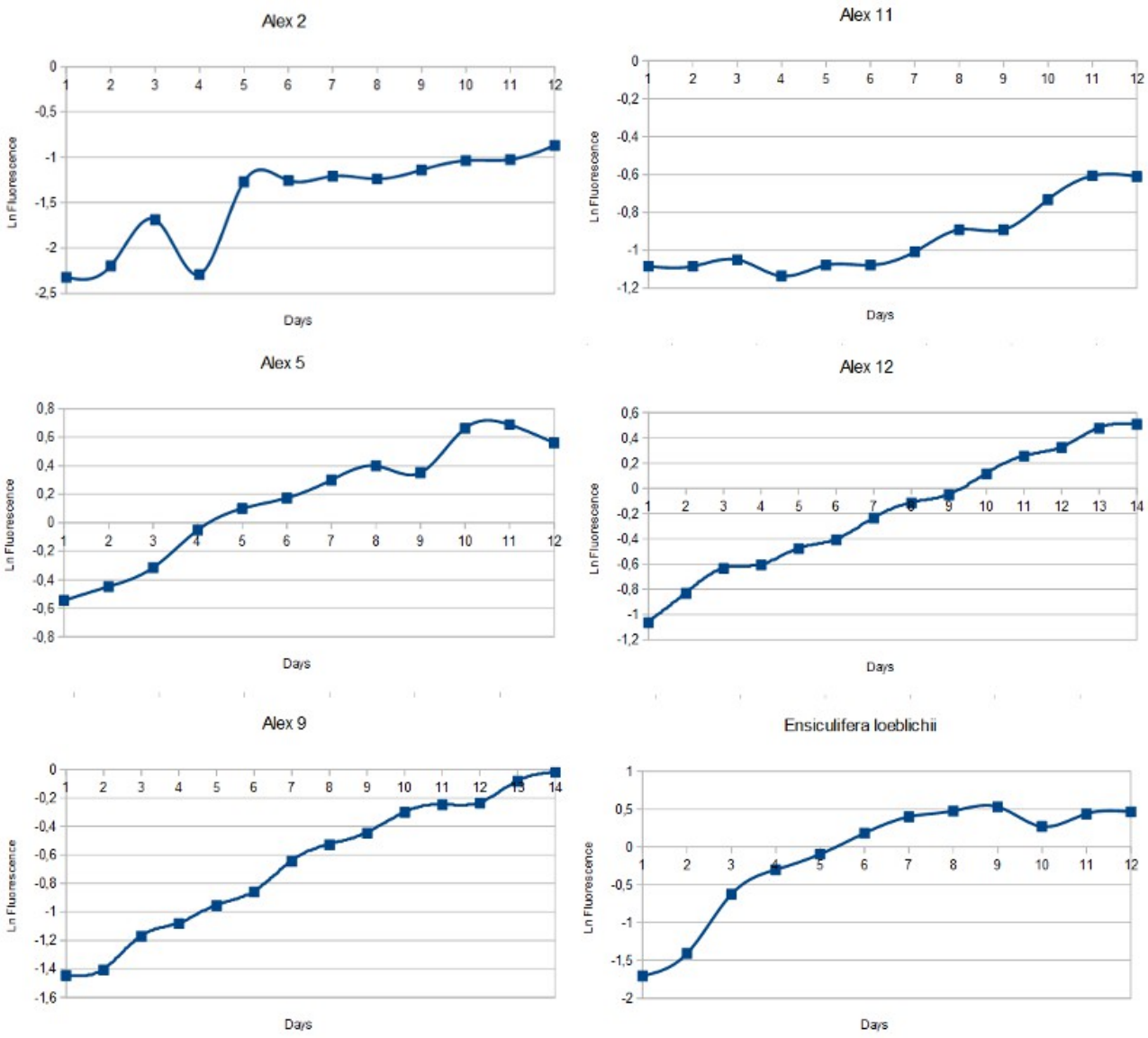


Fig.31. Semilogarithmic growth curves, using a natural logarithm for the Y-axis, of Alex 2, Alex 5, Alex 9 (*Alexandrium mediterraneum*), Alex 11 (*Alexandrium minutum*), Alex 12 (*A. tamutum*) and *Ensiculifera loeblichii* (was the negative control algae). The measurement of the fourth day of Alex 2 is probably an error.

4.3.1. Toxicity of *Alexandrium*

The toxicity bioassay on Artemia conducted with *Alexandrium mediterraneum* revealed toxic strains causing >60% larvae mortality of each strains, within 72 hours (Fig. 32).

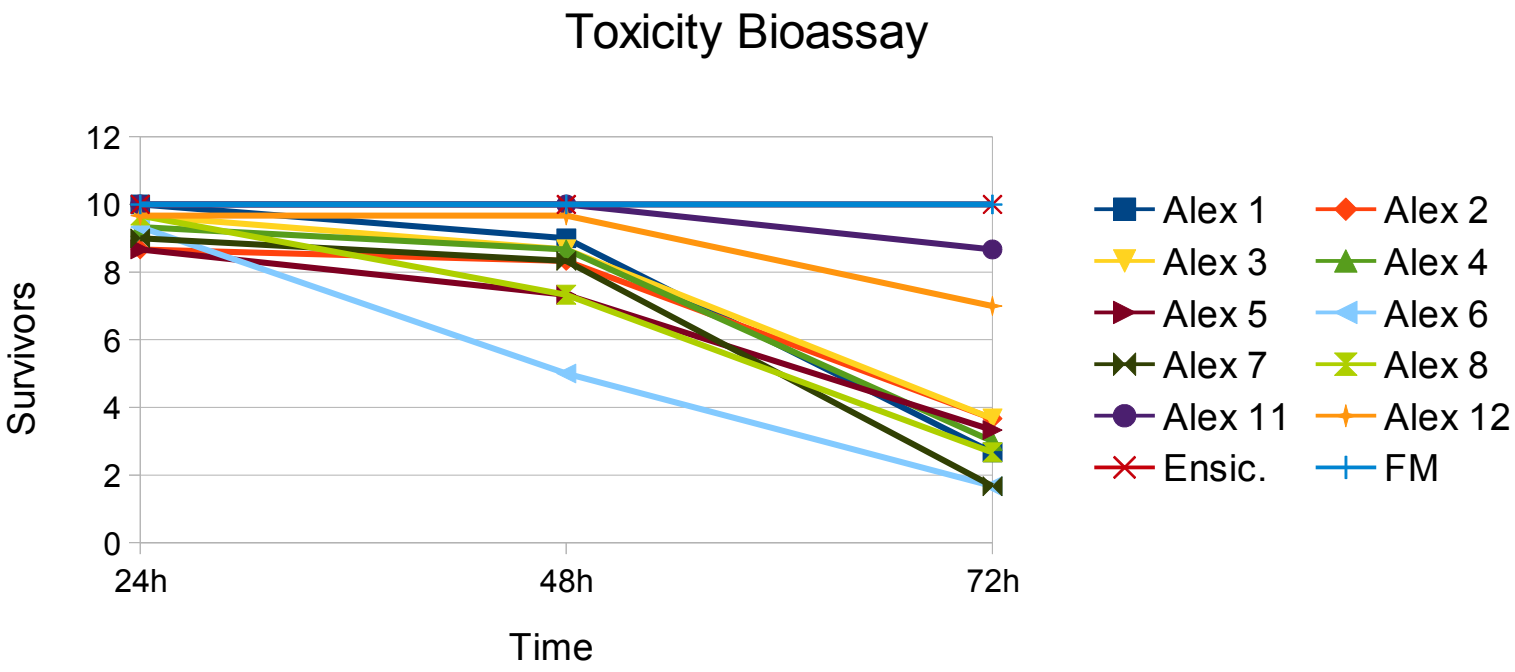


Fig.32. Mean of surviving nauplii exposed to different strains of *Alexandrium*. Two controls were used: filtered medium (FM), and *Ensiculifera loeblichii* (Ensic.). The numbers of survivors decreases with the exposure time for all the *Alexandrium* strains, whereas the number of the nauplii exposed to the two controls remained constant over the time.

The hypothesis of no interaction between strains and times, is rejected using both Wald-type statistics and Anova-type statistics with the p-value very close to zero (WTS) and 9.378 * 10 ^ -5 (ATS). The output is presented below:

WTS

```
               Statistic df       p-value
strains         1103.0892 10 1.138893e-230
hours            237.1397  2  3.204533e-52
strains:hours  12208.7249 17  0.000000e+00
```

ATS

```
 Statistic       df      p-value
strains         12.997923 5.379981 1.817265e-13
hours          131.961511 1.974713 2.423448e-57
strains:hours    3.949659 8.190058 9.377800e-05
```

To investigate the question of whether the hypothesis of no interaction is rejected, Kruskal-Wallis at each time point was performed (Table 5). Since the third time point (72h) was the most significant (p-value=0.003411), multiple comparison was performed between the strains, using Dunn's test. In order to reduce the family-wise error rate, also Holm's adjustment was performed. The results have been resumed on Table 6.

Table 5. Kruskal-Wallis performed on each time point. Df=degree of freedom.

	24 h	48 h	72 h
kruskal-wallis chi-squared	16,476	26,16	27,851
df	11	11	11
P-value	0,1243	0,006143	0,003411

Table 6. Multiple comparison against the control in filtered medium (FM) on the left. Holm's sequentially rejective procedure on the right. The latter method is based on placing in ascending order the p-values in the Dunn's matrix and assign them a mathematical notation (i). m=number of multiple comparison; α=0.05

Dunn's multiple comparison

Strains	F.M.
Alex 1	0,0151
Alex 11	0,5437
Alex 12	0,3996
Alex 2	0,0416
Alex 3	0,0501
Alex 4	0,0178
Alex 5	0,0379
Alex 6	0,0015
Alex 7	0,0015
Alex 8	0,0129
Ensiculifera	1

Holm's adjustment

Strains	m-i+1	α/(m-i+1)	significant
Alex 6	11	0,0045	*
Alex 7	10	0,0050	*
Alex 8	9	0,0056	
Alex 1	8	0,0063	
Alex 4	7	0,0071	
Alex 5	6	0,0083	
Alex 2	5	0,0100	
Alex 3	4	0,0125	
Alex 12	3	0,0167	
Alex 11	2	0,0250	
Ensiculifera	1	0,0500	

With exception of the strain Alex 3, all the *Alexandrium mediterraneum* strains showed significant differences with the control using Dunn's mutiple comparison; whereas using Holm's adjustment only two strains of *Alexandrium mediterraneum* (Alex 6 and Alex 7) showed significant differences. The hypothesis of no strains toxicity is not rejected for either *Alexandrium minutum* and *Alexandrium tamutum* using both Dunn's multiple comparison and Holm's adjustment.

The presence of *Alexandrium* cells inside the alimentary canal of *A. franciscana* was captured by camera during the observation with the microscope (Fig. 33).

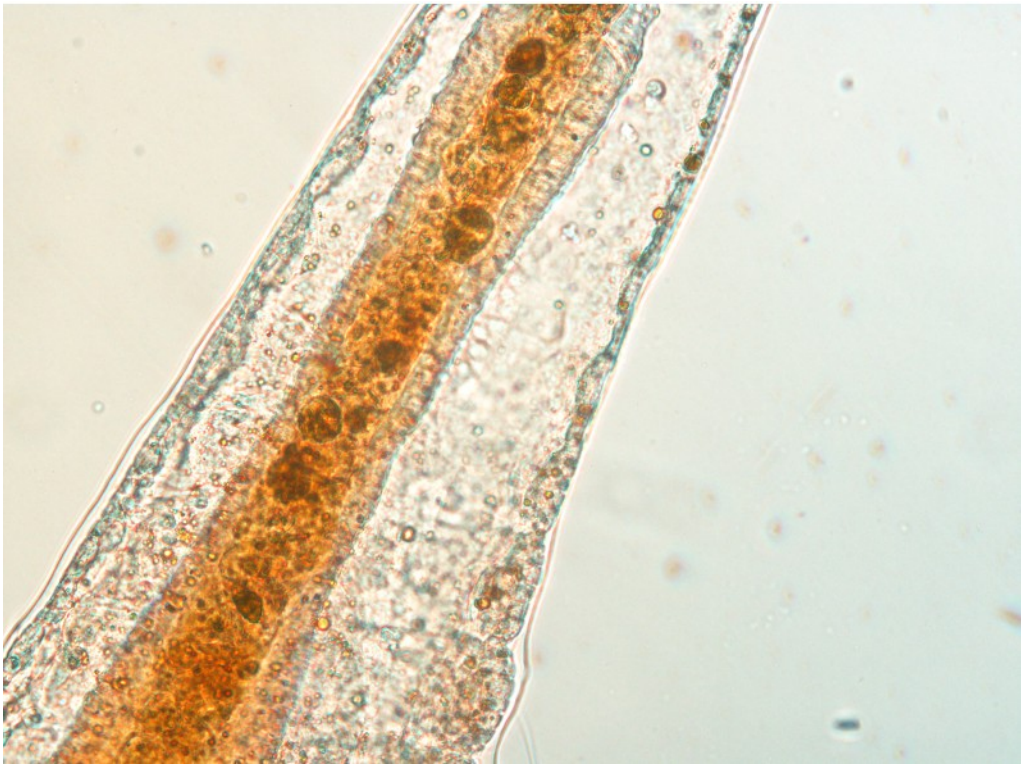


Fig.33. Live observation of *Artemia franciscana* with *Alexandrium minutum* (Alex 11) after 48 hours, captured by Leica DMRB (x20). Cells are clearly visible in the digestive tract showing that larvae feed *Alexandrium* cells.

5. DISCUSSION

In fifteen years of phytoplankton monitoring in Bilbao Harbour, the presence of at least 30 potentially harmful taxa, including diatoms, several dinoflagellates, haptophytes and raphidophyceans was revealed (Butrón et al., 2011). Strains of *Alexandrium minutum* were isolated from sample collected in May 2004 in the marina of Getxo (inner Abra of Bilbao) (Aylagas et al., 2014). *Alexandrium minutum* is a strong producer of PSP gonyautoxins (Oshima et al., 1989), these toxins can have negative impact on humans health, other mammals, birds and possibly fish (Guiry, 1991). Due to the high toxicity reported for *A. minutum* and other *Alexandrium* species, the identification at the species level was necessary in order to evaluate and prevent the risk of toxic bloom episodes. However, *A. minutum* was never been found in this area at high concentrations (Aylagas et al., 2014).

In this work, a first step towards the assessment of the potential threat of the toxigenic genus *Alexandrium* in the south-eastern Bay of Biscay was made by characterizing all the 12 *Alexandrium* strains isolated from the monitoring programme of the estuary of Bilbao from 2004 to 2015.

Regarding the size parameters (length and width), in this study highly significantly differences were observed among the three identified species *Alexandrium minutum* (Alex 10 and Alex 11), *A. tamutum* (Alex 12) and *A. mediterraneum* (Alex 1 – Alex 9). It's interesting to note that highly significant differences were determined following in the results of the multiple comparisons Dunn's test, among the groups of Alex 10, Alex 11, Alex 12 and all the others strains, with both the adjustments: without the adjustment of the p-value and with the Bonferroni adjustment, which is strongly conservative.

Balech (1995), in a review on taxonomic characters and criteria to identify *Alexandrium* species, reported: "except for a few species with some very peculiar characteristics, the genus *Alexandrium* is notably homogeneous and lacks those conspicuous elements, frequent in other genera such as *Gonyaulax s. str.*, that help to distinguish among species". Moreover, intraspecific variability in

some morphological traits was observed. For example, both cells with and without connecting pore were observed in Alex 2, Alex 4, Alex 6 and Alex 9. Also, the connecting pore on posterior sulcal plate (s.p.) was present in some, but not all cells, of Alex 10.

Strains Alex 1-9 morphologically fitted to *Alexandrium tamarense* complex using several morphological features like: the presence of a ventral pore between the plate 1' and 4', its median position along the right margin of plate 1', the shape of Plate 6", which was as wide as tall or slightly wider than tall and the anterior sulcal plate (s.a.) which was A-shaped. Measurements of all the diagnostic morphological features conducted by John et al. (2014) overlapped for the species of *Alexandrium tamarense* complex. Because of that, it was not possible to identify the species in the complex with the morphology alone.

Plate morphology of Alex 12 well corresponded to the description of *A. tamutum* provided in Montresor et al. (2004). In the present study it was found that Alex 12 had morphological characteristics shared with both *A. tamarense* complex and *A. minutum*. For instance, Alex 12 showed a spherical shape and dimensions similar to those observed for Alex 10 and Alex 11. It had a wide sixth precingular plate (6") that was similar to the plate 6" observed in strains of *A. mediterraneum*. Further, the anterior sulcal plate (s.a.) that was A-shaped like in *A. mediterranuem*. Alex 12 was smooth, with no observed grooves, as in Alex 10 and Alex 11.

Plate morphology of Alex 10 and Alex 11 coincided with that of *Alexandrium minutum*. No ventral pore between plate 1' and plate 4" was found in Alex 11 while it has been observed in Alex 10, constantly in the inferior part of the plate 1' as in the original description of *A. minutum*. Apparently, the presence/absence of the ventral pore (v.p.) was one of the criteria to distinguish among *A. lusitanicum*, *A. angustitabulatum* and *A. minutum*. The debate over the taxonomic utility of the ventral pore is not a new issue for *Alexandrium* taxonomy. The study of Hansen et al. (2003) also documented the presence of *A. minutum* cells in Denmark, and other European locations, that lacked a ventral pore. The results presented here show that the presence of a ventral pore is not a sufficient

taxonomic character to attribute the two strains (Alex 10 and Alex 11) to two different species. In fact, the maximum likelihood tree, demonstrated that these two strains belonged to the same species (*Alexandrium minutum*) and also to the same clade (Global clade).

In terms of practical use, *Alexandrium minutum* is so highly similar in size to *Alexandrium tamutum* that they cannot be distinguished during routine monitoring of cell counts. For this reason, when dealing with small-sized *Alexandrium minutum*-like cells during the monitoring in Bilbao, thecal plates' morphology should be analyzed, using the sixth precingular plate (6") as the diagnostic character to distinguish between the two species: being narrow in *A. minutum* and wide in *A. tamutum*.

Alexandrium minutum and *Alexandrium mediterraneum* presented in this study, showed intraspecies variability in morphology.

A molecular identification was performed using the sequences of the D1-D2 region of the LSU rDNA of the 12 strains. The resulting phylogenetic tree revealed three species among the local strains. It confirmed the previous morphological identifications of Alex 12 as *Alexandrium tamutum*, which is a species recently described from the North West Mediterranean Sea and included in the *Alexandrium minutum* complex (Montresor et al., 2004) and Alex 10 and Alex 11 as *Alexandrium minutum*. Strains Alex 1-9, that morphologically fitted to the *A. tamarense* complex, were revealed to belong to the recently described *A. mediterraneum* (group II of the complex; John et al. 2014). It includes all 7 strains isolated from the *Alexandrium* bloom of 2015.

Based on genetically identified isolates, *Alexandrium mediterraneum* had previously been found only in the Mediterranean Sea from the coastal waters of Greece, Italy, France and Spain (John et al., 2003; Lily et al., 2007; Penna et al., 2008). Here we presented strains from Alex 1 to Alex 9 that belonged to *Alexandrium mediterraneum* and that were recorded for the first time out of Mediterranean Sea.

The Basque Coast, in the south east of the Bay of Biscay has warmer waters than adjacent coasts

and the presence of other warm-water dinoflagellates has been reported there (Laza-Martinez et al., 2011). For example, *Ostreopsis*, which is present in the Mediterranean Sea and in the southern Portugal, disappears to the north of the Atlantic Iberian Peninsula but appears again in these waters (David et al. 2012).

In this study, a recently described species has also been characterized. In fact, in 2009 *Alexandrium tamutum* has been recovered for the first time from the Bay of Biscay, since it was firstly described by Montresor et al. (2004) in Italy, then it had been recorded in Britain (Brown et al., 2010), Spain (Figueroa et al., 2007; Penna et al., 2008) and China (Liu, 2008).

Using Artemia's larvae, Alex 12, which was identified as *Alexandrium tamutum*, did not appear to be toxic and this result is consistent with results of Montresor et al. (2004). Other publications, which used molecular tools to identify the *Alexandrium tamutum* toxin profile, supported the non toxicity of this species (Touzet et al., 2008; Gu et al., 2013; Figueroa et al., 2007).

Alex 11, which was identified as *Alexandrium minutum*, also did not appear to be toxic. The presence of non-toxic strains is not unfamiliar in *Alexandrium minutum*. In fact, as we anticipated, within the Global Clade of *Alexandrium minutum* group, there are both toxic and non-toxic strains (Lily et al., 2005). Moreover, within a given species there is a high degree of plasticity with respect to toxin content (Alpermann et al., 2010; Tillmann et al., 2009), as shown for Alex 10 (Aylagas et al., 2014) and Alex 11. Similar associations of toxic and non-toxic populations of *Alexandrium minutum* in different areas of the coastal waters of Ireland was described by Touzet et al. (2007).

The toxicity bioassay conducted on *Alexandrium mediterraneum* revealed toxic strains causing >60% larvae mortality on each strains, within 72 hours. The recent identification of genes involved in the production of saxitoxins (SXTs), which is the principal toxin responsible for PSP, represents a significant opportunity for the development of novel detection tools (Murray et al., 2011; Stüken et a.., 2011; Zhang et al., 2007; Kellmann et al., 2008). Using genes detection no strain of *Alexandrium mediterraneum* has been reported to produce SXTs up to now (John et al., 2003; John

et al., 2014). Several *Alexandrium mediterraneum* strains from this study, showed significant differences respect the control. These significant differences were observed only after 72 h of exposure and not in all the strains using both Dunn's test and Holm's adjustment, showing that toxicity was not high. This result could indicate that even if a toxic effect was revealed this could be not associated to PSP toxins production.

In fact, even if a species does not produce PSP toxins, it is possible that other types of toxins are present. Among the approximately 30 species that belong to *Alexandrium*, at least half are considered potentially harmful. Of these, 12 are known to be PSP toxins producers, while others produce different type of toxins and antifungal substances, or exhibit haemolytic activity (IOC Taxonomic Reference List of Harmful Micro Algae; Sampedro et al., 2013; Anderson et al., 2012).

6.0 CONCLUSIONS

The study presented here, allowed establishing morphological guidelines to be used in monitoring programme for the studied area, which is based on microscopy.

Three species of *Alexandrium*, *A. minimum*, *A. tamutum* and *A. mediterraneum*, have been identified in the study area. Only the latter species, which is not considered a PSP-toxins producer, was identified from the bloom event from 2015. One of the strains of *A. minutum* was toxic (Aylagas et al., 2014). However, this species has never been found in the area at high abundances. It is suggested that, from the available data, *Alexandrium* does not pose a major shellfish-intoxication threat in the area.

Despite they do not appear as PSP-toxins producers, strains of *Alexandrium mediterraneum* tested for the presence of toxic effect revealed low, although significant, toxicity towards Artemia larvae.

Although not contributing to PSP intoxication through shellfish consumption, the observed toxicity of *A. mediterraneum* could affect the health status of its grazeers and may have ecosystem-level consequences. Future research should investigate the nature of the toxicity detected during this study and it should include alternative methodologies for the detection of toxic strains in comibantion with different parameters (nutrients, salinity and light) and environmental conditions.

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