TRABAJO DE FIN DE MÁSTER

Risks for environmental health in coastal zones investigated from eDNA.

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ABSTRACT

Alexandrium genus (Dinophyceae, Gonyaulacales) includes a number of species producing neurotoxins responsible of a human disease when infected shellfish is consumed: paralytic shellfish poisoning (PSP), whose effects are muscular paralysis, neurological symptoms and even death. This genus is broadly distributed and have produced severe impacts on both human health and shellfish aquaculture in several countries. Procedures regarding the detection of *Alexandrium* species include microscopic observation of phytoplankton community, which is time-consuming and requires well-trained personnel. Within this frame, molecular tools might be a good helper to accurately detect harmful organisms in given water samples. This study makes use of already developed primers to elucidate whether toxic dinoflagellates of *Alexandrium* genus are present in Asturias (Northern Spain) through environmental DNA (eDNA) techniques.

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1. Introduction

Algal blooms are natural phenomenon (Bianchi et al., 2000; Hallegraeff, 2003) which consist in the proliferation of plankton algae up to millions of cells per liter (Hallegraeff, 1993). Since these microscopic planktonic algae are an important primary producer and food resource for marine filter-feeding shellfish (*e. g.*, oysters, mussels, clams), crustaceans and finfish, algal blooms are supposed to be beneficial for all the marine ecosystem, the aquaculture and the fishing industry (Hallegraeff, 1993; Duarte and Cebrián, 1996). However, algal blooms can have a harmful nature and produce negative effects, usually reflected in both human health impacts and economic losses (Hallegraeff, 1993; Hoagland and Scatasta, 2006). This kind of blooms are called “harmful algal blooms” (HABs) and its hazard lies in the production of toxins, oxygen depletion (leading to hypoxia or even anoxia), fish gills clogging and other effects (Ochumba, 1990; Zingone and Enevoldsen, 2000; Reis-Costa, 2016).

Moreover, some species can produce toxins that are expelled to the surrounding water and are in-taken by the living organisms inhabiting the area (Hallett et al., 2016; Bhunia, 2018). One of the most studied groups of toxin-producers is the genus *Alexandrium* Halim, 1960 (Dinophyceae, Gonyaulacales) (John et al., 2014), which contains 30 morphologically defined species, among them nearly a half are recognized as harmful (Anderson et al., 2012; Sildever et al., 2019) due to both toxin production and massive occurrences related to fish mortality. The species belonging to the genus *Alexandrium* are producers of different kinds of neurotoxins (gymnodimines, saxitonines and spirolides) that can produce a serious syndrome: Paralytic Shellfish Poisoning (PSP) (Otero et al., 2010; Van de Waal et al., 2015). PSP toxins produced by genus *Alexandrium* have been widely investigated and characterized (Lawrence et al., 2005; Sayfritz et al., 2008; Humpage et al., 2010) since these toxins might be accumulated in the tissues of the shellfish when *Alexandrium* HABs or red tides take place, and after they will be used as food by humans. The consumption by humans of PSP-contaminated shellfish leads to both neurological and gastrointestinal symptoms and even death (Anderson, 1997; Galluzzi et al., 2005; Wang, 2008; Anderson et al., 2012), so it’s a serious problem for human health (Grattan et al., 2016; Morabito et al., 2018). Red tides of *Alexandrium* spp. are also a major problem that fishing industry has to deal with due to the economic losses produced by the leakage of resources because of the PSP-intoxication of the cultures (Conte, 1984; Hallegraeff and Bolch, 2016; Díaz et al., 2019).

Alexandrium spp. blooms are well known to occur in temperate coasts throughout the world (Galluzzi et al., 2005), but in the last years the geographic range of the genus *Alexandrium* has been increasing on both regional and global scales (Hallegraeff, 1993; Scholin et al., 1995; Lilly et al., 2002). Until 1970, *Alexandrium* HABs were only known in some localities from temperate coasts of North America, Northern Europe, Japan and South Africa (Hallegraeff, 1993). However, several studies have reported the presence of PSP-producers from genus *Alexandrium* in new areas and have led to an increase of the knowledge about the geographic range of this group during the last decades. Nowadays, genus *Alexandrium* has been also reported in Central and South America (Almandoz et al., 2014; Kremp et al., 2014), in the Mediterranean Sea (Vila et al., 2001; Bravo et al., 2008), in several areas from South and Southeast Asia (Gu, 2011) and in Australia (MacKenzie et al., 1996; MacKenzie et al., 2004).

Already in 1989 Anderson listed potential reasons to explain the increasing range of genus *Alexandrium* and other HABs-producers: (a) potentially toxic species introduced in unaffected

new areas as non-native species by human vectors, such as transport in ballast water (Bolch and de Salas, 2007) of ships or with shellfish stocks; (b) new populations of toxic species arrive to unaffected areas by natural vectors such as marine currents (Genovesi et al., 2015) or zoochory (Tesson et al., 2018); (c) increasing nutrient availability due to pollution and coastal eutrophication provides HABs-forming species the chance to emerge from "hidden flora" status; and (d) the developing of new methodologies and tools, along with the arisen awareness of HABs, leads to an increase of the effectivity during the researches and easier detection of the target groups or species (Anderson, 1989). In addition, new vectors, such as drifting plastic debris (Masó et al., 2003; Kiessling et al., 2015), are earning relevance as dispersal vectors in the framework of the current "global change" process. Smayda (2007) alluded to the global warming and its associated climatic perturbations (North Atlantic Oscillation and El Niño-ENSO events) as mechanisms underlying the increase in HABs (Smayda, 2007). However, although knowing the mechanisms involved in the spread of genus *Alexandrium*, it's not easy to determine which main operative driver is triggering each given situation (Lilly et al., 2002; Smayda, 2007).

Given the potential toxicity of these dinoflagellates, its apparent geographic expansion would be a major threat for human health and for fishing industry in a growing number of coastal areas (Scholin et al., 1995; Anderson, 1997; Medlin et al., 1998; Penna et al., 2002; Gao et al., 2015). Therefore, early detection of HABs-producers, as *Alexandrium* spp., becomes important in order to anticipate HABs eruptions before shellfish and humans suffer PSP.

The detection of *Alexandrium* spp. in a given sample of water can be done through direct observation by using a microscope and making use of morphological features as diagnostic characteristics for the identification of the different species (Kim et al., 2017). However, in most cases the morphological identification requires well-trained personnel with taxonomic skills and is time-consuming (Penna and Magnani, 1999; Nagai et al., 2016; Shin et al., 2017). Therefore, more suitable and rapid methods for the identification of the different HABs-forming groups or species would be especially useful (Galluzzi et al., 2005). Accordingly, several different molecular techniques have been developed to accurately identify *Alexandrium* species from a given sample (Scholin and Anderson, 1994; Kamikawa et al., 2005; Nagai, 2011; Nagai et al., 2016; Ruvindy et al., 2018; Hatfield et al., 2019), usually taken the ribosomal DNA (rDNA) as target genetic material, due to the presence of highly conserved regions interspersed with variable regions, which makes it a useful tool for molecular studies over a wide range of taxonomic levels (Adachi et al., 1996). By the same token, different regions of the rDNA have been used as targets for PCR amplification in microalgae (Scholin and Anderson, 1994; Zardoya et al., 1995; Saito et al., 2002). Regarding to the genus *Alexandrium*, the 5.8S rRNA region is well conserved among the different *Alexandrium* species (Galluzzi et al., 2005), so that makes it an appropriate region to be amplified by PCR in order to detect *Alexandrium* in seawater samples taken in the field (Galluzzi et al., 2005; Galluzzi et al., 2010).

Moreover, the recently emerged environmental DNA (eDNA) methodology has been shown to be useful to detect target organisms in environmental samples (Ficetola et al., 2008) and has been used in many types of studies: biodiversity monitoring (Bohmann et al., 2014), detection of invasive species (Dejean et al., 2012) and harmful species (Antonella & Luca, 2013) and even identification of ancient biodiversity (Thomsen & Willersley, 2015). eDNA consists in DNA that can be extracted

directly from environmental samples (such as soil, water or air), without need of isolating any organism, but amplifying the remaining DNA from dead parts of such organisms, secretions, etc (Lodge et al., 2012).

Therefore, combining the already collected knowledge about *Alexandrium*'s 5.8S region with the environmental DNA techniques, this HABs-forming genus presence might be detected even when blooming wasn't occurring, which would provide useful information about phytoplankton communities and its harm before irrevocable damages were suffered.

Here, seawater samples were taken from an estuary located in the northwestern coast of Asturias (southeastern Biscay Bay; Spain): Eo's Estuary. The objective of this paper is to test the presence or absence of genus *Alexandrium* in this coastal ecosystem by rapid molecular methods: 5.8S region amplification using genus-specific primers (Galluzzi et al., 2005). Positive controls were made using known samples containing *Alexandrium* cells and purified DNA. The interest in this study area arose since these problematic dinoflagellates have been detected in several localities within the Biscay Bay and even in the north coast of Spain (Maestrini et al., 2000; Lilly et al., 2007; Seoane et al., 2012). In addition, extensive oyster's aquaculture is being developed in this area, being important to determine if these cultures are susceptible or not to intoxication by PSP-toxins in order to avoid economic losses and severe health problems.

2. Material and methods

2.1. Study area

Geographically, this work is set into the frame of an Estuary within the Biscay Bay, concretely in the Eo's Estuary (43°31′55″N 7°01′45″O; Fig. 1). This one is a shallow mesotidal system with a total area of 9.6 km2 and a bathymetry ranging between 2 and 7 m depth (Encinar & Rodríguez, 1983). Since 1970 the Eo's Estuary has suffered anthropic pressure due to shellfish farming: the cultured species have varied from the beginning of the farming (*Ostrea edulis*) to nowadays (*Crassostrea gigas* and *Ruditapes philippinarum*) because of the introduction of a parasite affecting the farmed shellfish species (*Bonamia ostreae*) in the early 80s (Cigarría et al., 1995). These aquaculture infrastructures would be susceptible to suffer economic losses if a HAB emerged into the estuary due to the intoxication of the shellfish. Standardizing the use of tools that easily provide information about the early presence or absence could avoid those economic losses drift from HABs occurring.

2.2. Marine water sampling for testing

Marine water was sampled in duplicate in each site (five sampling sites were randomly chosen surrounding the shellfish farms; Fig. 1C) in order to obtain one sample to be analyzed by using eDNA analysis methodology and another one to do *de visu* observation of the target organisms (*Alexandrium* spp.). The samples were taken with 2 L bottles previously sterilized and properly labelled (time and place of sampling). After the sampling, the bottles were stocked in the fridge to conserve the material as intact and no-degraded as possible.

2.3. Marine water and dinoflagellate samples as positive controls

In order to test the genus specific primers for *Alexandrium* described in Galluzzi et al. (2005), known *Alexandrium*-containing (*A. tamarense* and *A. minutum*) samples granted by Isabel Bravo (Instituto Español de Oceanografía) and from the ballast water of the Expedition PS102 of R/V Polarstern (granted by Alba Ardura, from Functional Biology Department of the University of Oviedo) were analyzed as positive controls for determining the effectiveness and specificity of the primer used to detect *Alexandrium* spp. within the Eo's Estuary samples.

Fig. 1. Map of the study area. **A**: Biscay Bay; **B**: Eo's Estuary; **C**: highlighting where sampling sites in Eo's Estuary (e_1, e_2, e_3, e_4 and e_5) and shellfish farms are located (red: Recursos Asturianos Linera; green: Acuicultura del Eo S.L.). Geographical data obtained from https://www.mapa.gob.es/.

2.4. Environmental DNA

Water samples collected in Eo's Estuary were filtered by using a filtration pump. The filters (Pall Corporation Supor® 0.2µm 47 mm PES 100 7pk) used to retain the biological material had a pore size of 0.2 µm (more than enough to catch the 10-50 µm target dinoflagellates). Five filters were immersed in ethanol 96% into Falcon tubes, which were those reserved for DNA isolation and then for doing genetic analysis. The remaining five filters were set into Petri dishes and were reserved for *de visu* identification by microscope of target organisms.

2.5. DNA extraction, quantification and PCR analysis

The five filters allocated for the genetic analysis of eDNA were manipulated according to the protocol of the DNA isolation kit "DNeasy PowerWater Kit" from Qiagen (protocol can be checked in https://www.qiagen.com/us/resources /download.aspx?id=bb731482-874b-4241-8cf4-c15054e3a4bf&lang=en). Purified DNA from *in vivo* samples was obtained using the "DNeasy Blood & Tissue Kit" from Qiagen (protocol can be checked in http://www.bea.ki.se/documents /EN-DNeasy%20handbook.pdf), running the overnight digestion after the second step of the protocol. In step 7 and 8, the elutions were done with 100 µl and with 50 µl respectively. After the procedure, the tubes were stored at 4 °C for immediate DNA amplification and the aliquots were stocked at -20 °C for long-time conservation.

The PCR was performed on an Applied Biosystems 2720 Thermal Cycler in a final volume of 20 µl containing 10 µl of template DNA and 10 µl of PCR mix. The PCR mix composition was: 4 µl of 5x Green Go Taq® Flexi Buffer, 2 µl of MgCl2 (25mM), 1 µl of dNTP mix (0.025 µl of each dNTP (from EURX 100mM Ultrapure dNTPs Set) plus 0.9 µl of distilled water (Laboratorios Serra Pamies)), 0.4 µl of both Primer F and Primer R (primer designed detailed in Galluzzi et al., 2005), 0.25 µl of Go Taq® G2 Flexi DNA Polymerase (5 u/µl), 1 µl of BSA (Bovine Serum Albumin; 50 mg/ml; from EURX) and 1.05 µl of distilled water. BSA was added because this addition has been proved to improve the amount of yield in PCR amplification of DNA found in extracts from marine water (Farrel & Alexandre, 2012).

The PCR scheme (Fig. 2) used in this work followed the proposal of Galluzi et al. (2005), but including a modification regarding the annealing temperature, in which step three different temperatures were set in order to determine which of them provided better DNA yield. So that, the samples were processed as follows: 7 min at 95 °C, 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension of at 72 °C for 7 min. Negative controls (no-template) were included in each PCR reaction.

Amplified DNA was electrophoresed in a 2% agarose gel at 120 V for 10 min in a BioRad Power Pac 300 Supply. A double-stranded DNA ladder (PerfectTM 100-1000 bp DNA ladder 50 µg (0.5 µg/µl) from EURx) was included on the gels as a size standard. PCR products were visualized under UV light in a Gene Flash-Syngene Bio Imaging.

The PCR products from *in vivo* samples that provided positive results in the gel were also quantified with a Qubit® Flurorometer in order to measure how much DNA can be amplified using the genus specific primers for *Alexandrium*.

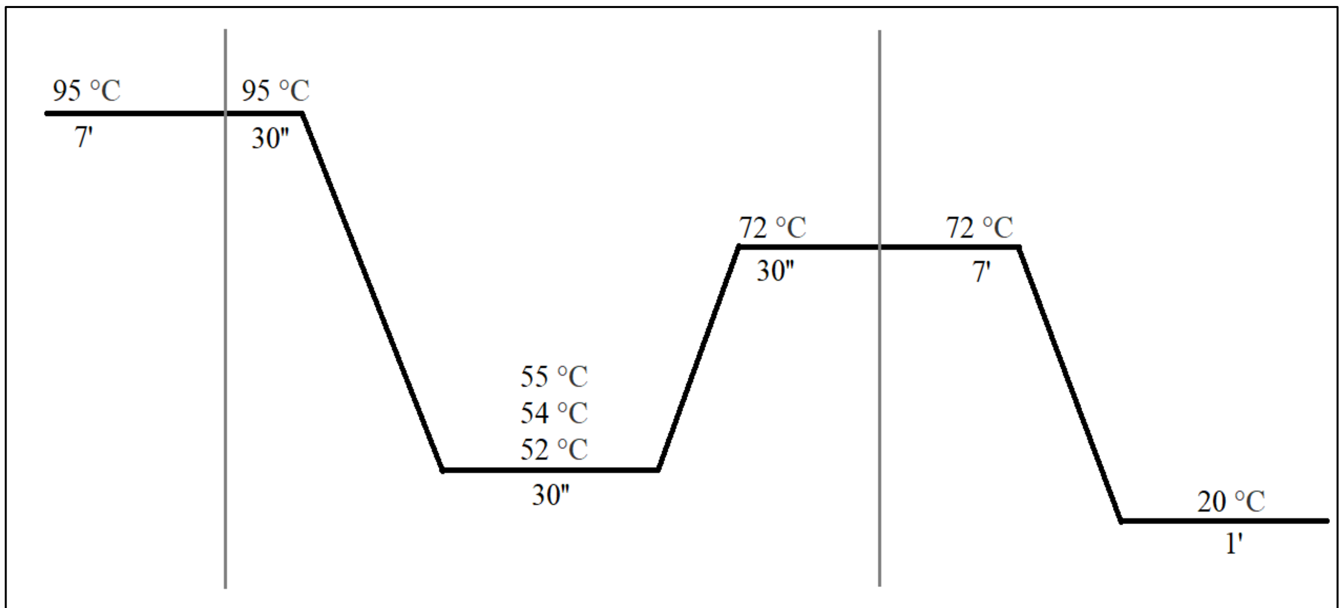

Fig. 2. PCR scheme employed along the amplification processes. Obtained and edited from Galluzzi et al. (2005).

3. Results

3.1. Primer performance: effectiveness and sensitivity

The primers employed for this study were the ones described by Galluzzi et al. (2005) for *Alexandrium* genus. This primers are supposed to provide PCR products of 135 bp. The effectiveness of the primer was confirmed by the positive controls: purified DNA from *in vivo* samples (Fig. 3) of *Alexandrium* (Fig. 4A) and purified DNA from the ballast water of the R/V Polarstern (Fig. 4B). After the PCR, amplification products of the expected size were visible in both positive controls (Fig. 4), which confirmed the capability of the primers to specifically detect the presence of *Alexandrium* cells even within a mixture of different algae species. Regarding the sensitivity of the employed primers, serial dilutions of the purified DNA from *in vivo* samples of *Alexandrium* (Fig. 4A) revealed positive amplification until 1.01×10^{-4} ng/ µl for *A. tamarense* samples and until 1.80×10^{-5} ng/ µl for *A. minutum* samples. Negative controls never showed any kind of band and the marker was observed as expected.

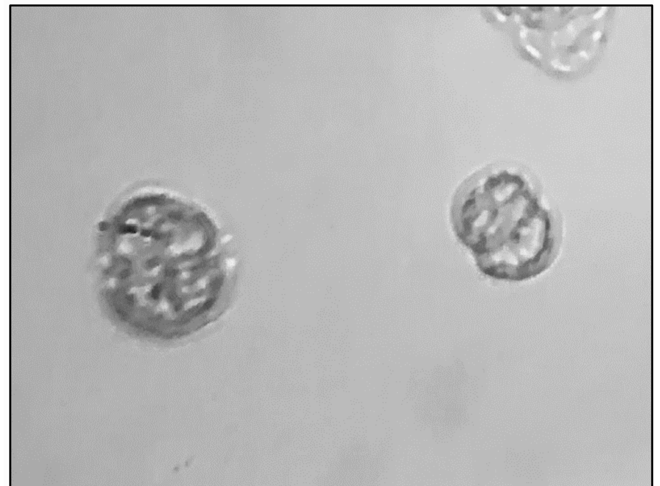

Fig. 3. *Alexandrium tamarense* cells from *in vivo* samples directly observed by an optical microscope at x40 zoom.

The ballast water from the R/V Polarstern had been previously analyzed by massive parallel sequencing (MPS), also known as NGS (Next Generation Sequencing), obtaining some given detections of *Alexandrium* spp. susceptible of amplification by the genus specific primer employed in this study. Here, those entrances were compared with the ones obtained after analyzing the same samples with the genus specific primers, concluding that both methods

allowed accurately to detect *Alexandrium* presence in the same way.

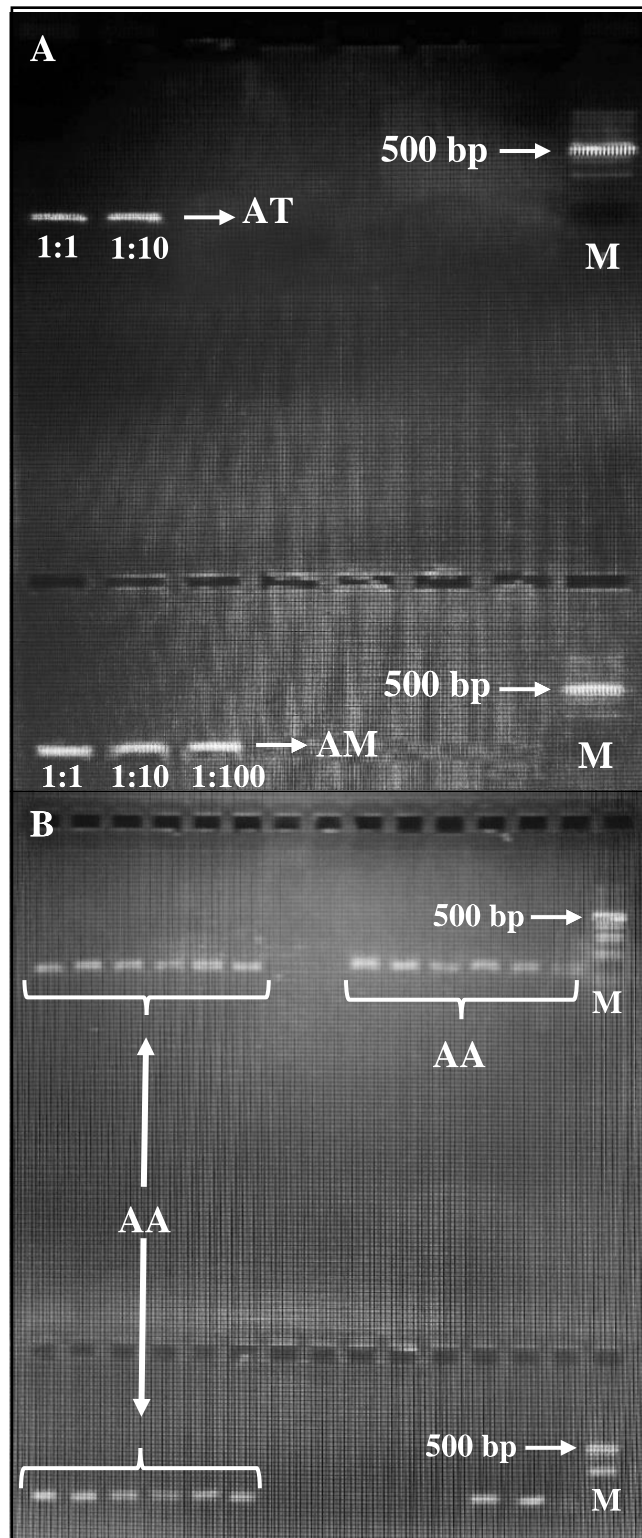

Fig. 4. **A**) Agarose gel of PCR products obtained with *Alexandrium* genus specific primers from serial dilutions from 1:1 to 1:10^5 of purified DNA extracted from in vivo samples. **B**) Agarose gel of PCR products obtained with *Alexandrium* genus specific primers from ballast water samples. AA: *Alexandrium*; AM: *Alexandrium minutum*; AT: *A. tamarense*. M: Marker.

In addition, the specificity of the primers was tested by cross-amplification of purified DNA from different phytoplankton groups (*Neoceratium platycorne*, *Nitoktra spinipes* and *Coscinodiscus* spp.) as described in Galluzzi et al. (2005); in this case, the PCR products were undetectable (Fig. 5).

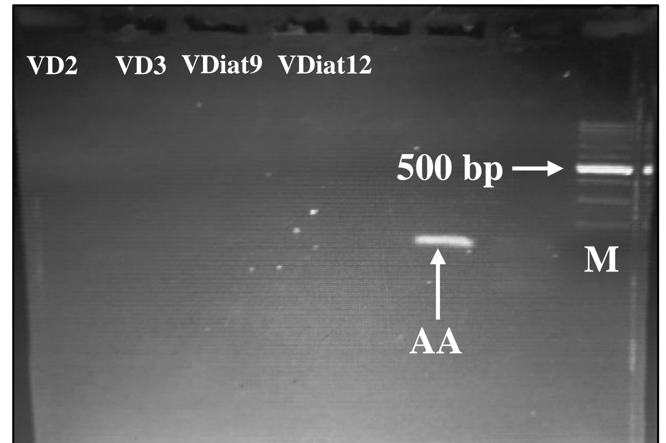

Fig. 5. Agarose gel of PCR products obtained with *Alexandrium* genus specific primers from a cross-amplification with other phytoplankton organisms. VD2: *N. Platycorne*; VD3: *N. spinipes*; VDiat9: *Coscinodiscus* spp.; VDiat12: *Coscinodiscus* spp.; AA: *Alexandrium*; M: Marker.

Furthermore, the sequence specificity of the primers was tested using the MegaBlast tool from BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Obtaining an "Identity" that ranged from 88.04% to 98.53% for *Alexandrium* spp.

3.2. Optimal annealing temperature

Different annealing temperatures were set for the PCR reaction in order to test which of them provided the best results. This change regarding the methodology described for the employed primers is due to the nature of the samples: eDNA appears in lower concentrations compared with tissue-DNA, which implies that some variations of the typical methodology should be considered. After a series of negative cases (not shown), 52 °C was chosen as the most appropriate annealing temperature.

3.3. Eo's Estuary samples

3.3.1. PCR results

The water samples collected from Eo's Estuary provided negative PCR results for every sampling site (Fig. 6), what indicates that at least *Alexandrium* suspended cells were not present in the estuary when the samples were taken. Negative control didn't provide any band and the DNA marker worked well. Different replicas (not shown) were done and the same results were obtained, so nor can an error of the PCR reaction take the blame for these results.

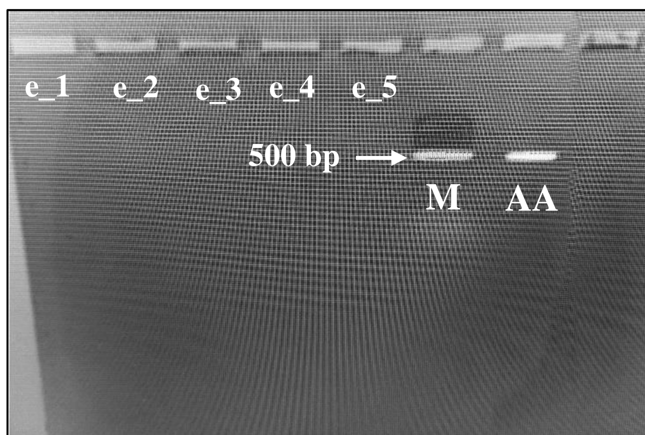

Fig. 6. Agarose gel of PCR products obtained with Alexandrium genus specific primers from the samples collected in the Eo's Estuary. e_1-5: Eo's Estuary samples; AA: *Alexandrium*; M: Marker.

3.3.2. De visu observation

In neither of the five filtered water samples collected in the Eo's Estuary *Alexandrium* cells were found.

4. Discussion

Eo's Estuary samples seemed to be free of *Alexandrium* floating cells, but it didn't provide definitive answers to the question about the presence or absence of this HABs-forming group since these dinoflagellates has been described as cyst-forming when environmental conditions are not optimal (Ichimi et al., 2001; Garcés et al., 2004). These cysts are buried and accumulated in the bottom sediments until appropriate conditions to proliferate come (Anderson et al., 2005). Given this, the possibility of underestimating the presence of *Alexandrium* in the Eo's Estuary should be considered. The results provided in this study are correct within an order of magnitude: since only water samples were taken, it can be said that there were not suspended cells of *Alexandrium* when the sampling were done, but definitive answers would be given only with the addition of sediment's samples analysis as Anglès et al. (2010) or Fertouna-Bellakhal (2015) did in different localities of the Mediterranean Sea. This proceeding would provide information about the whole profile distribution of *Alexandrium* cells (both floating and resting cysts) within a given water and sediment column.

After all, not just negative results were obtained with this study: the genus-specific marker designed by Galluzzi et al. (2005) has been tested as sensitive and accurate in all those Galician and Polastern's samples and in the cross-amplification, which provides useful information about both the employed primers and the previous analysis of those samples. Moreover, comparing the amplification results obtained with the genus specific primer with those obtained with the MPS of the ballast water of the R/V Polarstern, it can be said that both employed methods: NGS and genus specific primers are useful when looking for harmful species in a given water sample. Recently, the benefits drift from the MPS has been clearly proved from the metagenomics (Escalante et al., 2014), but when the target organisms are well-known and appear clustered taxonomically, genus specific primers seems to be a quite cheaper and less time-consuming option that provide as suitable results as NGS techniques (Lodge et al., 2012). Several are the authors alluding to a combination of NGS and eDNA (Lodge et al., 2012; Shokralla et al., 2012; Thomsen et al., 2012), due to the increasing in accurate estimations of species richness that would be achieved.

Therefore, as a cheap and quick procedure, the protocol exposed in this work and in the previous ones (Martin and Rygiewicz, 2005; Ficetola et al., 2008; Thomsen et al., 2012) could be easily standardized as a tool to periodically analyze water samples to easily detect the presence of harmful species that might produce impacts on both human and environment health. This, combined with a deeper knowledge of the HABs-forming species (dynamics, life cycle, and phenology in general terms), would lead important social and economic losses to be avoidable.

From a different perspective, this genetic tools could be used to analyze the ballast water of merchant vessels and harbors (important vectors and hosts of non-native and potentially invasive species; Bolch and de Salas, 2007) in order to avoid the entrance of non-native and harmful species to not-touched communities. Finding incipient populations early would provide managers with options to act before a harmful species achieved high abundance (Robinson et al., 2011; Lodge et al., 2012). This might avoid both problems: biodiversity homegenezation due to the pressure that introduced species could produce and the human health threat drift from the presence of toxin-producing species.

It's clear that much additional work will be required before a definitive answer regarding *Alexandrium* occurrence in Eo's Estuary occurs, but in the other hand it's hoped that this study will stimulate further investigations in this field in order to definitively determine whether Eo's Estuary is under the harm of *Alexandrium* blooms. Such investigations could regard the sediment's analysis with eDNA methodology, sampling in different dates in order to also understand the potential dynamics of *Alexandrium* in this area and even include abiotic measures to correlate with the presence or absence of *Alexandrium*.

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