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Unsuspected intraspecific variability in the toxin production, growth and morphology of the dinoflagellate *Alexandrium pacificum* R.W. Litaker (Group IV) blooming in a South Western Mediterranean marine ecosystem, Annaba Bay (Algeria)

Imen Hadjadji a,b, Mohamed Laabir b,* , Hocine Frihi a, Yves Collos b, Zhao Jun Shao c, Patrick Berrebi c, Eric Abadie b, Zouher Amzil d, Nicolas Chomerat e, Jean Luc Rolland f, Fabien Rieuvilleneuve b, Estelle Masseret b

a Laboratoire « Bioressources marines », Université Badji-Mokhtar-Annaba, BP 12, El-Hadjar, 23000, Annaba, Algeria

b Univ Montpellier, MARBEC, UMR 9190, IRD, CNRS, IFREMER, F-34095 Montpellier, France

c Univ Montpellier, CNRS, ISEM, IRD, EPHE, Montpellier, France Montpellier, CC065, Place E. Bataillon, 34095, Montpellier, Cedex 5, France

d Laboratoire Environnement, Microbiologie et Phycotoxines, Ifremer, Rue de l'Île d'Yeu BP 21105, Nantes, CEDEX 3 44311, France

e IFREMER, Laboratoire Environnement et Ressources Bretagne Occidentale, Station de Biologie Marine, Place de la Croix, 29900, Concarneau, France

f IHPE, Université de Montpellier, CNRS, Ifremer, UPVD, Montpellier, France

Abstract

Physiological plasticity gives HABs species the ability to respond to variations in the surrounding environment. The aim of this study was to examine morphological and physiological variability in *Alexandrium pacificum* R.W. Litaker (Group IV) (former *Alexandrium catenella*) blooming in Annaba bay, Algeria. Monoclonal cultures of up to 30 strains of this neurotoxic dinoflagellate were established by the germination of single resting cysts from the surface sediment of this southern Mediterranean marine ecosystem. Ribotyping confirmed formally for the first time that *A. pacificum* is developing in Eastern Algerian waters. Toxin analyses of *A. pacificum* strains revealed substantial intraspecific variability in both the profile and toxin amount. However, the toxin profile of most strains is characterized by the dominance of GTX6 (up to 96 mol %) which is the less toxic paralytic molecule. The toxin concentrations in the isolated strains varied widely between 3.8 and 30.82 fmol cell⁻¹. We observed an important variation in the growth rate of the studied *A. pacificum* strains with values ranging from 0.05 to 0.33 d⁻¹. The lag time of the studied strains varied widely and ranged from 4 to 20 days. The intraspecific diversity could be a response to the selection pressure which may be exerted by different environmental conditions over time and which can be genetically and in turn physiologically expressed. This study highlights, for the first time, that the sediment of a limited area holds an important diversity of *A. pacificum* cysts which give when germinate populations with noticeable physiological plasticity. Consequently, this diversified natural populations allow an exceptional adaptation to specific environmental conditions to outcompete local microalgae and to establish HABs which could explain why this dinoflagellate is successful and expanding worldwide

Keywords

Alexandrium pacificum ; Mediterranean ; Toxin profile ; Growth ; Intraspecific variability

1. Introduction

Anthropogenic activities lead to changes in marine coastal ecosystems by altering the composition of primary producers' communities (Glibert et al., 2005) and by accentuating dispersal and settlement of various invasive marine species (Smayda, 2007). Thus only 16% of marine ecoregions are thought to be free from invasive species (Molnar et al., 2008). This dispersal phenomenon is irreversible. Many species of the Dinoflagellate genus *Alexandrium* are able to produce potent paralytic shellfish toxins (PSTs) and are responsible of toxic blooms affecting wide marine systems throughout the world (Lilly et al., 2007; Anderson et al., 2012; Laabir et al., 2013). The Paralytic Shellfish Poisoning (PSP) syndrome is caused by different groups of molecules: Carbamate toxins including Saxitoxin (STX), Neosaxitoxin (NeoSTX) and Gonyautoxin (GTX), the Decarbamoyl toxins including dcSTX, dcNeoSTX, the Decarbamoyl Gonyautoxins with dcGTX2, dcGTX3, and the N-Sulfocarbamoyl toxins covering the "B" toxins: B-1 (GTX-5) and B-2 (GTX-6) and "C" toxins 1 to 4 (Chang et al. 1997) (Fig. 1). There have been several studies on toxin profiles of *Alexandrium* from different geographic origins (Oshima et al., 1982; Maranda et al., 1985; Kim et al., 1993; Touzet et al., 2008; Aguilera-Belmonte et al., 2011; Anderson et al., 2012; Varela et al., 2012; Laabir et al., 2013; Hallegraeff et al., 1991). Most of the studies on the ecophysiological characteristics and on toxins in a species took into account very few strains and more often only one. When several isolates were taken into account, the toxin composition and content were often found to vary between strains (Aguilera-Belmonte et al., 2011). Alpermann et al. (2010) demonstrated the presence of a wide variability in PST composition amongst a large number of *A. tamarense* strains (group I) isolated from vegetative cells belonging to a single geographical population in the Scottish waters of the North Sea. Moreover, a significant intraspecific diversity in the growth rate was observed among this group (Tillmann et al., 2009), as well as the capacity to grow on various culture media and the shape of the growth curves of *A. tamarense* strains (group III) isolated from a Mediterranean lagoon (Hadjadji et al., 2012). Now, it is assumed that a wide diversity exists between isolates of a single species and it is essential to take into account several isolates to study the physiology and the ecology of any species in a given region.

Many *Alexandrium* species develop in Mediterranean waters (Gomez, 2003) among them the neurotoxic *Alexandrium catenella* which was renamed *Alexandrium pacificum* by John et al. (2014). *A. pacificum* (former *A. catenella*) is widely distributed (Molnar et al., 2008) in reason probably to its capacity to produce resting cysts which could be introduced in virgin marine ecosystems via ballast waters and shellfish translocation (Wyatt and Jenkinson, 1997; Laabir et al., 2007; Anderson et al., 2012). *A. pacificum* blooms occur on the Italian, Spanish, French and Tunisian coastal waters (Garces et al., 1999; Luglie et al., 2003; Laabir et al., 2011; Penna et al., 2005; Turki et al., 2007; Fertouna-Bellakhal et al., 2015). *Alexandrium minutum* blooms has been described in Mediterranean Moroccan waters (Daoudi et al., 2012). Most of the studies on toxicity and ecophysiological traits of Mediterranean *Alexandrium* species were performed on strains of *A. minutum* isolated from Spain (Da Costa et al., 2008), *A. pacificum* from France (Collos et al., 2004, 2006; Laabir et al., 2012, 2013) and also for *Alexandrium andersonii* and *Alexandrium ostenfeldii* from Italian waters (Ciminiello et al., 2000; 2010). However, these works have been performed on one strain or very few number of isolated strains. Nowadays, no information about physiological parameters is available for south Mediterranean *A. pacificum* strains except for one Tunisian strain developing in Bizerte Lagoon (Fertouna-Bellakhal et al., 2015). Until now, few studies have been carried out on toxic phytoplankton in south Mediterranean in general and in Algerian coastal waters in particular. *A. pacificum* bloom was reported in the Bay of Annaba (Algeria) during 2002 (Frehi et al., 2007) but no molecular characterization of this reported species was conducted. Since then, this dinoflagellate was observed during a field study (Hadjadji et al., 2014).

In the present study, we carried out the first formal description of *A. pacificum* blooming in Annaba Bay (South Mediterranean) using up to 30 isolates established from sediment samples after cyst germination. The strains have been characterized morphologically, genetically and their toxin content and growth rate have been determined. Intraspecific trait diversity can be determinant in the success of a species in the ecosystem where it develops becoming more competitive in the framework of changing environmental conditions. Our objective was to highlight morphological and physiological (growth rate and toxin production) diversity of this neurotoxic species evolving in Mediterranean. These data are crucial for understanding the population dynamics of *A. pacificum*, a species whose toxins are increasingly impacting the components of the marine ecosystem with a serious threat of socio-economic activities and human health in the Mediterranean.

2. Material and methods

2.1. Study site and sampling stations

The Gulf of Annaba is located at the Algerian extreme eastern coast (South-Western Mediterranean), in a zone limited by cape Rosa (36°56'07"N, 8°13'47"E) and Cape de Garde (36°58'51"N, 7°47'19"E). The Annaba bay (Fig. 1) is located in the western part of the Gulf, between the Seybouse Oued (River) in the East and De Garde Cape in the West. The maximum depth of the Gulf of Annaba is 50 m. Sea bottom is steeper in the North of the bay than in the South. The continental shelf is very narrow and hilly especially near the two capes. It is limited (4.5 miles) in the north of De Garde Cap then widens into the Gulf until 14.5 miles (Vaissiere and Fredj, 1963).

2.2. Sediment sampling

The sediment samples were taken at two stations (Fig. 1) where important blooms of *A. pacificum* have been observed previously (Frehi et al., 2007). The locations of sampling stations are shown in Fig. 1. The station 1 (36°54'40"N, 7°46'36"E) is shallow (5 m depth) with a sandy bottom. It is located behind the commercial harbor of Annaba in front of the Gassiot beach, sheltered from the dominant winds (North West), and receives urban waste. The station 2 (36°53'09"N, 7°47'22"E) with a mean depth of 13 m is in front of the harbor. It receives continental inputs throughout two rivers, the Seybouse Oued which is considered as the second largest river in Algeria with an estimated 5900 km² watershed and the Boudjemaa river which receives wastewater of Annaba city and industrial effluents. This area is considered as eutrophic zone (Frehi et al., 2007), with a muddy bottom.

The sampling was carried out on March 24, 2010. Sediment samples were taken using an Eckman grab. At each station, 3 sediment samples were taken at the three angles of a triangle of 1 m long of each side. The top 3 cm of sediment were sliced out (Cho and Matsuoka, 2001) and stored at 4° C in the dark until the cysts were isolated and germination experiments performed.

2.3. Isolation of the single cells and the establishment of the clonal cultures

Monoclonal cultures were obtained from cyst germination using two methods. The germination of *Alexandrium* strains were obtained by incubating 1 g of the sediment in sterile flasks, each containing 30 ml of Enriched Natural Sea Water (ENSW) culture medium (Harrison et al., 1980). Germanium (10% final) was added to the culture medium in order to prevent the growth of diatoms. The cultures were grown at 20 ± 1 °C and $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a 12h: 12h light: dark cycle, a salinity of 36 and were checked every day using an inverted microscope for the presence of germling cells. When cysts germinated, the obtained vegetative cells were isolated individually using Pasteur pipettes and incubated in 96-well culture plates. In order to prevent the isolation of several progenies from the same resting cyst, only one monoclonal culture from the division of a single isolated vegetative cell was established from each plate corresponding to each flask where we have incubated 1 g sediment. The established cultures were named ANN xx-y.

In another set of laboratory experiments, *Alexandrium* cysts were extracted from the sediment using the gradient density method (Yamaguchi et al., 1995) and single cysts were incubated individually in 96-well culture plates. When cells started to divide, the young monoclonal cultures named Annaba xx-y were transferred to 48-well culture plates and later to flasks of 40 ml filled with ENSW medium when cell concentration increased.

2.4. Morphological characterization and biometry

The morphology of *Alexandrium* cells from the established monocultures was investigated using light and epifluorescence microscopy. Plate tabulation and arrangements of thecal plates were studied on a large number of vegetative cells (≥ 30 individuals) after dissecting the thecae using 5% sodium hypochlorite solution and staining with a solution of calcofluor white (1% final). Tabular notations were used following Balech's (1985) nomenclature. The length and width of 29 strains of *A. pacificum* strains were determined for cultures harvested in the exponential phase of growth on a large number of live cells (≥ 45) using a digital flow cytometer (FlowCAM, Fluid Imaging Technologies, Yarmouth, ME, USA), which combines flow cytometry, light microscopy, and fluorescence detection to provide rapid particle enumeration and analysis. We used 10X magnification level.

2.5. Molecular identification and phylogeny

2.5.1. DNA extraction and PCR

Total genomic DNA was extracted from 20 monoclonal cultures using the classical phenol-chloroform method (Sambrook et al., 1989). Ribotyping was performed with the two specific primers D1R and D2C described by Scholin et al. (1994). The PCR mixture contained 10 ng of DNA, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 μM of each primer, 1U *Taq* polymerase in 1X polymerase buffer (Promega, Madison, WI, USA). The PCR conditions were 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 45 °C for 1.5 min, 72 °C for 1 min and a final step at 72 °C for 5 min. The separation of the aqueous and organic phases was performed by centrifugation. The aqueous phase contains the DNA which was ultimately recovered in solid form as a result of precipitation in ethyl alcohol. DNA was then resuspended on ultra-pure water. All reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA. Sequencing was performed by a private company (Genoscreen, Lille, France).

2.5.2. Phylogeny

For the phylogenetic analysis, the sequence of the 20 Algerian strains were aligned together with 64 LSU sequences of other *Alexandrium* species and 1 *Goniodoma polyedricum* (as outgroup) retrieved from GenBank using the multiple sequence alignment program MUSCLE version 3.7 (Edgar, 2004). The alignment was refined by eye. The data matrix obtained (85 LSU rDNA sequences, 605 characters) was analyzed by two methods of phylogenetic reconstruction: maximum likelihood (ML), using PhyML v.3.0 software (Guindon and Gascuel, 2003) and Bayesian inference (BI) using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). The most suitable model of substitutions was first selected using the software jModeltest v 0.1.1 (Posada, 2008). The General-Time Reversible model (GTR β I β G) was chosen as indicated by the Hierarchical Likelihood Ratio Tests (hLRTs), Akaike Information Criterion 1 (AIC1), Akaike Information Criterion 2 (AIC2) and Bayesian Information Criterion (BIC) tests implemented in jModeltest. Bootstrap values (support for branches) of trees were obtained after 1000 iterations in ML (Tamura et al., 2011). For Bayesian inference, four Markov chains were run simultaneously for 2×10^6 generations with sampling every 100 generations. On the 2×10^4 trees obtained, the first 2000 were discarded (burn-in) and a consensus tree was constructed from the remaining trees. The posterior probabilities corresponding to the frequency with which a node is present in preserved trees, were calculated using a coupled Monte Carlo Metropolis approach e Markov Chain (MCMC).

2.6. Toxin quantification by Liquid Chromatography/Fluorescence Detection

Batch cultures of 26 *A. pacificum* strains were harvested during their exponential growth phase. Cultures were centrifuged at 3000 g, 8 min, 4 °C, culture medium discarded and the pellet resuspended in 1 ml of 0.1 N acetic acid, then the samples frozen at 20 °C until analysis. To release the cell toxins, the samples were sonicated for 5 min in a water bath three times, and centrifuged at 17 000 g for 10 min at 4 °C. The supernatants were used for the subsequent LC/FD PSTs analyses, which were performed using the method of Oshima (1995). Toxins were separated by reverse chromatography using a C8 column (5 μm Develosil, 4.6 mm i.d. X 250 mm) with a flow rate of 0.8 ml min⁻¹. Eluent pH and/or column temperature were calibrated to optimize the separation of gonyautoxins. Toxins were quantified using certified standards provided by CNRC (Halifax, Canada). GTX6 and C2-toxins were detected and quantified indirectly after acid hydrolysis (HCL 0.4 N at 97 °C for 5 min) (Masselin et al., 2000). The limit of detection (LOD) and limit of quantitation (LOQ) of toxins are shown in Table 1 and the common PSTs are shown in Fig. 2.

2.7. Growth characteristics

Eighteen monoclonal cultures of *A. pacificum* maintained in ENSW (Enriched Natural Sea Water) medium were acclimated to the ESAW (Enriched Seawater Artificial Water) medium (Harrison et al., 1980) during 5 weeks. ESAW medium was used to minimize organic matter and to obtain better reproducibility of growth in order to avoid bias related to the variability of the quality of *in situ* water from Thau lagoon (France, Mediterranean). The salinity was adjusted to 36. Growth was monitored using three replicates batch culture of 35 ml and the experiments lasted 35 days. Sampling was done every two days in order to mini mize

detrimental effects of stirring, as *Alexandrium* is very sensitive to shaking (Therriault et al., 1985; Collos et al., 2004). *In Vivo* Fluorescence (IVF) was measured with a spectrofluorometer equipped with a microplate reader (TECAN INFINITE 2000). Three measurements were made on each of the two replicate cultures. Growth rate was estimated from linear regressions of natural log of IVF values vs time (Guillard, 1973). The duration of lag phase represents the number of days before the exponential phase.

2.8. Statistical analysis

Statistical analyses using Pearson and Spearman tests were applied to test if there were correlations between toxin content, growth rate and cell size of *A. pacificum*. Before applying statistical tests the normality of the data was verified. SigPlot Stats V10.0 software was used.

3. Results

3.1. Morphology and biometry

During culture assays, monoclonal *A. pacificum* cultures were obtained only from station 2 characterized by a muddy sediment rich in resistant cysts.

For morphological investigations, up to 30 cells were isolated from each monoclonal culture. The examined cells appeared as single cells or forming small chains of 2, 4 or 8 cells, the latter mainly present in exponential growth phase. Cells are globular, slightly wider than long and are antero-posteriorly compressed. They have rounded apex and a slightly concave antapex. The epitheca and hypotheca are equal in length. Plate formula Po (apical pore plate), 4' (apicals), 6'' (precingular), 6c (cingular), 8s (sulcal), 5''' (postcingular), 2'''' (antapical) refers to the genus *Alexandrium*. For the studied strains, all the observed cells (>30 individuals) were characterized by the absence of the ventral pore on the suture between first (1') and fourth (4') plates. The size characteristics of *A. pacificum* strains were examined using Flow CAM on up to 45 live cells for each strain (F). Cells ranged in size between 23 and 30 μm in width and 24–32 μm in length (Fig. 3, Table 2). Altogether, the obtained data are close to those observed for other strains of *A. pacificum* developing in other marine systems (Fukuyo, 1985; Hallegraeff et al. 1991; Laabir et al., 2013).

3.2. Ribotyping and phylogeny

All the sequences obtained for the 20 examined strains, randomly selected, were identical and were also identical to sequences identified as *A. pacificum* group IV (called Temperate Asian clade) from Genbank. The phylogeny inferred from LSU rDNA showed that all these sequences clustered in a highly supported clade which indicated that the LSU of the strains from Annaba is identical to the strains also found in Japan, Mediterranean area (South of France, Tunisia) and also to the strain ACPPO1 from Australia and recently described as '*A. pacificum*' (Fig. 4).

3.3. Toxin content and profile

The PSTs were determined by Liquid Chromatography/Fluorescence Detection (LC/FD) allowing to separate carbamoyl toxins (STX, NeoSTX, GTX1, GTX2, GTX3 & GTX4), N-sulfocarbamoyl toxins (C1, C2, C3, C4, B1 = GTX5 & B2 = GTX6) and decarbamoyl toxins (dc-STX, dc-GTX2 & dc-GTX3), the structure of these toxins is reported in Fig. 2. The total cell toxin content varied widely from 3.8 fmol.cell⁻¹ for the strain AN G4-4 to 30.82 fmol.cell⁻¹ for AN D6-2 (Fig. 5). The strains showed mainly two different toxin profiles including all types of the carbamate toxins GTX 1, 2, 3, 4, and STX, NeoSTX, as well as two toxins belonging to the N- Sulfocarbamoyl toxins group (GTX5, GTX6). The first toxin profile was that dominated by GTX6 followed by C2 and GTX5 and concerned the largest number of the strains (ANB4-7, AN D9-1, AN G4-6, AN G4-8, Annaba C3-5, Annaba C10-5, Annaba C11-5, Annaba E10-4, Annaba F3-6, Annaba G3-6, AN B4-6, AN D2-8, AN D2-9, AN D4-6, AN G4-10 and AN G4-4) (Figs. 5 and 6). The second profile was characterized by the domination of C1 followed by GTX1, GTX6 or GTX5, and displayed by the strains AN B4-3, AN B4-5, AN D 4–5, AN D6-1, AN D6-2, AN G4-1, AN G4-9 and AN G5-1 (Fig. 5). The strain AN D4-2 showed a completely different profile with the domination of GTX1 followed by Neo-STX mainly. STX was absent or present at low concentrations (<0.1 fmol cell⁻¹). NeoSTX was present in most of the strains but in low concentrations (<1.623 fmol.cell⁻¹). The calculated toxicity of the 26 Annaba strains varied widely, the most toxic strain was AN D6-2 (1.87 pg STX equiv cell⁻¹), while the less toxic was AN B4-3 (0.08 pg STX equiv cell⁻¹) (Fig. 7).

3.4. Growth characteristics

A. pacificum strains cultivated on artificial seawater medium showed a large variability in growth rates (Fig. 8). Values ranged from 0.05 d⁻¹ (AN D4-1) to 0.33 d⁻¹ (AN B4-1). We observed a high variability in the duration of the lag phase among the strains. Values ranged between 4 and 17 days (Fig. 9).

3.5. Correlations results

There are no significant relationships using both Pearson ($P \geq 0.851$) and Spearman ($P \geq 1$) comparison test between any pair of variables (Toxin content, growth rate and cell width). For the analyzed strains of *A. pacificum* toxin content is therefore not related to growth rates or cell size.

4. Discussion

4.1. Species identification and morphology

The main morphological characters which allow distinguishing between *A. pacificum* and *A. tamarensis* species are the shape of the apical pore plate (Po), the position of posterior attachment pore (p.a.p) in sulcal posterior plate and the presence or absence of the ventral pore (v. p) (Fukuyo, 1985). This author stated that the ventral pore is the most reliable character since other morphological differences change with the growth phase and the culture conditions. Number of cells in the chain is also used to distinguish the two species. According to Balech (1985), *A. pacificum* forms long chains and cells are anterior-posteriorly flattened, lacking a ventral pore on the plate 1' and this latter is connected to Po. In our study, photonic observations of a large number of the vegetative cells of all of examined cultures showed clearly the absence of the ventral pore. All of the cultures exhibited chains formed by 2–8 cells during the exponential growth phase. The observed morphological characteristics and the ability to form routinely chains allowed us to identify the dinoflagellate species blooming in Annaba Bay as *A. pacificum*. Our morphological study was confirmed by ribotyping which showed that *A. pacificum* strains isolated from Algerian coastal waters as those from other ecosystems of western Mediterranean were closely related to the *A. pacificum* strains blooming in temperate Asian coastal waters (Lilly et al., 2002; Penna et al., 2005; Masseret et al., 2009). Sequencing of the D1-D2 region and LSU rDNA is a strong taxonomic marker allowing the determination of the species and its bio-geographical origin (Scholin et al., 1994). However, the analysis of these sequences provides specific information but not population nor individual ones and cannot respond to questions related to morphological and physiological intraspecific variability (Aguilera-Belmonte et al., 2011).

A. pacificum appeared for the first time in Catalonia (Spain) in 1983 (Margalef and Estrada, 1987) and its presence was later reported in the Mediterranean Thau lagoon of France (Abadie et al., 1999; Lilly et al., 2002), Bizerte Lagoon (northern Tunisia) (Turki et al., 2007; Fertouna-Bellakhal et al., 2015), Santa Giusta Lagoon (Italy) (Satta et al., 2013), many Spanish Catalan harbors (Vila et al., 2001) and Annaba Bay (Algeria) (Frehi et al., 2007). Our ribotyping results confirmed that Eastern Algerian waters contain *A. pacificum* from Group IV which constitute additional information to the study of Frehi et al. (2007). This study was only based on morphological classical identification. There is no mollusc culture in the Bay of Annaba that could be responsible for the accidental introduction of *A. pacificum* due to molluscs translocation. This species may have been introduced through ballast water via the international port of Annaba. Hence, Cheniti et al. (2018) showed the presence of *A. tamarensis* complex in the ballast waters of bulk carrier ship coming from Castelon, Spain. Some Studies based on LSU rDNA D1-D2 sequencing (Lilly et al., 2002) and microsatellite markers (Masseret et al., 2009) suggested the introduction of *A. pacificum* into Mediterranean waters from Japan due to human activity (ballast water discharges and/or shellfish translocation). *A. pacificum* blooms have not been observed earlier than 1983 in the Mediterranean coasts. Most of the marine waters (as Thau lagoon in France, Spanish coast: Lilly et al., 2002; Vila et al., 2001) where this species develops are close to commercial harbors as is Annaba Bay. It is possible that ballast water carrying cysts of this species has contaminated the eutrophic station 2. This has facilitated the establishment and recurrence of blooms fueled by a large bank of cysts that lets us assume that *A. pacificum* was introduced in this zone before the first observation by Frehi et al. (2007)

4.2. Toxins diversity and amounts

Many *Alexandrium* species are known to produce saxitoxin and its derivatives. Strains belonging to the same species and appearing morphologically identical can have different toxin profiles. This has been observed in species of the *A. tamarensis* complex (Touzet et al., 2008; Alpermann et al., 2010; Aguilera-Belmonte et al., 2011; Varela et al., 2012). Here, the toxin analyses of *A. pacificum* strains originating from Annaba bay reveal a noticeable diversity in the toxin profile and amount of each strain. The toxin profiles of most strains (18 strains among 26 analyzed) were characterized by the dominance of GTX6 (up to 83.82 mol % for the strain Annaba B10-6) and noticeable amounts of C1 (10.9–34.05 mol %). Hallegraef et al. (1991) observed that the principal toxin in the Australian *A. pacificum* was GTX6 (30–57%). The prevalence of N-Sulfo-Carbamoyl toxins (GTX5-6) has been reported in many other studies on *A. pacificum* strains from different locations: Thau lagoon in French Mediterranean (Lilly et al., 2002; Laabir et al., 2013), China (Xu et al., 2012; Li et al., 2011), Chile (Varela et al., 2012), and New Zealand (Mckenzie et al., 2004). The Tunisian strain ABZ1 isolated from Bizerte lagoon (far from Annaba bay only 286 km away) showed a very similar profile to many of the examined Algerian strains with the dominance of GTX6 and C1 representing 97.2%, whereas Neo-STX was a minor toxin (2.8%) (Fertouna-Bellakhal et al., 2015). Two strains of *A. pacificum* isolated in 1998 from Thau Lagoon (Mediterranean, France) namely ATTL01 and ATTL02 showed a dominance of C1,2 and GTX1,4, 5. Another strain ACT03 from Thau Lagoon, isolated in 2003, was characterized by the dominance of C2, C4, GTX4 and GTX5 (Laabir et al., 2013). *A. catenella* (Group I), renamed *Alexandrium fundyense* by John et al. (2014), developing in southern coast of Chile was characterized by high amounts of C1,2 or GTX1,4 depending on the strain (Varela et al., 2012).

Strains AN B4-3, AN B4-5, AN D 4–5, AN D6-1, AN D6-2, AN G4-1, AN G4-9 and AN G5-1 are distinguished by the dominance of GTX1 (up to 80.4% for AN D4-2) and C1 (up to 94.72% for AN D6-2), a highly toxic PST. Such toxin profiles have been shown for other strains of *A. pacificum* from Hong Kong (Siu et al., 1997) as well as for *A. pacificum* in Southern Chile (Aguilera-Belmonte et al., 2011). In addition, among these strains, the strain AN D4-2 produces mainly GTX-1 and Neo-STX, which have a toxicity equivalent to that of STX. The concentrations of toxins in the strains from Annaba Bay ecosystem varied between 3.8 and 30.82 fmol cell⁻¹. These values are not different from those observed for other *Alexandrium* species (Lilly et al., 2002; Mckenzie et al., 2004; Li et al., 2011; Aguilera-Belmonte et al., 2011; Laabir et al., 2013). The total toxicity of the strains was between 0.08 and 1.87 pg STX equiv cell⁻¹, this degree of toxicity having also been observed in strains of *A. pacificum* from New Zealand (1.3–6.6 pg STX equiv cell⁻¹; Mckenzie et al., 2004) as well as in *A. pacificum* cells from Irish waters (2.3–23.8 pg STX equiv cell⁻¹; Touzet et al., 2008). However, these levels of toxicity are relatively low compared to those observed for other *A. pacificum* strains blooming in Chile (63.65 pg STX equiv cell⁻¹; Varela et al., 2012) and *A. pacificum* blooming in the Beagle Channel, Argentina (325 pg STX equiv cell⁻¹; Benavides et al., 1995).

Several studies showed that the toxin content varies with the environmental conditions and the growth phase (for a review see Anderson et al., 2012; Laabir et al., 2013). To a smaller extent, changes in some abiotic factors could influence the toxin composition such as inorganic nitrogen which has been shown to influence the type of toxin produced by the cells in *A. tamarensis* from Southern Chinese waters. In medium with low nitrogen, cells tend to produce C1 and C2 toxins whereas in environments with low phosphorus concentration GTX2 and GTX3 dominated (Wang and Hsieh, 2005). It has also been shown that the abundance and the diversity of bacteria associated with *Alexandrium* cells could play a role in the diversity of toxin profiles among strains isolated from the same geographical location (Uribe and Espejo, 2003). The toxin content may vary depending on environmental conditions but not the toxin profile which is more stable and can be different among strains and species (Wang et al. 2006). It has been suggested that toxins can increase competitiveness of *Alexandrium* towards predators (Touzet et al., 2008). Some environmental conditions could select some phenotypes of the blooming

populations which supply a cyst bank with different phenotypes (Anderson et al., 1994; Alpermann et al., 2009). This phenomenon can explain the toxin variability observed in the present study among the 26 analyzed strains.

4.3. Growth and lag time characteristics

Growth rate is an important determining factor of the bloom success; this physiological parameter can vary between strains (Tillmann et al., 2009; Hadjadji et al., 2012). Several studies revealed a significant intraspecific growth rates variability as for *Karlodinium veneficum* strains (Calbet et al., 2011). Tillmann et al. (2009) highlighted a similar variability in the growth rates of several *A. tamarensis* (group I) strains isolated from the East coast of Scotland. Hadjadji et al. (2012) demonstrated high intraspecific variability in oligo-nutrients requirements and growth rates among strains of *A. tamarensis* isolated from the same bloom which occurred in Thau lagoon (France). Thessen et al. (2009) showed a high diversity in the growth rate of 19 strains of *Pseudo-nitzschia* from mid-Atlantic Coast of the United States. The variations in the lag time (4 and 20 days) obtained for *A. pacificum* strains in the present work have been observed for other *A. pacificum* strains developing worldwide. The lag time varied between 0 and 6 days for *A. catenella* from southern Chile (Navarro et al., 2006), was of 14 days for strains from Hong Kong (Siu et al., 1997). The same variability in the lag time was showed by Hadjadji et al. (2012) for *A. tamarensis* from Thau lagoon. Variability in the ability of strains to adapt to the ESAW medium may explain the variability in duration observed of lag time.

Here, we observed an important variation in the growth rate of the studied strains of *A. pacificum*, with values ranging from 0.05 to 0.33 d⁻¹. These measured growth rates of *A. pacificum* Algerian strains were slightly lower than those of other *A. pacificum* strains developing in other marine systems around the world. For example, Laabir et al. (2011) reported a growth rate of 0.4 d⁻¹ for *A. pacificum* blooming in Thau lagoon (France), Aguilera-Belmonte et al. (2011) showed that the Chilean *A. catenella* strains have growth rate ranging from 0.42 to 0.73 d⁻¹ and finally Fauchot et al. (2005) reported a growth rate of 0.55 d⁻¹ of *A. catenella* isolated from St Lawrence estuary. The observed differences highlight the intraspecific variability of the growth capacity of the studied strains.

5. Conclusion

This study confirms that to gain more insight in the plasticity of a species and thus its adaptive potential, we have to investigate the physiology of high number of strains. It also corroborates previous works showing significant phenotypic differences and high degree of plasticity in regards to toxin content, growth rate and size among strains of many HABs species developing in different marine waters (e.g., Alpermann et al., 2010; Kremp et al., 2012; Calbet et al., 2011; Tillmann et al., 2009; Thessen et al., 2009; Hadjadji et al., 2012; Sampedro et al., 2013; Brandenburg et al., 2018; Cruzat et al., 2018; Park et al., 2018). This is the first time that this plasticity is demonstrated for monoclonal cultures obtained from single resting cysts suggesting that the cyst banks even originating from a restricted geographic area (one station in the Gulf of Annaba) holds an unsuspected richness of strains differing in their physiology and probably genotypes.

The observed intraspecific diversity could be a response to the selection pressure that can be exerted by different environmental conditions over time and which can be genetically and physiologically expressed. Our work suggests that whatever sites and strains studied, there is a wide variability within *A. pacificum* populations. Therefore the amazing fitness of this species could explain its dispersion capacity and its exceptional adaptation to the contrasted colonized environments. Finally, given the significant diversity of the physiological traits of this species in the studied Mediterranean ecosystem, making it highly adaptable to the changing environment, we must expect its spread and repeated blooms.

Ethical statement

We agree with Toxicon Ethical Statement. I confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

Imen Hadjadji: Methodology, Investigation, Formal analysis, Data curation, Writing - original draft. **Mohamed Laabir:** Formal analysis, Writing - original draft, Validation, Writing - review & editing. **Hocine Frihi:** Formal analysis, Writing - original draft, Methodology, Validation. **Yves Collos:** Methodology, Writing - original draft, Formal analysis, Conceptualization. **Zhao Jun Shao:** Methodology, Formal analysis, Writing - original draft. **Patrick Berrebi:** Methodology, Formal analysis, Writing - original draft. **Eric Abadie:** Methodology, Formal analysis, Writing - original draft. **Zouher Amzil:** Methodology, Investigation. **Nicolas Chomerat:** Methodology, Investigation. **Jean Luc Rolland:** Methodology, Formal analysis, Writing - original draft. **Fabien Rieuvilleneuve:** Investigation. **Estelle Masseret:** Conceptualization, Funding acquisition, Investigation, Project administration, Methodology, Writing - original draft, Formal analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxicon.2020.04.005>.

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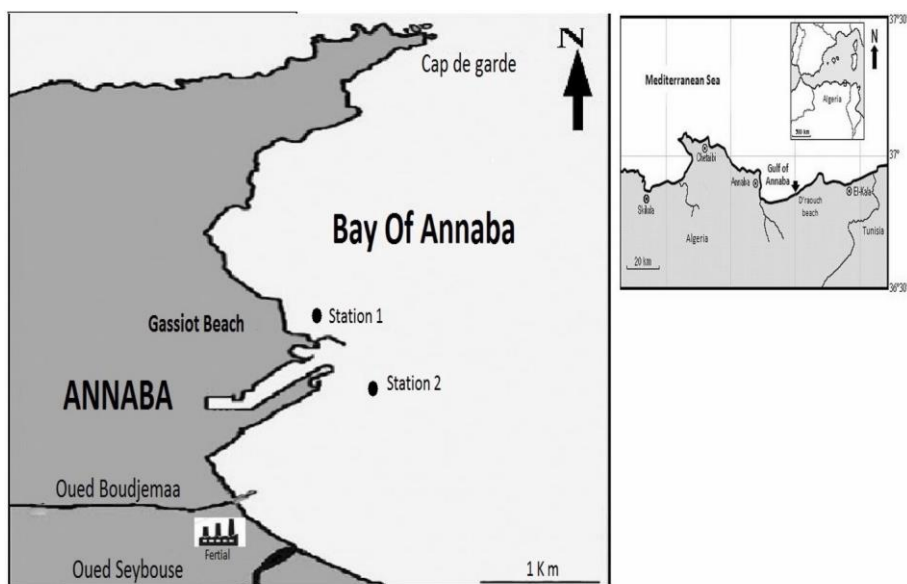
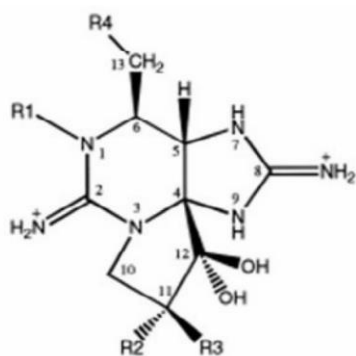


Fig. 1. Location of the sampling stations in Annaba bay (Mediterranean, Algeria).



R1	R2	R3	R4: -CONH ₂	R4: -CONHSO ₃	R4: -OH	R4: -H
H	H	H	STX	GTX5	dcSTX	doSTX
OH	H	H	NeoSTX	GTX6	dcneoSTX	-
OH	OSO ₃ ⁻	H	GTX1	C3	dcGTX1	-
H	OSO ₃ ⁻	H	GTX2	C1	dcGTX2	doGTX2
H	H	OSO ₃ ⁻	GTX3	C2	dcGTX3	doGTX3
OH	H	OSO ₃ ⁻	GTX4	C4	dcGTX4	-

STX: saxitoxin; GTX: gonyautoxin; dc: decarbamoyl; do: deoxydecarbamoil

Fig. 2. Common structure of Paralytic Shellfish Toxins.

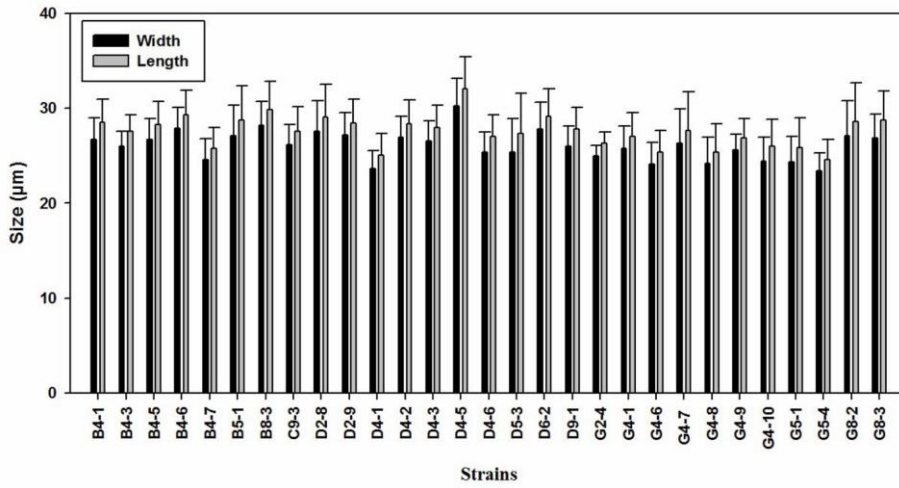


Fig. 3. Biometry (length and width in μm) of *Alexandrium pacificum* strains using Flow CAM device on up to 45 live cells for each monoclonal culture.

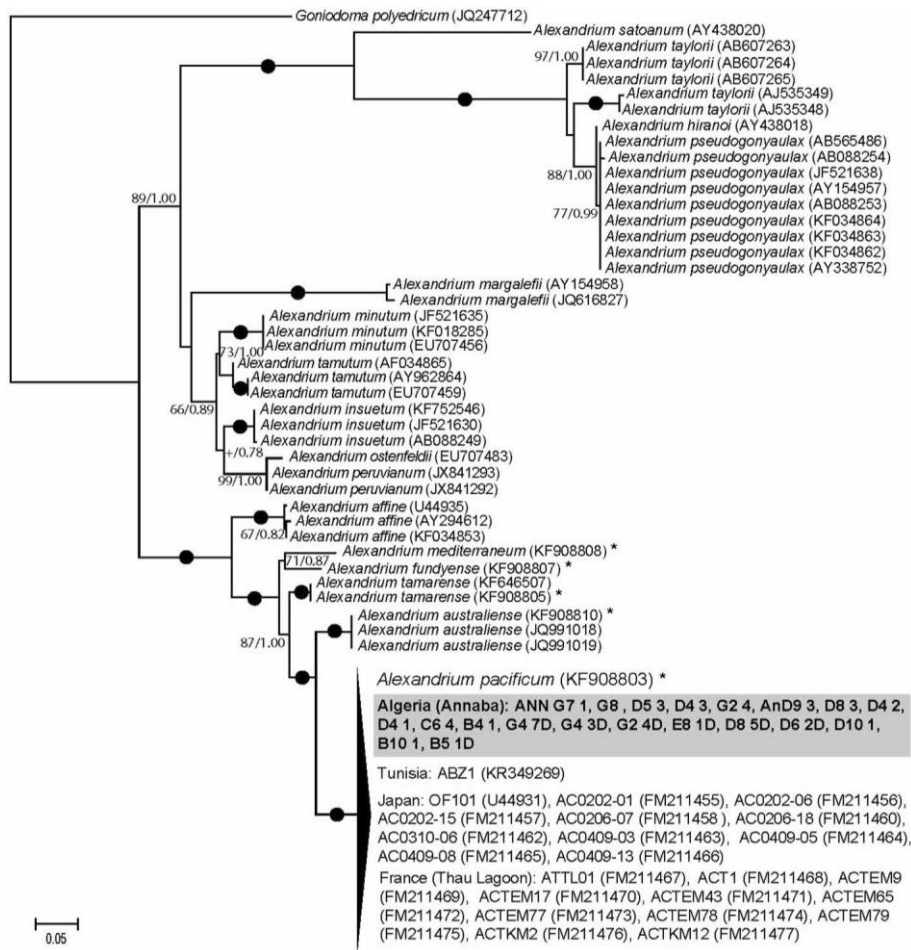


Fig. 4. Maximum likelihood phylogenetic tree of *Alexandrium pacificum* strains inferred from partial LSU rDNA (matrix of 85 taxa and 605 positions). Filled black circles indicate full statistical support of branches (100/1.00). Values associated with branches correspond to bootstrap support and posterior probabilities (ML/BI).

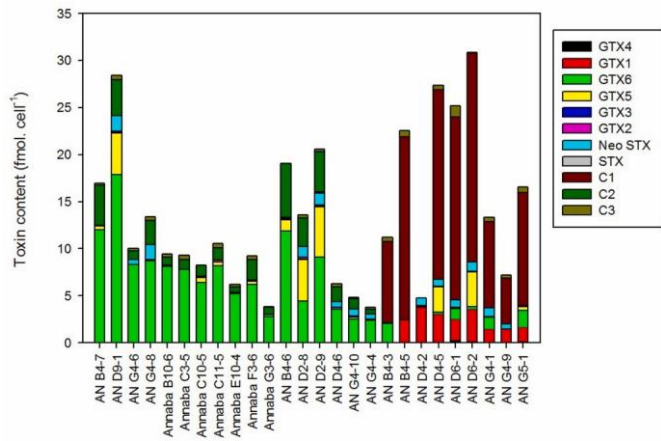


Fig. 5. Toxin content (fmol cell⁻¹) of twenty six strains of the toxic *Alexandrium pacificum*.

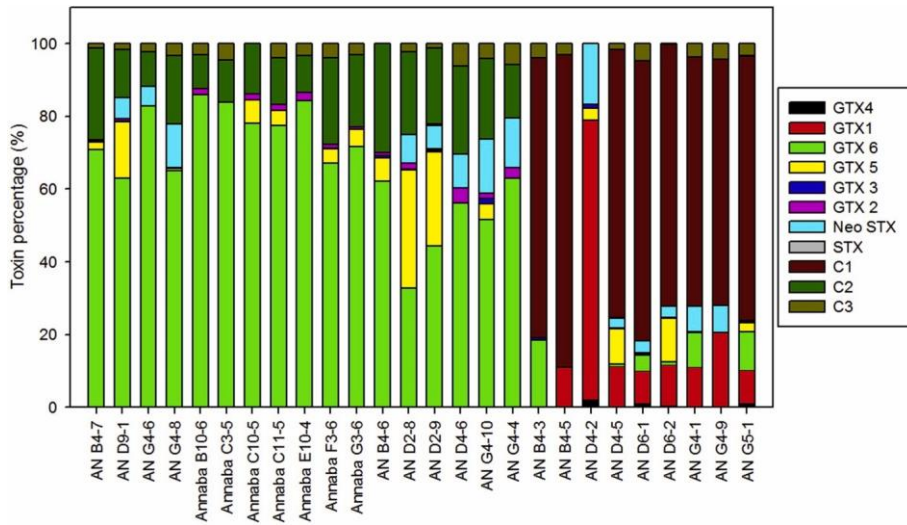


Fig. 6. Percentage of each toxin in the studied strains of *Alexandrium pacificum*.

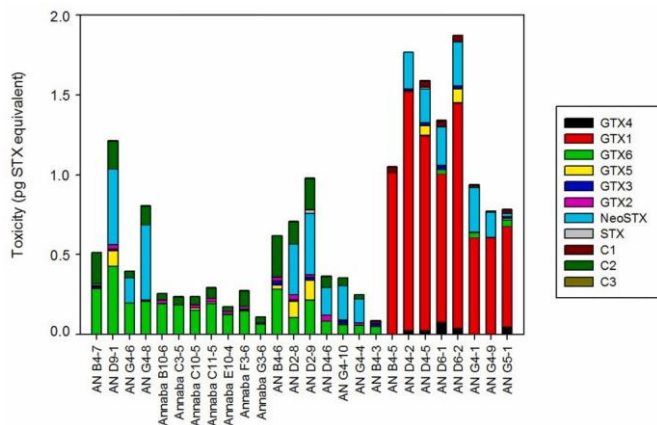


Fig. 7. Toxicity (pg STX equivalent cell⁻¹) of twenty six strains of the toxic *Alexandrium pacificum*.

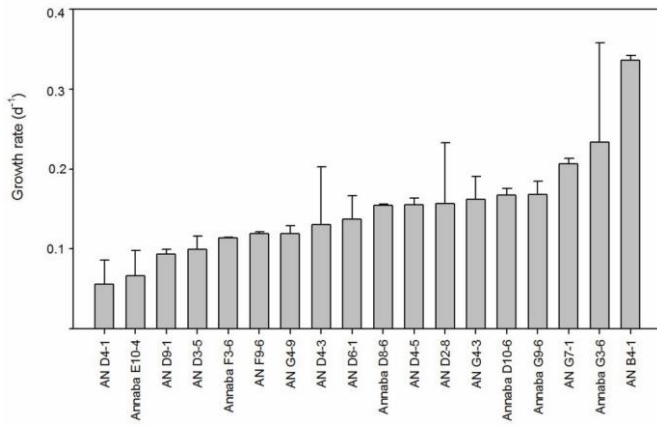


Fig. 8. *Alexandrium pacificum* growth rates (day⁻¹) variability of different strains.

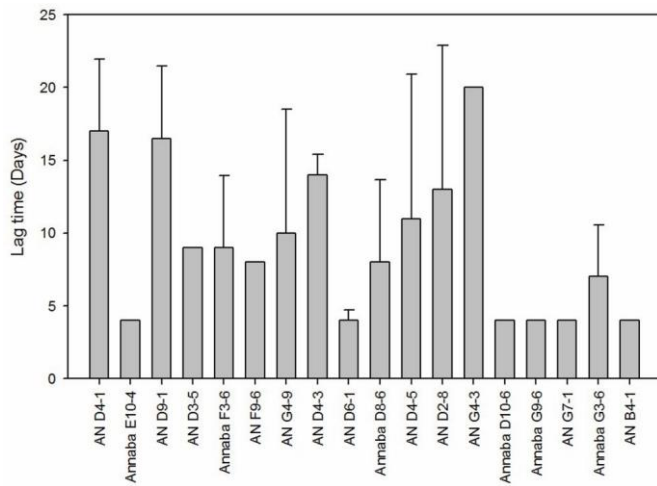


Fig. 9. Lag time (days) observed during the growth of *Alexandrium pacificum*.

Table 1

The limit of detection limits (LOD) and limit of quantification limit (LOQ) of PSTs..

Toxins	LOD ($\mu\text{mol L}^{-1}$)	LOQ ($\mu\text{mol L}^{-1}$)
GTX4	0.030	0.090
GTX1	0.035	0.105
GTX3	0.010	0.030
GTX2	0.015	0.045
dc-GTX3	0.003	0.009
dc-GTX2	0.012	0.036
GTX5	0.040	0.120
NeoSTX	0.030	0.090
dc-STX	0.018	0.054
STX	0.020	0.060

Table 2Cell size in μm (width and length) of *Alexandrium pacificum* strains.

Strain	Mean width	Min	Max	Std Dev	% CV	Mean length	Min	Max	Std Dev	% CV	Number of cells analyzed
AN B4-1	26.68	23.48	30.86	2.28	8.55	28.5	24.89	33.28	2.46	8.65	39
AN B4-3	25.97	22.92	27.85	1.57	6.05	27.59	24.6	30.23	1.74	6.31	31
AN B4-5	26.75	23.03	31.5	2.17	8.11	28.27	24.41	33.67	2.47	8.73	32
AN B4-6	27.86	21.43	34.49	2.26	8.13	29.32	22.21	35.83	2.61	8.89	36
AN B4-7	24.6	20.05	29.58	2.2	8.93	25.74	20.93	31.28	2.26	8.77	55
AN B5-1	27.07	20.23	35.25	3.25	12	28.79	21.39	39.25	3.6	12.49	36
AN B8-3	28.2	22.31	36.13	2.49	8.84	29.88	23.08	39.28	2.95	9.88	29
AN C9-3	26.14	22.37	30.92	2.16	8.28	27.6	23.13	33.67	2.54	9.19	57
AN D2-8	27.54	22.43	35.3	3.25	11.8	29.05	23.8	37.54	3.49	12.02	49
AN D2-9	27.16	21.49	32.29	2.41	8.86	28.42	22.65	34.21	2.57	9.06	35
AN D4-1	23.65	20.17	28.25	1.91	8.06	25.08	21.22	31.23	2.25	8.97	42
AN D4-2	26.96	23.75	30.73	2.18	8.09	28.33	24.23	33.45	2.56	9.04	42
AN D4-3	26.53	23.17	32.96	2.12	7.98	27.95	23.44	36.37	2.38	8.51	45
AN D4-5	30.26	24.14	39.29	2.91	9.63	32.06	25.29	42.32	3.4	10.61	52
AN D4-6	25.41	20.84	28.79	2.09	8.23	27.04	22.16	30.73	2.29	8.46	33
AN D5-3	25.37	20.04	34.54	3.55	14	27.35	21.3	38.38	4.25	15.54	37
AN D6-2	27.8	22.66	33.8	2.85	10.3	29.15	24.39	35.26	2.88	9.89	40
AN D9-1	26.02	22.84	31.21	2.09	8.04	27.84	24.06	33.18	2.28	8.18	33
AN G2-4	24.95	23.09	27.06	1.11	4.45	26.34	24.41	28.53	1.15	4.36	29
AN G4-1	25.74	21.75	31.4	2.39	9.29	26.99	22.85	32.66	2.51	9.3	40
AN G4-6	24.14	20.25	29.79	2.25	9.31	25.4	21.43	30.72	2.29	9.03	43
AN G4-7	26.31	20.21	37.51	3.65	13.9	27.68	21.2	41.22	4.02	14.51	35
AN G4-8	24.2	19.28	31.76	2.74	11.3	25.37	20.68	32.94	2.95	11.65	36
AN G4-9	25.59	21.96	28.74	1.68	6.55	26.85	23.17	32.46	2.03	7.57	41
AN G4-10	24.45	19.65	30.5	2.5	10.2	26.02	20.86	32.35	2.82	10.84	34
AN G5-1	24.38	18.95	31.75	2.66	10.9	25.84	19.6	34.42	3.13	12.1	45
AN G5-4	23.39	19.13	27.05	1.87	7.98	24.63	19.63	29.03	2.1	8.54	42
AN G8-2	27.06	21.78	36.3	3.76	13.9	28.62	22.99	38.52	4.07	14.21	35
AN G8-3	26.84	19.73	33.36	2.51	9.36	28.78	20.83	37.2	3.04	10.57	33