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Influence of environmental parameters on *Karenia selliformis* toxin content in culture

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Abstract: *Karenia selliformis* strain GM94GAB was isolated in 1994 from the north of Sfax, Gabès gulf, Tunisia. This species, which produces gymnodimine (GYM) a cyclic imine, has since been responsible for chronic contamination of Tunisian clams. A study was made by culturing the microalgae on enriched Guillard f/2 medium. The influence of growing conditions on toxin content was studied, examining the effects of (i) different culture volumes (0.25 to 40 litre flasks), (ii) two temperature ranges (17-15°C et 20-21°C) and (iii) two salinities (36 and 44). Chemical analyses were made by mass spectrometry coupled with liquid chromatography (LC-MS/MS). Results showed that (i) the highest growth rate (0.34 ± 0.14 div d^{-1}) was obtained at 20°C and a salinity of 36, (ii) GYM content expressed as pg eq GYM $cell^{-1}$ increased with culture time. The neurotoxicity of *K. selliformis* extracts was confirmed by mouse bioassay. This study allowed us to calculate the minimal lethal dose (MLD) of gymnodimine (GYM) that kills a mouse, as a function of the number of *K. selliformis* cells extracted.

Résumé : *Influence des paramètres environnementaux sur la teneur en toxine d'une souche cultivée de* Karenia selliformis. La souche *Karenia selliformis* GM94GAB a été isolée en 1994 au nord de Sfax dans le golfe de Gabès (Tunisie). Depuis, cette espèce, productrice de la gymnodimine (GYM, imine cyclique) est responsable de la contamination chronique des palourdes en Tunisie. L'étude de l'influence des conditions de croissance sur le contenu toxinique de *K. selliformis*, en culture avec le milieu f/2 de Guillard, a porté sur (i) les volumes de culture (0.25 à 40 L), (ii) deux gammes de température (15-17°C et 20-21°C), (iii) deux salinités (36 et 44). L'analyse chimique a été réalisée en Chromatographie Liquide couplée à un Spectromètre de Masse (CL-SM/SM). Les résultats montrent que (i) le taux de croissance le plus élevé (0.34 ± 0.14 div j^{-1}) est obtenu à la température de 20°C pour une salinité de 36, (ii) le contenu en GYM exprimé en pg eq GYM $cell^{-1}$ augmente en fonction de l'âge de la culture. De plus, la neurotoxicité sur souris des extraits de *K. selliformis* a été confirmée. La dose minimale mortelle (DMM) de GYM pour la souris a été déterminée en fonction du nombre de cellules de *K. selliformis* extraites.

Keywords : Karenia selliformis ● Growth ● Toxicity ● Gymnodimine ● Mouse bioassay

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Introduction

The Gabès gulf in southern Tunisia is an important area for natural stocks of the grooved carpet shell, *Ruditapes decussatus* (Linné, 1758) for both local and national fisheries. Exploitation of these beds is a long tradition that supports around 8000 families and represents a source of income for the country. Unfortunately, over the last fourteen years, the production of this shellfish has been repeatedly affected by phycotoxin contaminations. The problem has been particularly recurrent at Boughrara (Hamza et al., 1999; Hamza, 2003). In 1991, Guerloget signaled the presence of a toxic alga on the south Tunisian coasts. The species was first identified as *Gyrodinium aureolum* (Guerloget, 1991), then in 1994 as *Gymnodinium sp*. (Arzul et al., 1995). Coloration of the waters of the Gabès gulf was seen for the first time in 1994, due to a cellular concentration of 60×10^6 cells L^{-1}, and mass fish mortality occurred (Hamza & El Abed, 1994; Hansen et al., 2004). It was only in 2004, however, that this microalga was renamed *Karenia selliformis* by Haywood et al. (2004). The same species was also associated with high fish mortality on Kuwaiti coasts in 1999 (Heil et al., 2001).

In the gulf of Gabès different toxic dinoflagellates were detected (Turki el al., 2006; Fiki et al., 2008; Dammak-Zouari et al., 2009). Moreover, several studies reported that *K. selliformis* is considered as the most problematic species in this gulf, causing 67 to 70% of toxicity cases (Fiki et al., 2008; Dammak-Zouari et al., 2009). During the past 10 years, the routine monitoring of this toxic dinoflagellate in the gulf of Gabès was correlated with abiotic conditions (Fiki et al., 2008). These last authors showed that blooms of *K. selliformis* were observed at three temperature levels (< 18°C, between 18 and 22°C and > 22°C) and with preference for high salinities (42 and 50 in the Boughrara lagoon). Moreover, Dammak-Zouari et al. (2009) confirmed that blooms of *K. selliformis* are detected at high temperatures (> 19°C) in all sampling stations except the Boughrara lagoon, which is characterized by high salinity (up to 52) and where blooms coincide with temperatures of 13 to 16°C.

Figure 1 shows the structure of the basic toxin gymnodimine A (GYM), and its analogues (Seki et al., 1995; Biré et al., 2002; Miles et al., 2000 & 2003). This phycotoxin was discovered for the first time in 1994, in oysters (*Tiostrea chilensis* Philippi, 1845) from the Foveaux strait, New Zealand (Mackenzie et al., 1995 & 1996). In Tunisia, the relation between *Karenia* and GYM was confirmed by the work of Biré et al. (2002) on bivalve contamination.

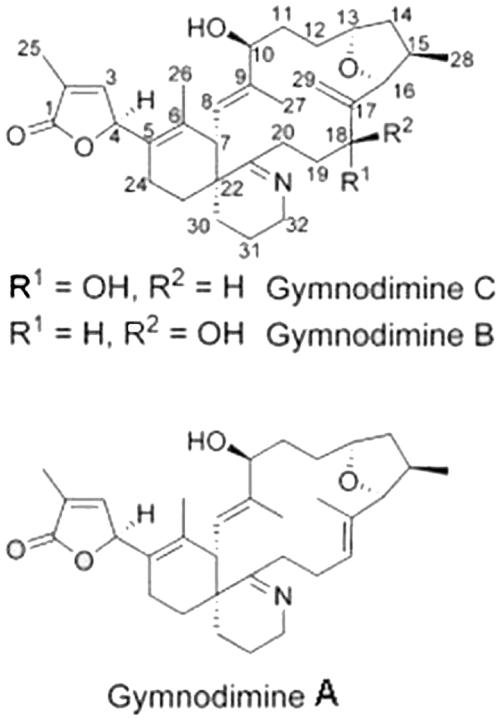

Figure 1. Gymnodimine A structure and its analogues B and C.

Figure 1. Structure de la Gymnodimine A et de ses analogues B et C.

These contaminations have meant that the harvest and consumption of clams has been banned and their production has fallen, which has had a serious economic impact for those working in this industry. A monitoring network, in place since 1995, guarantees products for export to the European Community and those for the home market. It relies on shellfish toxicity testing according to the official mouse test for lipophilic toxins (Yasumoto et al., 1978), and on the detection of the presence of any toxic species.

* Determined as the difference between cell density at the beginning of growth and at maximum cell yield

Different letters indicate significantly different values (P=0.05) according to ANOVAs test.
(I): Effect of culture volume on the growth rate of *K. selliformis* at 16°C and 36.
(II): Effect of temperature and salinities on growth rate of *K. selliformis* in Erlenmeyer culture of 1.5 L.
(III): Effect of salinities on the growth rate of *K. selliformis* in tank culture.
(IV): Effect of culture volume on the growth rate of *K. selliformis* at 20°C and 36.

The strain GM94GAB of *K. selliformis*, isolated by E. Erard-Le Denn (Ifremer), was put into culture at the INSTM Centre de Monastir in June 2005.

In the aim to reduce the economic impact of GYM contamination in clams, a project was started to study the feasibility of clam decontamination: *'Elevage Suivi Sanitaire et Détoxification des Animaux Filtreurs'* (ESS-DAF) - Cultivation, sanitary supervision and detoxication of filtering animals.

Data were therefore needed on contamination kinetics. However, before any controlled contamination experiments could be done, a full technical understanding of *K. selliformis* culture was needed with the objective i) to produce *K. selliformis* cells of known toxicity and ii) to perform further contamination/decontamination trial on edible shellfish.

We examined the influence of the alga in culture environment, two temperature scales and two salinities, on the toxin content of cultured *K. selliformis*. The neurotoxicity of GYM was determined by the minimum lethal dose (MLD) of GYM by intraperitoneal (ip) administration to mice, using extracts of cultured *K. selliformis*. Measurement of toxin content was made by chemical analysis using Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS).

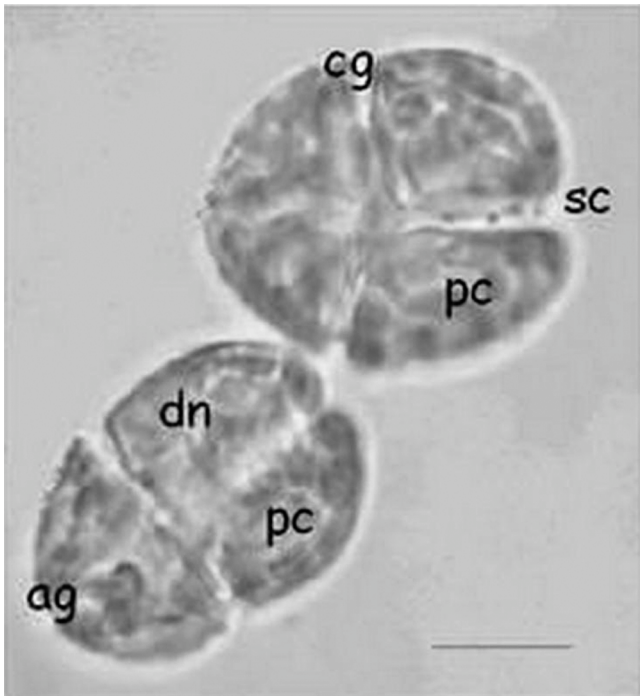

Figure 2. *Karenia selliformis*. Photo in light microscopy of cg: cingulum, sc: sulcus, pc: peripheral chloroplasts, dn: dinocaryon-loged in the posterior right of hypoteca, ag: apical groove. Scale: 10 µm

Figure2. *Karenia selliformis*. Vue microscopique, cg : cingulum, sc : sulcus, aps : assise périphérique des chloroplastes, dn : dinocaryon logé dans la partie postérieure droite, ca : carène apicale. Echelle : 10 µm

Table 1. *Karenia selliformis*. Culture conditions, growth and toxin content.
Tableau 1. *Karenia selliformis*. Conditions de culture, croissance et teneur en GYM.

	Culture vessel	Volume (mL)	Temperature (°C)	Salinity	Maximum cell yield (cell mL^{-1})*	Maximum cell production rate (cell mL^{-1} d^{-1})		µ (d^{-1})	Standard deviation	GYM (pg cell^{-1}) D7 - D14 - D21
I	**Erlenmeyer**	250	16	36	17330	19300	D21	0.11 [a]	0.05	8.3- 9.3 - 22.4
		1000			24930	27000	D21	0.12 [a]	0.06	5.6 - 12.1 - 12.9
		2000			17000	19100	D21	0.11 [a]	0.04	5.3 - 10.5 - 8.1
		8000			2570	4600	D14	0.06 [b]	0.07	2,1 - 5.6
										exp φ / decline φ
II	**Erlenmeyer**	1500	15	36	12874	13317	D29	0.11 [c]	0.06	2.1
			17		17475	17934	D32	0.14 [c]	0.07	1.2 - 3.4
			20		18722	19166	D11	0.34 [d]	0.14	5.1 - 10.05
			15	44	7960	8502	D29	0.10 [c]	0.06	6.4 - 18.2
			17		10670	11164	D24	0.13 [c]	0.06	1.6 - 12.6
			20		10201	12320	D11	0..3 [d]	0.17	9.5 - 18.2
III	**Tank**	40000	21	36	8380	8900	D18	0.15 [e]	0.05	Not done
				44	3960	4380	D18	0.13 [e]	0.07	
IV	**Erlenmeyer**	1500	20	36	18722	19166	D11	0.34 [f]	0.14	Not done
	Flask	15000	19.8		2973	5000	D20	0.09 [g]	0.05	
	Tank	40000	21		8380	8900	D18	0.15 [h]	0.05	

Material and Methods

Culture and growth conditions

The strain GM94GAB of *K. selliformis*, isolated by E. Erard-Le Denn (Ifremer) and in culture at the INSTM Centre de Monastir since June 2005, was used in the present study (Fig. 2). A certified standard solution of GYM was supplied by the Conseil National de Recherche Canadien (CNRC), Halifax, Canada.

K. selliformis was cultivated in natural seawater at two salinities: 36 and 44. All seawater was filtered at 1 µm and autoclaved. The cultures were set up in f/2 culture medium (Guillard & Ryther, 1962; Guillard, 1975), without aeration, in flasks and tanks of different sizes: 0.25, 1, 1.5, 2, 8, 15 and 40 L (Table 1). Experiments released in small and larger cultures were performed three times (n = 3).

The experiments were done at two temperature ranges 15-17 °C and 20-21°C, under a light intensity of 45 µmol photons m^{-2} s^{-1} provided by «cool white» fluorescent tube with a photoperiod of 12 h light/12 h dark.

To isolate other clones of *K. selliformis*, water samples were taken from different stations situated in the Gulf of Gabès. The choice of the temperature and salinity is based on the measures of these two factors during the sampling from coastal water of this Gulf.

Every 2 to 7 days, temperature and pH were measured and *K. selliformis* cell counts were made under the microscope using a Sedgwick Rafter® counting cell. Maximum cell yield was calculated as the difference between initial cell concentrations (at D_0) and final cell concentrations (Mountfort et al., 2006).

Sampling and extraction of cultured K. selliformis

For the chemical analysis of gymnodimine (GYM), three samples (n = 3) of 10 to 100 mL were taken, then extracted and pooled every 7 days from cultures of 250 mL, 1, 2 and 8 L. Concerning culture used in 1.5 L, samples were taken: at the end of the exponential phase (D_{30} at 15°C, D_{32} at 17°C and D_{11} at 20°C) and in the decline phase (D_{35} at 15°C, D_{40} at 17°C et D_{13} at 20°C).

For the mouse bioassay, three samples (n = 3) of 100 to 300 mL were taken, then extracted and pooled from culture of 1.5 L during each of these two phases: at the end of exponential growth (D_{32} at 17°C and D_{11} at 20°C) and during the decline phase (D_{40} at 17°C and D_{13} at 20°C).

The two types of sample were immediately centrifuged at 4000 rpm for 25 min. GYM was extracted from the cellular residues with 1 mL methanol (4 x 250 µL), followed by 20 min treatment with an ultrasound probe to ensure cell disruption.

Conditions of extract conservation depended on the method of analysis: i) for chemical analyses, conservation was done in methanol at -80°C; ii) for bioassays, the methanol was evaporated and the sample taken up in 1 mL Tween 60 at 1% (polyoxyethylene sorbitan monostearate) conservation at -20°C.

Mouse Bioassay

The bioassay used was the official mouse test for detection of lipophilic toxins (including 'diarrhetic shellfish poisoning' DSP toxins) (Yasumoto et al., 1978). These tests were done in an officially approved laboratory at INRVT-Centre régional de Sfax. For each mouse test, three mice (Swiss females) of 19 to 21 g were used. Each received 1 mL of the test solution by ip administration. The treated mice were kept under observation to note any symptoms and the duration of their survival.

To determine the minimum lethal dose (MLD) of GYM that can kill a mouse, different dilutions of the extracts of cultured *K. selliformis* were made in Tween 60 at 1%. The MLD was calculated from the GYM concentration per cell of *Karenia* determined by chemical analysis and given in µg GYM per Kg mouse weight. A series of doses was tested on mice to determine the MLD of GYM: 75, 80, 84, 86, 95, 100, 107, 120, 141, 150, 157, 175, 187, 200, 228, 250 µg Kg^{-1}.

Chemical analysis by LC-SM/SM

The chemical analysis of GYM levels in cultured *K. selliformis* was made by liquid chromatography coupled to a triple quadrupole mass spectrometer API 2000 (LC-SM/SM) according to a method modified from Quilliam et al. (2001). Concentrations were expressed in picograms of GYM per cell of *K. selliformis.* (pg eq GYM $cell^{-1}$).

Statistical analysis

Experimental data were analysed using Statistica 5.5 software. During all experiment, the effects of temperature, salinity and volume culture on the growth of the toxic dinoflagellate *K. selliformis* used in this study was were assessed using one or two factorial ANOVAs. The variance homogeneity of the data was checked using box M test. The growth rate was arcsin(x) transformed. For P-values less than 0.05, differences between growth rates were considered statistically significant at a 95% confidence level.

Results

Cellular growth of K. selliformis cultures

Data on culture conditions, growth (Fig. 3) and cellular GYM concentrations (eq. GYM-A by cell) are given in table 1. The growth (growth rate, cell yield and cellular concentration) of the toxic dinoflagellate was studied by examining the effects of volume, salinity and temperature. The growth rate of *K. selliformis* cultivated in 0.25, 1 and 2 L at 16°C and at a salinity of 36 (I: Table 1) showed a significant difference ($P < 0.05$) from the one cultivated in 8 L. The maximum cell yield and concentration of *K. selliformis* were obtained at D_{21} (24930 cell mL^{-1} and 27000 cell mL^{-1}, respectively) in a culture volume of 1 L at a salinity of 36.

In the same way, the growth rates of *K. selliformis* cultivated in 1.5, 15 and 40 L in the range of temperature 20-21°C at a salinity of 36 (IV, table 1) were significantly different ($P < 0.05$). The highest growth rate was found in 1.5 L. However the lowest was seen in culture volume of 40 L.

The maximum cell yield and concentration of *K. selliformis* obtained at D_{11} (18222 cell mL^{-1} and 19166 cell mL^{-1} respectively) in culture volume of 1.5 L at 36.

In the two ranges of temperature 15-17°C and 20°C (II, table 1) we saw no significant difference ($P > 0.05$) in the growth rate of *K. Selliformis* cultivated in 1.5 L at two salinities (36 and 44) (II, table 1). However, a significant difference ($P < 0.05$) was obtained in the growth rate between these temperature ranges. At 20°C, maximum division rates of 0.30 and 0.34 $div.d^{-1}$ were seen at salinity of 36 and 44, respectively, in a final culture volume of 1.5 L, with maximum cell yield and concentration reached at D_{11}. Also, when *K. selliformis* cultivated in larger culture of 40 L (III. table 1) at two salinities (36 and 44), there was no significant difference in growth rate ($P > 0.05$).

Toxicity

Cellular GYM concentrations, measured by LC-MS/MS chemical analysis, are shown in table 1. An example of analytical results for cultured *K. selliformis* is given in figure 4, showing the presence of GYM A in all samples, and GYM B at trace levels (< 0.3%). Our results showed that at all temperatures and salinities used cellular GYM concentrations increased with the age of the *K. selliformis* cultures. At D_{21} (Table 1), the maximum GYM concentration, 22 pg eq GYM $cell^{-1}$ at 36, was obtained in the smallest experimental volume (0.25 L). However, minimal concentrations were obtained in the 8 L culture:

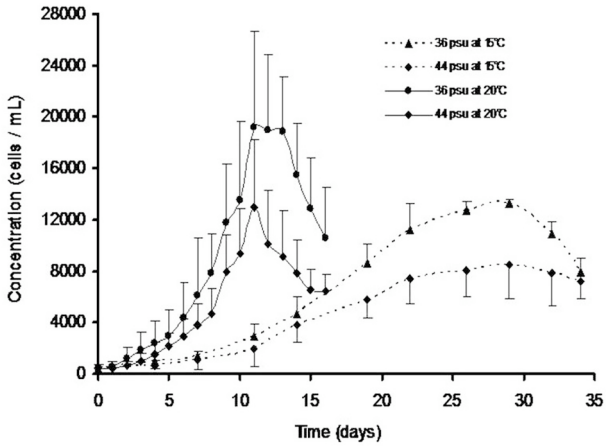

Figure 3. *Karenia selliformis.* Growth in volume of 1.5 L at two temperatures and two salinities: 15 and 20°C, 36 and 44.

Figure 3. *Karenia selliformis.* Croissance en volume de 1,5 L à 15 et 20°C, aux salinités 36 et 44.

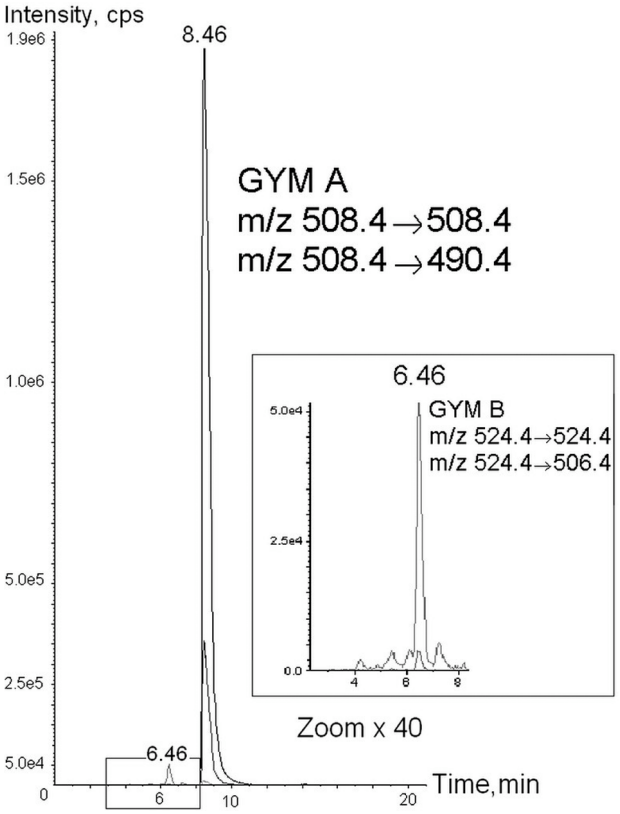

Figure 4. *Karenia selliformis.* Culture extract: CL-SM/SM result as showing the presence of the GYM-A and the B-GYM at trace (< 0.3%).

Figure 4. *Karenia selliformis.*Extrait de culture : résultat analytique en CL-SM/SM montrant la présence de la GYM-A, ainsi que la GYM-B à l'état de trace (< 0,3%).

5.6 pg eq GYM cell^{-1} at the same day of culture.

Table 1 showed that the maximum GYM concentration at decline phase was higher in the cultures grown at a salinity of 44 (18.2 pg at eq GYM cell^{-1}) than those at a salinity of 36 at the same phase (2 to 10 eq GYM cell^{-1}).

The MLD of the GYM for mouse was determined from *K. selliformis* culture extracts with variable cellular GYM concentration. Table 2 shows the doses of GYM that were lethal to mice, according to the growth phase, salinity and temperature of the algal cultures. Whatever the salinity (36 or 44) or the temperature (17 or 20°C), the number of cells needed was high during the exponential phase than in the decline phase.

Depending on the culture conditions, the MLD to a mouse varied from 84 to 228 eq GYM Kg^{-1} mouse weight (Table 2). However, when considering the same culture, the MLD (eq GYM Kg^{-1}) obtained in the exponential phase was similar to that obtained in the decline phase.

Moreover, our studies showed that the MLD of cultures grown at 20°C was higher than those of culture grown at 17°C (Table 2).

The symptoms of toxicity observed in the mice were stretching, hyperactivity, jumping immediately after injection, stiff tail, slowed movements, paralysis of the hind legs, breathing difficulties and exophthalmoses. These symptoms were followed either by death 3 to 7 minutes later at the MLD or by a recovery to a normal state after about 20 minutes.

Discussion

Growth

In the Gabès gulf, *K. selliformis* occurs in waters of different salinities: 36-37, 39-40, 41-44 and 47-52. There is also a salinity gradient running from the north to the south of the gulf. The lowest salinities are found to the north of Sfax, while the highest salinities are found further to the south. At Boughrara, salinity in the winter period is 41-44 and can reach 47-52 in summer. These data which were recorded during our samplings are confirmed by the work of Fiki et al. (2008) and Dammak-Zouari et al. (2009). These authors showed that blooms of *K. selliformis* are observed at various temperatures (< 18°C, between 18 and 22°C, > 22°C) and with preference of high salinity (> 42). For this reason we choose to study algal growth and toxicity at these two salinities (36 and 44) and at these two temperature ranges (15-17°C and 20-21°C). Although the acclimation of *K. selliformis* to salinity of 36-52 causes no particular problems or fundamental changes in the behaviour of these cells, this microalga does seem highly sensitive to temperature variation.

Several studies have been made on the effect of salinity on growth and toxicity of dinoflagellates such as *Alexandrium ostenfendii* (Maclean et al., 2003), *Karenia brevis* (Magana & Villareal, 2006) and *Karenia spp* (Maier Brown et al., 2006), but no specific study had been made on the effect of salinity on the growth and toxicity of *K. selliformis* before now. Our study shows that these microalgae can grow at a similar rate at 36 and 44 if the temperature is within the same range. The maximum growth rates at salinities 36 and 44 were found at 20°C (0.34 and 0.30 div d^{-1}, respectively); though the rates for these same salinities were considerably lower at 17°C (0.14 and 0.13 div d^{-1}, respectively) in the same culture volume. In a similar way, MacKenzie et al. (2002) reported a maximum growth rate of *K. selliformis* (0.18 div d^{-1}) at 18°C.

Under the conditions used to culture *K. selliformis* in this study (Table 1), maximum growth (growth rate, cellular concentration) was obtained in small volumes (0.25 to 2 L), while the lowest growth was observed in the largest volumes (8 to 40 L).

These results agree with the study by Mountfort et al. (2006), who showed that the highest growth rate and maximum cell yield of *K. selliformis* (0.27 and 210.2 × 10^3 cell mL^{-1}, respectively) were obtained in volumes of 5 mL (plate wells) rather in the larger volumes used (15 L) (0.04,

Table 2. *Karenia selliformis.* Minimum lethal doses (MLD) for mouse expressed in numbers of cells subjected to extraction and in µg GYM Kg^{-1} mouse weight.

Tableau 2. *Karenia selliformis.* Doses minimales mortelles (DMM) pour la souris exprimées en nombre de cellules (extrait injecté aux souris), ainsi qu'en µg GYM Kg^{-1} de souris.

Salinity - temperature	Exponential phase		Decline phase	
	Number of cells ×10^3	GYM µg Kg^{-1}	Number of cells ×10^3	GYM µg Kg^{-1}
36 - 20°C	700 ± 53	175	485 ± 15	157
44 - 20°C	404 ± 17,5	187	251 ± 23	228
36 - 17°C	1 440.36 ± 98	86	770 ± 26.5	84
44 - 17°C	1 340 ± 95	107	224 ± 24	141

and 41.4 × 10^3 cell mL^{-1}). Even though the maximum cell yield obtained in plate wells is higher than any obtained in the volumes tested in the present study, 5 mL is not a practical volume for future contamination tests, which will require a very high biomass of *K. selliformis*.

In this present study, culture of *K. selliformis* during all experimental conditions did not show any effects of photo-inhibition because the light intensity (45 µmol m^{-2} s^{-1}) used in all experiments was lower than the one observed in areas (Boughrara lagoon) where blooms of *K. selliformis* occurred. Moreover, to determine optimal light for growth of *K. selliformis*, further experiments will be needed with different light intensities.

It is generally known that the cultures of dinoflagellates are more fragile than other microalgae currently cultivated in batch or continuous flow culture (photobioreactors) with considerable evidence of negative effects from aeration condition and as such offer a challenge to microalgal biomass production.

In this study, the low growth of *K. selliformis* obtained in larger cultures without aeration was attributed to the culture volume (I, IV, table 1). Indeed, White (1976) and Pollingher & Zemel (1981) were the first to demonstrate experimentally that agitation, shaking, aeration and stirring inhibit dinoflagellate growth rate and can lead to mortality, if sustained or intensified. These last authors found also, that turbulence disrupts the cellular clock, mitotic cycle, and alters nucleic acid concentrations.

Toxin content

Under the conditions used to culture *K. selliformis* in this study, the lowest cellular concentration of GYM was found in the largest volume we analysed (8 L). It should be noted that this concentration appears to decrease as the culture volume increases.

GYM production by *K. selliformis* has already been described elsewhere (Seki et al., 1995; Miles et al., 2000; Mackenzie et al., 2002). The analysis of GYM in the particulate (cellular) and dissolved fractions of sampled batch culture of this microalga (Mackenzie et al., 2002) show that the toxin is produced at a relatively stable rate over most of the algal growth cycle. Up to 50% of the GYM remains in the dissolved form in the culture medium at the start of the growth phase, but this proportion rapidly falls during the exponential and stationary phases. More than 70% of the toxin is associated with cells during the exponential and stationary phases. Indeed, the GYM is characterized by its instability at neutral and high pH (Miles et al., 1999), it is likely that a significant decomposition of this toxin takes place during the experiment when the pH reaches the range 8.1-8.7. Such GYM decomposition was confirmed and estimated at a rate of approximately 5% per day (Mackenzie et al., 2002). These authors indicated that the cause of the gym dissolution in the surrounding environment is unknown suggesting that it is the result of either an active process of *K. selliformis* cells excretion, a passive loss of these cells, or a release of the GYM in the middle due to cell lysis.

In addition, these same authors reported that the GYM seems as a rather stable component in the cell, but its presence in significant amounts in the dissolved form in the medium suggest the possibility of an ectocrine function which confers an ecological advantage to *K. selliformis*.

In our own study, GYM was only quantified in the cellular fraction. However, the concentration of GYM seems to increase with the age of the cultures and was higher during the decline phase (senescence). We could expect a higher toxicity in coastal waters when a proliferation of this alga is prolonged. A higher toxicity could then to risk therefore arise when clams were exposed to long lasting blooms.

In the same way, brevetoxin (measured in cultured *K. brevis*) showed a higher level in the stationary phase, due to a higher accumulation of this toxin in senescent cells (Magaña & Villareal, 2006). The authors also suggested that as a natural bloom ages it becomes more toxic and thus becomes a greater public health risk.

Effects of environmental factors including salinity on cellular growth and toxin production have been studied for several species of dinoflagellate (Cembella, 1998; Grzebyk et al., 2003). In our study, salinity may affect the toxin content of *K. selliformis*. Indeed, the maximal toxicity was obtained at the highest salinity (44) examined (Table1).

Salinity is a factor that affects brevetoxin production in the raphidophyceae. In *Heterosigma akashiwo,* low salinities correspond to the highest levels of brevetoxins (Haque & Onoue, 2002a), and two *Chattonella* species show a higher toxicity at medium salinity (25) and low toxicity at high salinities (Haque & Oneoue, 2002b). In *K. brevis*, however, the effect of salinity on toxin production is complex, as some studies have shown that maximum toxicity is achieved during the stationary phase for tested salinities (20, 30 and 40) (Kim & Martin, 1974; Maier Brown et al., 2006).

GYM is a cyclic imine whose structure was first elucidated by nuclear magnetic resonance (Seki et al., 1995), then confirmed by X ray crystallography (Stewart et al., 1997). It has a number of characteristics in common with other active compounds like the spirolides (Hu et al., 1995), pinnatoxins (Uemura et al., 1995) and prorocentrolides (Hu et al., 2001). Although GYM toxicity to mice is low by oral administration (LD50 = 755 µg Kg^{-1}) it is higher and more rapid following ip administration (LD50 = 96 µg Kg^{-1}) (Miles et al., 1999, Munday et al., 2004). This method of injection is used in surveillance of lipophilic toxins (including diarrhetic toxins). According to

these authors, the MLD by ip injection to mice varied from 450 µg Kg^{-1} (Seki et al., 1995) to 700 µg Kg^{-1} (Stewart et al., 1997). However, the MLD found for mice in the present study varied from 84 to 228 µg Kg^{-1}. Though this range is large, the maximum values recorded are clearly lower than those cited in the literature.

As GYM is extracted directly from *K. selliformis* cells, our results reflect the purity of our material, which could partially explain this difference. In the same way, the combined effect of salinity and temperature on toxin production appears to be complex, which could contribute to explaining the observed variation in MLD. Also, this variation could be explained by the nature of the bio test. For this reason, further studies will be needed to investigate experimental plan with two factors (salinity and temperature) on the MLD of gymnodimine to mice.

Additionally, in contrast to the amount of toxin produced, the number of cells needed to reach the MLD seems to be higher in the exponential phase than in the decline phase.

The values we obtained are close to the cellular concentrations reported to have caused shellfish contamination (alert conditions) in the Gabès gulf or mortality of marine organisms in the Sfax region: 1000 and 60×10^6 cells L^{-1}, respectively (Hamza & El Abed, 1994)

One characteristic of GYM, like other spiroimines including GYM B, is the rapid mortality they induce in ip injected mice bioassays (Miles et al., 2000). For this reason they are known as "fast-acting toxins" (FAT) (Munday et al., 2004). The same authors also reported that mortality occurred less than one minute after administration of lethal doses, but that there was no further mortality after 10-11 min. Seki et al. (1996) noted that death of mice occurred between 5 and 15 minutes after the administration of the lethal dose. In the present study, mortality occurred between 3 and 7 minutes for MLD, preceded by the same symptoms as those described by Seki et al (1996) and Munday et al. (2004). There seems to be a great similarity between these intoxication symptoms and those recently described for 13-desmethyl-C-spirolide (Gill et al., 2003).

During the mouse tests we performed, some injected doses were not lethal even though they caused characteristic FAT symptoms over the first 10 minutes. The symptoms gradually disappeared and the mice presented a normal state after 20 min. The same observations were made by Seki et al. (1996) and referred to as "sub-lethal" by Munday et al. (2004).

In conclusion, the results we gathered produced a dataset on *K. selliformis* growth and toxicity determined under the following experimental conditions: culture volumes 0.25 to 40 L, two temperatures ranges(15-17 or 20-21°C), two salinities (36 and 44), a single light level (45 µmol photons m^2 s^{-1}), and a photoperiod of 12h light/12h dark. Overall, growth rates were similar for the different salinities tested, but varied depending on the temperature (0.11-0.10, 0.14-0.13 and 0.34-0.30 div^{-1} at 15, 17 and 20°C, respectively, Table 1). Toxicity of the cultures seems to be increase with age. The neurotoxicity of this dinoflagellate was confirmed by the bioassay test, and the MLD for mouse was determined as a function of the number of *K. selliformis* cells on which the extraction was made. In contrast to the quantity of toxin produced, the number of cells necessary to reach the MLD may be higher in the exponential phase than in the decline phase.

It would be interesting to continue this study with a comparison of different techniques of mass culture of *Karenia* (in tanks, a horizontal photobioreactor or in static culture with or without CO_2). Moreover, further studies will be needed to investigate the relationship between growth and the production of gymnodimine at different levels of light intensity and salinity with the aim of optimising the production of this microalga in the laboratory. Biomass of *K. selliformis* could then constitute a good support to study the experimental contamination and detoxification kinetics of the grooved carpet shell *R. decussatus* (Medhioub et al., 2009) in order to develop, validate and propose to shellfish farmer a detoxification procedure within a short time . Such study would limit the economic impact at the closure of production areas affected by *K. selliformis* .

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