Limnetica, 29 (2): 257-268 (2010)
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Is Microtox® toxicity related to potentially harmful algae proliferation in Mediterranean salt marshes?

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Received: 25/8/09 Accepted: 24/2/10

ABSTRACT

Is Microtox® toxicity related to potentially harmful algae proliferation in Mediterranean salt marshes?

Mediterranean salt marshes are ecosystems that are highly influenced by sea changes and freshwater inputs from runoff. In these ecosystems, toxic and non-toxic algae blooms often produce large and unpredictable biomasses of phytoplankton. The Microtox® test has been described as a successful, quick method for detecting toxicity in various phytoplankton taxa. Our study sought to test the efficiency of Microtox® in detecting toxic HAB in Mediterranean salt marshes. The results showed that the Microtox® test was able to detect toxic substances in the particulate matter of several lagoons in the Empordà salt marshes. This Microtox® toxicity coincided with periods when potentially harmful cyanobacteria, dinoflagellates and haptophytes had a high biomass. The results suggest that potentially harmful phytoplankton cannot be ruled out as a source of Microtox® toxicity or as a source of other organism kills and sub-acute effects in Mediterranean salt marshes.

Key words: Toxicity assay, coastal lagoons, bloom, phytoplankton, confinement.

RESUMEN

¿Está la toxicidad detectada por Microtox® relacionada con las proliferaciones de algas potencialmente tóxicas en las marismas mediterráneas?

Las marismas mediterráneas son ecosistemas altamente influenciados por la entrada de agua marina y de agua dulce, procedente de la escorrentía. En estos ecosistemas, las proliferaciones de algas tóxicas y no tóxicas sin patrón temporal o espacial, alcanzan altas e impredecibles biomasas de fitoplancton. El ensayo Microtox® ha sido descrito como un rápido y eficiente método para detectar la toxicidad de diferentes taxones de fitoplancton. Nosotros hemos hecho una primera aproximación para determinar la eficiencia del test Microtox® en la detección de proliferaciones algales tóxicas en las marismas mediterráneas. Se ha podido concluir que hay substancias tóxicas, en la materia particulada de diversas lagunas de las marismas del Empordà, que el método Microtox® es capaz de detectar. Esta toxicidad detectada por Microtox® se da en periodos en que cianobacterias, dinoflagelados y haptófitos potencialmente tóxicos presentan alta biomasa. Los resultados sugieren que el fitoplancton potencialmente tóxico no puede ser descartado como causante de la mortalidad o de efectos sub-agudos a otros organismos de las marismas mediterráneas.

Palabras clave: *Ensayo de toxicidad, lagunas costeras, proliferación, fitoplancton, confinamiento.*

INTRODUCTION

Mediterranean salt marshes are ecosystems that are highly influenced by sea changes and freshwater inputs from runoff (Britton & Crivelli, 1993). The composition of the phytoplankton community in these areas is largely determined by hydrological variability (Quintana & Moreno-Amich, 2002; López-Flores *et al.*, 2006a). The hydrological pattern leading to phytoplankton production is characterised by sudden inputs during sea storms that cause the lagoons to be flooded, mainly by seawater. Later, these salt marsh basins become confined, leading to high concentrations of organic matter, nutrients and organisms (this process is most extreme during summer; see Quintana *et al.*, (1998). In these ecosystems, toxic and non-toxic algae blooms often and unpredictably produce large biomasses of dinoflagellates, cryptophytes, haptophytes or cyanobacteria species (Quintana and Moreno-Amich, 2002; López-Flores *et al.*, 2006a). The proliferation of harmful phytoplankton in nearby Mediterranean beaches and harbours has been reported by several authors (Garcés *et al.*, 2000; Vila *et al.*, 2001). Some authors have also reported HAB (Harmful Algal Blooms) events in other Mediterranean coastal lagoons (e.g., Comín & Ferrer, 1978; Sarno *et al.*, 1993). However, López-Flores *et al.* (2006b) showed that, rather than reaching high concentrations of biomass and becoming potentially harmful, the taxa proliferating in the coastal lagoons were not the same as those taxa forming blooms in the nearby marine waters. Thus, the species composition and abundance variability in these ecosystems make it difficult to monitor toxicity because one needs a method that provides extensive and efficient measurements.

The Microtox® test has been described as a successful, rapid method for detecting cyanobacterial toxicity (Lawton *et al.*, 1990; Volterra *et al.*, 1992; Bruno *et al.*, 1994; Campbell *et al.*, 1994), dinoflagellate toxicity (Bruno *et al.*, 1990; Giacobbe & Yang, 1999; Derby *et al.*, 2003), and diatom toxicity (Derby *et al.*, 2005). Thus, the test could be a feasible method for taking an initial measurement of an ecosystem's toxicity if the ecosystem has characteristics similar to the Empordà salt marshes, where the variety of waterbodies (Trobajo *et al.*, 2002; Gascón *et al.*, 2005) and the high variability of the phytoplankton community in different environmental conditions (López-Flores *et al.*, 2006a; López-Flores *et al.*, 2006b; López-Flores *et al*., 2009) necessitate an extensive method of toxicity-detection.

The aim of this study was to perform an initial test of the efficiency of Microtox® in detecting toxic HAB in Mediterranean salt marshes. In order to reach this objective, we analysed the composition of phytoplankton taxa during periods of high biomass development and its relationship to the results obtained through the Microtox® test.

METHODS

Study site

The study was undertaken in the Empordà wetlands. These wetlands include a group of Mediterranean coastal lagoons and salt marshes with variable depth (average depth of 0.60 m and maximum depth close to 2 m) located in the northeast NE Iberian Peninsula (Fig. 1). They show a typical Mediterranean hydrologic regime, which is greatly affected by the sea's influence (Quintana *et al.*, 1998). The hydrology of this area depends primarily on sudden and irregular intrusions during sea storms and/or intense rainfall. After sea storms, rainfall or freshwater inputs from rivers, the marshes remain confined (lacking water supply) for a long period and tend towards desiccation. Lagoons from two salt marshes, La Pletera marshes and Alt Empordà marshes, were included in this study.

The La Pletera salt marshes are situated between the urban centre of l'Estartit (Torroella de Mongrí, Girona) and the Ter River mouth. Five lagoons were sampled within these salt marshes (Fig. 1): two old (O1 and O2), more-eutrophic lagoons, and three new (N1, N2 and N3), less-eutrophic lagoons (Badosa *et al.*, 2006; López-Flores *et al.*, 2006a).

The Alt Empordà salt marshes include a group of coastal lagoons and salt marshes sit-

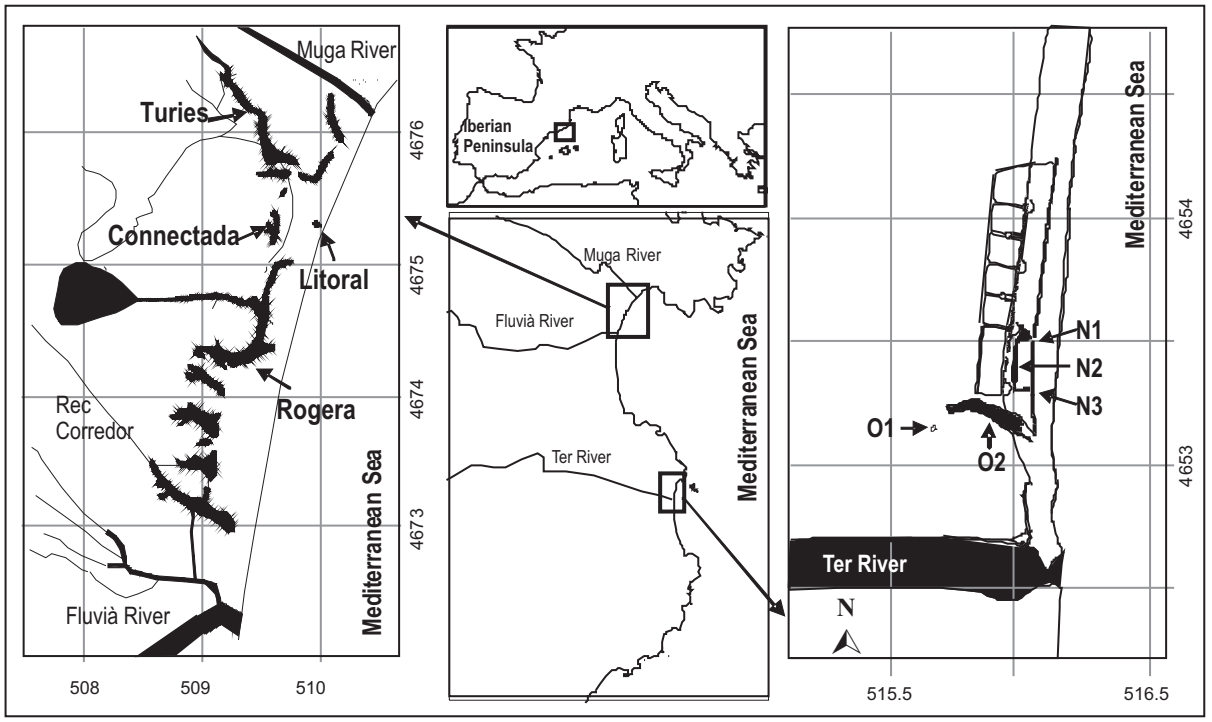

Figure 1. Sampling sites in the Alt Empordà (left) and La Pletera (right) salt marshes. Grid indicates UTM coordinates (1000 m). *Zonas de muestreo en las marismas del Alt Empordà (izquierda) y La Pletera (derecha). Las líneas indican las coordenadas UTM (1000 m).*

uated between the mouths of the Muga and the Fluvià. Four lagoons were sampled in these salt marshes (Fig. 1): Litoral and Rogera (more eutrophic), and Turies and Connectada (less eutrophic). More data about the limnological characteristics of these lagoons can be found elsewhere (Gascón *et al.*, 2005; Brucet *et al.*, 2005; López-Flores *et al.*, 2009).

Sampling

Samples were taken simultaneously with observations of water discoloration between August 2000 and December 2003 at a central point of each lagoon at a depth of 15-30 cm. Some additional samples were also taken during fish kill episodes (e.g., Rogera lagoon in August 2001). Electrical conductivity (CRISON 524, Crison Instruments, Barcelona, Spain), pH (CRISON 507, Crison Instruments, Barcelona, Spain), temperature and oxygen concentration (OXI 320, WTW, Munich, Germany) and water level were measured *in situ.* Filtered samples (Whatman GF/F) were frozen for later laboratory analysis of NH_4^+, NO_2^-, NO_3^- and soluble reactive phosphate (SRP). Filtered and unfiltered samples were refrigerated for total and dissolved organic carbon analysis (TOC and DOC, respectively). Inorganic samples and total nutrient samples were oxidised to nitrate and orthophosphate and then analysed via colourimetry following Grasshoff *et al.*, (1983). Total organic carbon was measured using a TOC analyser (TOC 5000 Shimadzu, Shimadzu Scientific Instruments, Columbia, USA).

Microphytoplankton counting

Inverted microscopy

Aliquots (150 ml) were fixed with lugol (1 % final concentration) for quantifying phytoplankton. The general procedure for identifying and quantifying phytoplankton cells involved sedimentation of a subsample in a settling chamber for 24 hours and then counting cells in an appropriate area (Utermöhl, 1958) using an inverted

microscope (Zeiss Axiovert 135, Göttingen, Germany). Several taxonomic works (Desikachary, 1959; Dodge, 1982; Sournia, 1986; Chrétiennot-Dinet, 1990; Tomas, 1997; Faust & Gulledge, 2002; Hallegraeff *et al.*, 2003) were used to determine the phytoplankton taxa.

Epifluorescence microscopy

After staining the thecate dinoflagellates with calcofluor white, a specific stain for cellulose, observations were conducted to identify the *Alexandrium* species (Hallegraeff *et al.*, 2003). A drop of calcofluor (2 mg/l) was added to the sample slide, which was then covered with a cover slip and examined under an epifluorescence lamp (HBO 50, Göttingen, Germany).

Scanning electron microscopy (SEM)

The identification of some athecated dinoflagellates, cyanobacteria and other flagellates was supported by SEM (Zeiss DSM 960A, Göttingen, Germany). Samples were centrifuged, dehydrated, dried to the critical point and gold-coated.

Biomass estimation

Biovolume estimations were obtained following Hillebrand *et al.* (1999). The formula developed by Reynolds (1984) was then utilised to transform biovolume to cell biomass dry weight.

Microtox® toxicity assays

The Microtox® bioassay was used for studying the toxicity of potentially harmful algae. This method is based on the fading of light emitted by the luminescent bacteria *Vibrio fischeri* when exposed to noxious substances.

Frozen filters from natural samples were extracted using a Pyrex tube with 5 ml of Milli Q water (Millipore). The tub was placed in an ultrasonic bath with ice water and then stored for 24 hours in the refrigerator (4 °C) to prolong extraction time. Extracts were then centrifuged at 4000 rpm for 10 minutes, and the supernatant was immediately analysed.

For this study, we used a Microtox® M500 Toxicity Analyser System from Microbics Corporation (Carlsbad, California). The system is equipped with a 30-well, temperature-controlled incubator block set at 15 °C and a storage cell kept at approximately 5 °C for the reconstituted bacteria before dilution. The Microtox® test was carried out according to the trademark directives. The light emission of the bacterial suspensions was measured before and after exposure to five different dilutions of the sample, including a control sample (diluent alone). Because the bacteria came from a marine environment, and in order to avoid an osmotic shock, 0.25 ml of 20 % NaCl were added to the sample (1.5 ml) before the dilution. The sample concentrations tested were 50 %, 25 %, 12.5 % and 6.25 % of the initial concentration (original extract). Sample dilutions were always prepared by adding saline solution (2 % NaCl aqueous solution (Microtox Diluent)) to provide osmotic protection.

Concentration values (%) and luminescence inhibition (τ) were entered into a logarithmic equation, which allowed us to compute concentration values for a decrease in the luminescence of 50 % (Volterra et al., 1992). The model for the computation of light emissions, where toxic effects are expressed as the ratio of activity lost to activity remaining, was developed and named gamma (τ) by Johnson *et al.* (1974). τ is computed by the formula,

$$\tau = \frac{lost\ light}{remaining\ light} = \frac{I_0}{I_5} - 1$$

where I_0 and I_5 were the luminescence at 0 and 5 minutes, respectively.

The concentration of the test chemical that causes τ to equal 1 was used to compute the EC50 value for the assay. The log transformation in the τ approach permits simple regression analyses to compute EC50 values (Johnson, 2005). Thus, high values of $EC50_5$ indicate low toxicity, whereas low values indicate high toxicity. Toxicity Units (TU_5) have been calculated as,

$$TU_5 = \frac{100}{EC50_5}$$

Toxicity was considered for values of $EC50_5$ lower than 100 % or $TU_5 > 1$.

We also analysed the soluble fraction of the samples for which the Microtox® results were positive for the particulate fraction. For the soluble fractions, none of the tested samples showed luminescent inhibition.

Heavy metals analyses and pesticides data

For metals samples, 500 ml of water was filtered through a Whatman GF/C filter and frozen for later analysis. Frozen filters from samples were digested for 6 hours by a mixture of 4 ml HNO_3 (1:1) + 10 ml HCl (1:4) and filtered be-

Table 1. List of phytoplankton taxa in the different lagoons studied. Potentially harmful taxons are in bold (Hallegraeff *et al.*, 2003). The symbol + indicates mean values $<10^5$ cells l^{-1}, and ++ indicates mean values between 10^5 and 10^6. Blooms are identified with b and B if the number of cells per litre is between 10^6-10^7 and $>10^7$, respectively. *Lista de taxones de fitoplancton de las diferentes lagunas estudiadas. Los taxones potencialmente tóxicos se muestran en negrita (Hallegraeff* et al.*, 2003). El símbolo + corresponde a valores medios $<10^5$ celulas/l, y ++ a valores medios entre 10^5y10^6. Los blooms algales se identifican con b y B, si el número de celulas por litro es entre 10^6y10^7, y $>10^7$, respectivamente.*

	Species	O1	O2	N1	N2	N3	Litoral	Turies	Rogera	Connectada
Diatoms	*Amphora* spp.	+			+		b	+		+
	Chaetoceros sp.			+		B				
	Cocconeis placentula Ehrenberg		++	+	++	b	++	+		
	Cyclotella spp.	+	++	+	+	+			++	
	Cylindrotheca closterium (Ehrenberg) Reimann & Lewin	++	++	B	++	++	b	b		
	Entonomeis spp.	+		+		+	++	+		
	Navicula spp.	++	++	+	++	+	+	++	++	+
	Nitzchia sp.	+	+	+	+	+	+	+		
Chlorophytes	*Nannochloris* sp.	B	B							
Chrysophytes	*Ochromonas* spp.		++			b	b	b		
Cryptophytes	*Chroomonas* sp.	b	b	++	+	+	b			
	Hemiselmis sp.						++	+		
Cyanobacteria	***Anabaena* spp.**	+	+					+		
	***Aphanocapsa* sp.**								B	
	Dactylococcopsis cf. *raphidioides* Hansgirg								B	
	Merismopedia spp.			+	++	++		+		
	***Synechococcus* sp.**					+		+		
Dinoflagellates	***Alexandrium pseudogonyaulax*** (Biecheler) Horiguchi			++	+	++				
	***Amphidinium* sp.**		B			+		b		
	Glenodinium foliaceum Stein		B	+	++		b	++		b
	***Gymnodinium* spp.**	B	B	b	b	+	++	++		
	Gyrodinium instriatum Freudenthal & Lee		++							
	Gyrodinium sp.				++					
	***Heterocapsa* spp.**	+		b	+					
	Oxyrrhis marina Dujardin	b	B	b	b	+	B	+		
	Prorocentrum micans Ehrenberg	+	+					+		
	Prorocentrum minimum (Pavillard) Schiller		+	B	B	B				
	Scrippsiella subsalsa (Ostenfeld) Steidinger & Balech		b	+		+				
	Scrippsiella spp.	+	++	+	b	+		++		
Euglenophytes	*Euglena* cf. *Próxima* Dangeard		+	+						
Prasinophytes	*Pyramimonas* spp.	b		B	b	b	B	b		
	Tetraselmis cf. *gracilis* (Kylin) Butcher	b			++			++		
Haptophytes	***Chrysochromulina* sp.**						+			
	Pavlova cf. *Lutheri* (Droop) Green							+		++
	***Prymnesium* sp.**	B		+	+	+				
	Number of samples	12	16	7	3	7	7	3	2	1

fore analysis (López-Flores *et al.*, 2003; Salvadó *et al.*, 2006). Flame atomic absorption spectrometry was employed to determine the metal concentration. A SpectrAA-300 and a GTA-96 electrothermal atomisation unit with an automatic injector were used (all supplied by Varian). Samples from La Pletera salt marshes were analysed in the present study (O1, O2, N1, N2 and N3), whereas the heavy metal concentration of the Empordà wetlands (Litoral, Connectada, Rogera and Turies) was extracted from previous works (López-Flores *et al.*, 2003; Salvadó *et al.*, 2006).

The pesticide data used in the present study were part of a larger study commissioned by the local government (López-Flores *et al.*, 2003; Salvadó *et al.*, 2006), 15 years after the declaration of the Integral Reserve figures; thus, a subsequent increase in the concentration of pesticides is improbable.

Statistical analysis

Environmental and biological variables were correlated with TU_5 using lineal correlation (Pearson coefficients; $p < 0.05$). Calculations and statistical analyses were performed with SPSS 15.0.

RESULTS

The phytoplankton taxa identified are shown in Table 1. The potentially harmful species belonged mainly to cyanobacteria and dinoflagellate taxonomic groups and were found in both salt marshes; however, they were not detected in all of the lagoons. Diatom species from the genus *Chaetoceros* were also detected, forming a bloom in the N3 lagoon during spring 2003; however, no fish kills were related to this genus. The harmful cyanobacteria genera identified were *Anabaena,* with low densities in different lagoons belonging to both salt marshes, and *Aphanocapsa*, whose blooms joined with *Dactylococcopsis* cf. *raphidiodes* blooms during a fish kill in Rogera in August 2001. Haptophytes from the genus *Prymnesium* were also found in high densities in O1 and with a lower abundance in the new lagoons (N1, N2, N3). *Alexandrium pseudogonyaulax* and *Prorocentrum minimum* were identified in the new lagoons of La Pletera salt marshes, the latter developing high biomass in all three lagoons. *Scrippsiella subsalsa* was found in O2 every summer between the years 2001 and 2003. At least two different species from the genus *Gymnodinium* were identified in most of the lagoons.

The Microtox® test obtained positive values for the particulate fraction in samples from the two salt marshes (Table 2); however, not all of the lagoons produced positive results. The results for the soluble fraction were always negative, indicating that positive toxicity results are only related to the particulate fraction.

Samples from the less-eutrophic lagoons (N1, N2, N3, Turies and Connectada) never had val-

Table 2. List of samples with TU_5 >1 ($EC50_5$ <100 %). Samples are sorted by TU_5. *Lista de muestras con TU_5 >1 ($EC50_5$ <100 %). Las muestras están ordenadas por TU_5.*

				Biomass (mg/l)				
Lagoon	**Season**		**TU_5**	*Gymnodinium* spp.	*P. minimum*	*S. subsalsa*	*Prymnesium* sp.	*Aphanocapsa* sp.
O2	Summer	(17.07.03)	36.54	—	—	0.01	—	—
O1	Summer	(17.07.03)	14.91	—	—	—	0.16	—
O2	Summer	(18.09.02)	12.75	172.65	—	—	—	—
O1	Summer	(18.09.02)	9.54	4.05	—	—	—	—
O1	Summer	(20.08.03)	6.42	—	—	—	0.15	—
O2	Spring	(18.06.03)	5.67	—	—	0.18	—	—
O2	Summer	(18.08.01)	4.10	—	—	9.35	—	—
N1	Spring	(14.05.03)	2.75	0.79	0.12	—	—	—
O2	Autumn	(15.10.02)	2.48	45.56	—	—	—	—
Litoral	Summer	(01.07.02)	1.99	24.24	—	—	—	—
O2	Summer	(15.07.02)	1.90	—	—	17.38	—	—
O2	Summer	(13.08.02)	1.92	5.69	—	—	—	—
Rogera	Summer	(18.08.01)	1.90	—	—	—	—	33.45

Table 3. Heavy metal concentrations in the studied lagoons. Microtox® test $EC50_5$ values extracted from (1) Villaescusa *et al.* (1996); (2) Villaescusa *et al.* (1997); (3) Ronco (1992); (4) Villaescusa *et al.* (1998). *Concentración de metales pesados en las lagunas estudiadas. Valores $EC50_5$ del test Microtox® extraidos de: (1) Villaescusa* et al.*, (1996); (2) Villaescusa* et al. *(1997); (3) Ronco (1992); (4) Villaescusa* et al. *(1998).*

Lagoon	*Cadmium (mg/l)*	*Chrome (mg/l)*	*Copper (mg/l)*	*Nickel (mg/l)*	*Lead (mg/l)*
O1	< 0.02	< 0.03	0.03	< 0.02	< 0.07
O2	< 0.02	< 0.03	< 0.02	< 0.02	< 0.07
N1	< 0.02	< 0.03	< 0.02	< 0.02	< 0.07
N2	< 0.02	< 0.03	< 0.02	< 0.02	< 0.07
N3	< 0.02	0.034	< 0.02	< 0.02	< 0.07
Litoral	< 0.02	0.149	< 0.02	< 0.02	< 0.07
Connectada	< 0.02	0.130	< 0.02	< 0.02	< 0.07
Rogera	< 0.02	0.089	< 0.02	< 0.02	< 0.07
Turies	< 0.02	0.148	< 0.02	< 0.02	< 0.07
$EC50_5$ (mg/l)	7.82(1)	4.30(2)	8.00(3)	10.00(4)	0.36(4)

ues of $TU_5 > 1$ ($EC50_5$ <100 %). Only in one sample of N1 in later spring (May 2003) was $TU_5 > 1$. Note that high TU_5 generally coincided with mid to late summer.

Heavy metal concentrations in the lagoons were compared to the corresponding $EC50_5$ found in the literature (Ronco, 1992; Villaescusa *et al.*, 1996; Villaescusa *et al.*, 1997; Villaescusa *et al.*, 1998) in order to measure their contribution to sample toxicity (Table 3). Generally, concentrations did not reach the method's detection limit. Only chrome concentrations were detectable in some lagoons; however, they never reached the $EC50_5$ values established in studies with Microtox®.

Often, high TU_5 coincided with a high biomass of cyanobacteria, dinoflagellates or haptophytes. However, the proliferation of some potentially harmful species was also identified in lagoons where TU_5 was close to zero (Table 4). In these samples, *P. minimum* was the main species found, but *A. pseudogonyaulax* and *Gymnodinium* spp. were also found.

TU_5 was positively correlated with the biomass of bacterioplankton ($r^2 = 0.521$; $p < 0.0001$) and phytoplankton ($r^2 = 0.392$; $p = 0.007$), chlorophyll *a* ($r^2 = 0.454$, $p = 0.005$), TOC concentration ($r^2 = 0.441$, $p = 0.002$), conductivity ($r^2 = 0.460$, $p = 0.001$) and temperature ($r^2 = 0.310$, $p = 0.038$), whereas the wa-

Table 4. List of samples with a high biomass of potentially harmful species and with TU_5 <1 ($EC50_5$ <100 %). *Lista de muestras con biomasa alta de especies potencialmente tóxicas y con TU_5 <1 ($EC50_5$ <100 %).*

			Biomass (mg/l)		
Lagoon	**Season**		*Gymnodinium* spp.	*P. minimum*	*A. pseudogonyaulax*
N1	Autumn	(15.10.02)	—	—	—
	Autumn	(12.11.02)	—	0.05	0.42
	Winter	(19.02.03)	0.76	0.10	—
	Winter	(17.03.03)	—	3.76	—
	Spring	(15.04.03)	—	4.48	—
	Spring	(18.06.03)	27.98	—	—
N2	Winter	(19.02.03)	0.83	0.42	—
	Winter	(17.03.03)	—	2.45	—
	Spring	(15.04.03)	—	8.96	—
	Spring	(18.06.03)	18.46	—	—
N3	Summer	(18.09.02)	—	0.02	0.12
	Winter	(19.02.03)	—	0.52	—
	Winter	(17.03.03)	—	24.23	—
	Spring	(15.04.03)	—	4.48	—

ter level was negatively correlated with TU_5 ($r^2 = -0.401$, $p = 0.019$). No significant correlations were found between TU_5 and the biomass of each harmful species or with the total harmful species biomass.

DISCUSSION

There are substances in the particulate matter of several lagoons in the Empordà salt marshes that produce toxicity detected by the Microtox® test. This Microtox® toxicity coincides with periods when potentially harmful cyanobacteria, dinoflagellates and haptophytes have a high biomass. In addition, in one case, HABs and Microtox® toxicity co-occurred with fish kills. In the Empordà salt marshes, Microtox® toxicity occurs during summer, coinciding with high temperatures, high bacterial biomass and high organic matter concentration as well as high densities of chlorophyll *a* and high pico- and nanophytoplankton biomasses. Although the correlations found are low, they suggest that toxicity events are related to confinement situations when water level decreases, increasing salinity, organic matter and phytoplankton biomass (López-Flores *et al*., 2006a). The toxicity measured was not caused by substances dissolved in the water because the Microtox® test was negative for this fraction and was not caused by heavy metals in particulate matter because their concentrations were under the $EC50_5$ for the metals analysed. Furthermore, previous studies have ruled out the presence of harmful concentrations of pesticides in the Empordà salt marshes (López-Flores *et al.*, 2003; Salvadó *et al.*, 2006). The lack of oxygen is one of the most likely causes of fish and zooplankton mortality in this ecosystem because, during summer, high phytoplankton biomasses occur and anoxia situations are frequent. However, the lack of oxygen in the water has no effect on Microtox® toxicity; thus, positive values cannot be attributed to anoxia.

Previous studies have found a relationship between Microtox® toxicity and harmful algae proliferation (Bruno *et al.*, 1990; Bruno *et al.*, 1994; Derby *et al.*, 2003). However, in the present study, high biomass densities developed by some harmful species could not be related to Microtox® toxicity (e.g. *P. minimum* proliferation). These negative Microtox® results could be due to the lack of toxic substances in the water, but also could be accounted for by their low toxicity for *Vibrio fischeri* or to the chemistry of toxins (because only hydrosoluble toxins were tested). The species *P. minimum* has often been associated with fish mortality and the production of venerupin, a hepatotoxin (Tangen, 1983; Faust & Gulledge, 2002). This species has also been shown to contain a water-soluble, neurotoxic component that can kill mice (Heil *et al.*, 2005). However, its toxicity is known to be conditioned by the species clone, and the toxic effects may be elicited under certain growth conditions (Glibert & Sellner, 2005). These contradictions bring into doubt the efficiency of the Microtox® method in detecting harmful algae toxins. Moreover, some authors criticise immersion exposure methods like Microtox® because they assume an indiscriminate, whole-organism exposure, whereas in natural conditions, exposure to toxins is mainly through the grazer's digestive system (Caldwell *et al.*, 2004). In addition, Microtox® toxicity detection is limited because not all phytoplankton toxins affect the viability of the tested bacteria in the same way. Other methods, such as high-performance liquid chromatography (HPLC), lipid emulsions and liposome or microparticulate zooplankter diets (Caldwell *et al.*, 2004), are more rigorous but are only recommended if taxonomic knowledge of the community composition is available and the chemical structure of the toxin is well known because the analysis is highly focussed on a particular toxin or organism. Even with perfect knowledge of the taxonomic composition, variations of the species strain (Saker *et al.*, 2005) or the nutrient availability (Granéli *et al.*, 1999; Johansson *et al.*, 2006) could lead to changes in the toxicity level. Moreover, these methods have the disadvantage of being more expensive and slower and are generally not available for non-qualified technicians. As an example, the harmful species *A. taylori* in Marina di Melilli (Sicily, Italy) was related to positive Microtox® results; however, no PSP

(Paralytic Shellfish Poison) toxins were detected using HPLC (Giacobbe and Yang, 1999), although PSP is the syndrome most related to this species.

The pulse rhythm of phytoplankton proliferation and the spatial variability of species composition did not allow us to correlate harmful species biomass to Microtox® toxicity. Despite this, we discovered a contemporaneity between HABs and Microtox® toxicity. The results suggest that potentially harmful phytoplankton cannot be ruled out either as a source of Microtox® toxicity or as a source of other organism kills or sub-acute effects in Mediterranean salt marshes.

ACKNOWLEDGEMENTS

The authors wish to thank E. Fulladosa and I. Villaescusa for Microtox® assistance. This work was supported by a grant from the Comisión de Investigación Científica y Técnica (CICYT), Programa de Investigación Fundamental (ref. 518 CGL2008-05778/BOS) and by a BR grant of the University of Girona.

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