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Do the levels of industrial pollutants influence the distribution and abundance of dinoflagellate cysts in the recently-deposited sediment of a Mediterranean coastal ecosystem?

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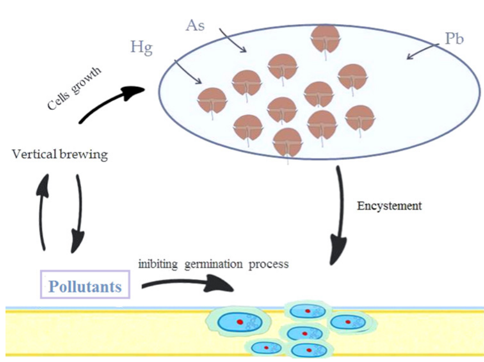
HIGHLIGHTS

- Impact of industrial pollutant on cyst abundance and distribution
- Statistical correlation between trace metal and cysts abundance
- Importance of highlighting dinoflagellate cyst assemblage within Bizerte Lagoon
- Levels of inorganic and organic contaminants within Bizerte lagoon

GRAPHICAL ABSTRACT



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ABSTRACT

We studied the relationships between sediment industrial pollutants concentrations, sediment characteristics and the dinoflagellate cyst abundance within a coastal lagoon by investigating a total of 55 sampling stations within the Bizerte lagoon, a highly anthropized Mediterranean ecosystem. The sediment of Bizerte lagoon is characterized by a high dinocyst abundance, reaching a maximum value of 2742 cysts$\cdot$g^{-1} of dry sediment. The investigated cyst diversity was characterized by the presence of 22 dominant dinocyst morphotypes belonging to 11 genera. Two dinoflagellate species dominated the assemblage: *Alexandrium pseudogonyaulax* and *Protoperidinium claudicans*, representing 29 to 89% and 5 to 38% of the total cyst abundance, respectively, depending on the station. Seven morphotypes belonging to potentially toxic species were detected, including *Alexandrium minutum*, *A. pseudogonyaulax*, *Alexandrium catenella/tamarense species complex*, *Lingulodinium polyedrum*, *Gonyaulax* cf. *spinifera* complex, *Prorocentrum micans* and *Protoceratium reticulatum*. Pearson correlation values showed a positive correlation ($\alpha = 0.05$) between cyst abundance and both water content and fine silt sediment content. Clustering revealed that the highest abundance of cysts corresponds to stations presenting the higher amounts of heavy metals. The simultaneous autoregressive model (SAM) highlighted a significant

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Organic/inorganic contaminants
Interaction pollutants/cysts

correlation ($\alpha = 0.05$) between cyst accumulation and two main factors: sediment water content and sediment content for several heavy metals, including Hg, Cd, Cu, Ni and Cr. These results suggest that the degree of heavy metal pollution could influence cyst accumulation patterns.

1. Introduction

Resting cysts are resistance forms produced sexually. They allow dinoflagellates to survive unfavorable environmental conditions, thus playing an important role in the population dynamics of harmful algal species (Anderson and Wall, 1978; Dale, 1983). Their capacity to be preserved in the sediment for long periods provides a reservoir of diversity and a suitable tool to study temporal changes in phytoplankton populations in a given area (Belmonte et al., 1997). Resting cysts are closely involved in the occurrence and spread of toxic blooms. Their distribution and abundance are considered to be valuable predictors of the formation of toxic blooms, particularly in semi-confined areas (Satta et al., 2013; Steidinger and Garcès, 2006; Bravo et al., 2006; Genovesi et al., 2009). A high cyst production rate ensures a continuous supply for the cyst bank and a regular inoculation of the water column (Zmerli Triki et al., 2015a, 2015b; White and Lewis, 1982).

Cyst distribution could be controlled by various factors, including sediment physical characteristics and hydrodynamics. Several studies suggest that cysts act like fine sediment particles and that the highest cyst abundance is correlated with the fine sediment fraction (<63 μm) (Anderson et al., 2005; Anglès et al., 2010; Horner et al., 2011). The cyst production rate and abundance are also influenced by many biological and environmental factors (temperature decrease, nutrient deficiency, turbulence). It has been suggested that nutrient supplies discharged through river runoffs from land or re-suspended from bottom surface sediment through upwelling could significantly increase the abundance of dinocysts by promoting diatom biomass increases and, consequently, also of heterotrophic predators such as dinoflagellates and their resting cysts (Thorsen and Dale, 1997; Matsuoka, 1999; Dale, 2001, 2009; Godhe and McQuoid, 2003). At the same time, higher water temperatures increase the vertical stability of the water column, influencing the growth rates and the metabolism of the autotrophic dinoflagellates by inducing indirectly the cyst production at the end of the bloom (Elshanawany et al., 2011; Godhe and McQuoid, 2003).

Some studies showed that trace metals are preferentially associated with the 5–80 μm fraction of plankton, mainly dominated by microphytoplankton (Rossi and Jamet, 2008). Chemical contaminants can affect negatively the metabolic activities of vegetative cells of various phytoplankton species, inhibiting their growth and survival at determined concentrations (e.g., Rai et al., 1998; Mosulén et al., 2003; Wang et al., 2005; Miao and Wang, 2006; Herzi et al., 2013). Until now, most of the ecotoxicological studies conducted on dinoflagellates investigated the effects of contaminants only on vegetative cells. Few studies examined their potential effect on cyst production and abundance (e.g., Pospelova et al., 2005; Godhe and McQuoid, 2003; Aydin et al., 2015; Liu et al., 2012). These authors studied the relationships between resting cyst abundance and the degree of heavy metal contamination, suggesting that trace metals could potentially affect the physiology of dinoflagellates by enhancing resting cyst production rates. Nevertheless, to date, no study to evaluate the potential effect of organic contamination on cyst abundance has been performed. In the present study, we evaluated statistically any possible correlation between resting cyst abundance and the degree of inorganic and organic contaminants in sediment of an anthropized Mediterranean lagoon. For this purpose, a high resolution mapping study was conducted to 1) investigate the diversity of the dinoflagellates having relatively high resting cyst densities ($\geq$50 cysts$\cdot$g^{-1} of dry sediment DS) and to 2) measure the concentrations of the main contaminants within the surface sediment, including trace metals, organotin compounds (tributyltin, TBT), Polycyclic aromatic hydrocarbons (PAHs) and polar pesticides (mainly herbicides). Field measurements and sampling were performed within Bizerte lagoon, located along the north-eastern coastline of Tunisia (Mediterranean Sea) which hosts important industrial activities and intense coastal urbanization.

2. Materials and methods

2.1. Study area and sampling

Bizerte lagoon (37° 8′–37°14′ N, 9°46′–9°56′ E) is a shallow (8 m average water depth) area covering 128 km^2 (Fig. 1). It's economically important, holding nine shellfish farms (*Mytilus galloprovincialis*, *Ostrea edulis* and *Crassostera gigas*). In the Bizerte lagoon, the surface current is stronger than the bottom current at the northern, western and southern parts and is relatively weak in the central part of the lagoon (Bejaoui et al., 2008). The phytoplankton assemblage is mainly represented by 51 diatom taxa and 31 dinoflagellate taxa (Bellakhal-Fartouna and Daly Yahia-Kéfi, 2004). In November 2007, a bloom of *Alexandrium catenella* was recorded in Bizerte Lagoon, with observed cell concentrations reaching levels of up to $20 \cdot 10^4$ cells$\cdot$L^{-1}. No additional blooms were recorded since 2007 (Turki et al., 2007).

The watershed of the lagoon currently hosts 277 industrial units (petrochemical, textiles, food, steel and plastics processing) and receives numerous industrial runoffs, either directly or through river discharge. The most polluting industries are the cement-production and oil-refinery ones along the northern shores, and shipbuilding, ship-repair and steel-production ones along the southwestern shores. Land bordering the Southeast sector is mainly exploited for intensive agriculture featuring cereal crops (7800 ha), vegetables (3400 ha) and tree crops (500 ha) (Mansouri, 1996). The main pollutants released in this lagoon are nitrates, phosphorus and trace metals, including iron, zinc and arsenic (Comete-IHE, 2003). TBT and PAH contamination levels within the lagoon are considered to be low to moderate (Mzoughi et al., 2005; Barhoumi et al., 2014b). In terms of pesticide contamination, Organochlorine pesticides (OCPs) were detected within sediments (Barhoumi et al., 2014a; Ben Salem et al., 2016) with iodosulfuron, mesosulfuron, 2,4-dichlorophenoxyacetic acid (2,4 D), glyphosate, and fenoxaprops being the most abundantly-recorded herbicides, and tebuconazole and epoxiconazole being the most abundant fungicides, and deltamethrin being the most widely-used insecticides.

2.2. Sediment sampling and analysis

Sediment sampling was carried out at 55 stations (Fig. 1) during July–August 2012 using cylindrical cores (26 cm long, 4 cm diameter) operated by SCUBA divers. The sampling station depth ranged from 4.5 to 16 m. Only the superficial sediment layer (0–3 cm) was used. Three replicates per station were collected and then stored in darkness at 4 °C. The sediment mean particle diameter size was analyzed using the laser particle sizer Malver Master sizer TM LSE (details are provided in Zmerli Triki et al., 2014).

2.2.1. Organic matter (OM) and water content (H_2O %) analysis

Wet sediment samples from Bizerte lagoon (Ww) was dried for 7 days at 60 °C to evaluate their H_2O %. The resulting dry sediment was weighted (Wd), then heated at 450 °C for 12 h to eliminate any OM. The final weight was registered as Wd1. The sediment water and organic content were calculated as follows: H2O % = [(Ww − Wd) ∗ 100] / Ww, OM % = [(Wd − Wd1) ∗ 100] / Wd1.

2.2.2. Contaminant analyses

They were carried out for 55 sampling stations, for trace metals and butylin compounds, and for over 20 sampling stations for PAHs and pesticides, since insufficient sediment was available to conduct all the chemical analyses for all the sampling sites.

2.2.2.1. Trace metal analysis. Trace metals were analyzed through inductively coupled plasma mass spectrometry (ICP-MS) using a X-Series II ICPMS equipped with a collision cell technology chamber (Thermo Fisher Scientific). Digestion of samples was carried out in a microwave oven (Discover SP-D Plus, CEM®) using the U.S. EPA method SW 846-3052. In brief, 100 mg of sediment samples were digested using a mixture of 2 ml HF (Suprapur 40%, Merck Millipore®) and 4 ml HNO_3 (Suprapur 65%, Merck Millipore®). Hg analysis was performed using the direct mercury analyzer Milestone DMA-80 (Milestone GmbH, Germany) according to U.S. EPA 7473 method (Mercury in Solids and Solutions by Thermal Decomposition, Amalgamation, and Atomic Absorption Spectrophotometry). Detection limits ($\mu g \cdot g^{-1}$) for trace metals adopted in this study are: 0.00600 for Hg, 0.02620 for Cd, 0.06638 for As, 0.05631 for Cu, 0.05631 for Cu, 0.05578 for Ni, 0.06092 for Pb and 0.07220 for Cr. Results were classified according to Long et al. (1995) into three categories, in terms of their degree of adverse biological effects: rarely (<Effects Range Low (ERL), occasionally (ERL-ERM) and frequently (> Effects Range Medium (ERM).

2.2.2.2. Butyltin compounds. The tin-containing compounds TriButyltin (TBT), DiButylin (DBT) and MonoButyltin (MBT) were analyzed using a gas chromatograph (Focus GC – Thermo Fisher Scientific®) coupled with an inductively-coupled plasma mass spectrometer (ICP-MS X Series II-Thermo Fisher Scientific®). The butyltin compounds analysis steps included a gentle extraction to avoid Sn speciation modification, followed by a derivatization step and extraction in the organic phase (isooctane), as described in Carlier-Pinasseau et al. (1996). The certified reference marine sediment from the Canadian National Research Council (National Research Council NRC, Canada), PACS-2, was used to check for analytical precision and accuracy in trace metals and organotin speciation.

2.2.2.3. Pesticide analysis. The ASE extraction (Dionex, France) was carried out for 15 min with a solvent mixture (hexane/acetone (50/50)) at 120 °C and at 1500 psi of nitrogen. The internal standard (Atrazine d5) was added to the sample prior the extraction step. After extraction, the extract was cleaned on a Strata SAX (8B-S008-JCH). The elution step was carried out with 3 ml of MeOH and with 3 ml of CH_2Cl_2 so to recover hydrophobic compounds. Purified extracts were completely evaporated under a gentle stream of nitrogen and were then dissolved in 1.5 ml of acetonitrile. All sample extracts were spiked before analysis with 120 µl of the deuterated internal standard simazine-d10 ($1.2\ mg \cdot l^{-1}$) and analyzed through HPLC-MS/MS.

Pesticide analysis was performed through HPLC-MS/MS using an Alliance HPLC system (Waters Series 2695). Analytic separation was achieved with a Kinetex C18 analytical column (100 mm ∗ 4.6 I.D ∗ 260 Å, Phenomenex). Acetonitrile (A) and ultrapure water (B), both with 0.05% formic acid, were used as mobile phases at the constant flow rate of $0.4\ ml \cdot min^{-1}$. The Linear gradient was started at 40% for 0.2 min, ramped to 80% for 8 min, then to 100% for 1 min and finally back to the initial conditions for 2 min. A triple quadrupole mass spectrometer (Micromass Quatro microTM, Waters) equipped with an electrospray ionization source (ESI) was used as the detector device. The spectrometer operated in positive ESI mode under the following conditions: capillary voltage (3.5 kV), source temperature (120 °C), desolvation temperature (300 °C), drying (600 l/h), and nebulization gas (N2) flow (30 l/h). Argon was used as the collision gas. Acquisition for each compound was performed in the multiple reaction-monitoring mode (MRM). Two transitions were retained: one was used for the quantification aspect and the other was used for the confirmation aspect.

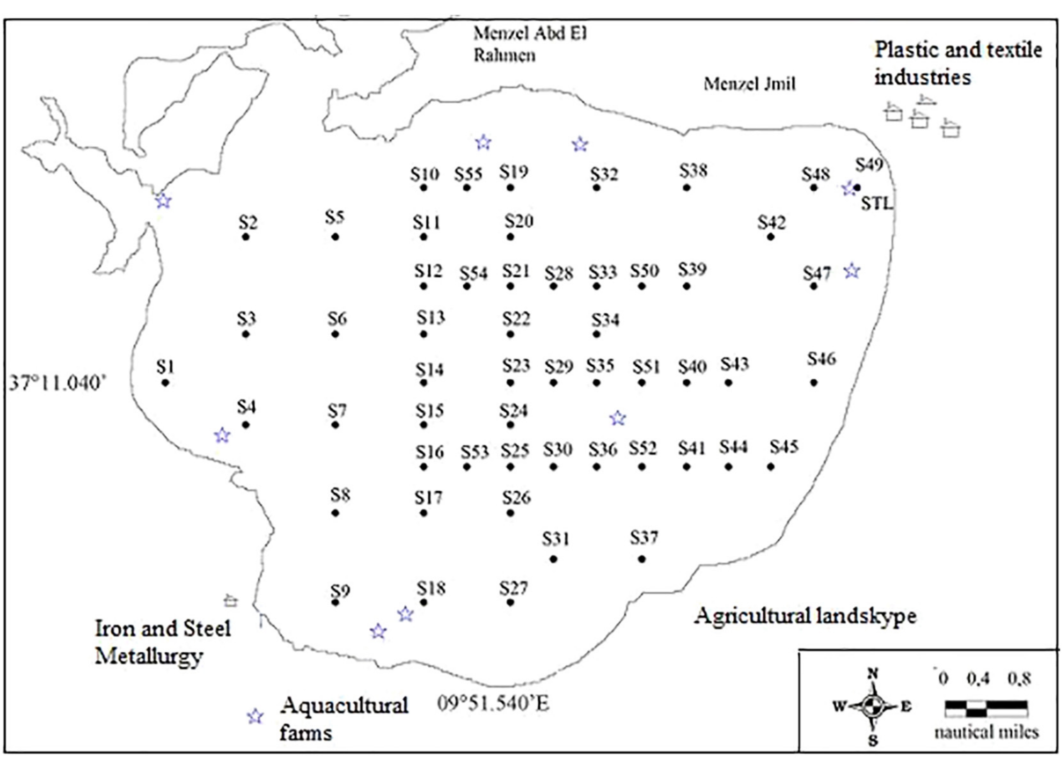

Fig. 1. The Bizerte Lagoon sampling stations adopted within this study.

2.2.2.4. PAH analysis. The ASE (350 Dionex, France) extraction was carried out for 15 min with a solvent mixture (hexane/acetone (50/50)) at 120 °C and at 1500 psi of nitrogen. The extract was then kept in contact with copper powder for 48 h in order to remove sulfur interferences. After extraction, the extract was cleaned on a Strata Florisil (FL-PR, 32138). The elution step was carried out with 5 ml Hexane. Purified extracts were analyzed through a GC–MS (Varian 450-GC and Varian 240 MS) working in the electron impact mode at 70 eV. A DB-5 ms (Agilent) chromatographic column (30 m, 0.25 mm ID and 0.25 μm of film thickness) was used. The Helium flow rate was fixed at 1 ml·min^{-1}. The extract (2 μl) was injected in the chromatographic column with a PTV 1079 injector maintained at 300 °C. The initial oven temperature was increased from 120 °C (1 min) to 160 °C at 6 °C min^{-1} (holding for 5 min); then the temperature was subsequently increased to 310 °C at 10 °C min^{-1} (holding for 5 min). The MS transfer line and the ion source were retained at 310 °C and at 220 °C, respectively. Acquisition was carried out in the single ion monitoring (SIM) mode using characteristic ions as standards for each target analyte. Internal standard calibration was performed with Acenaphthylene-D8, Acenaphthene-D10, Naphthalene-D8, Fluoranthene-D10 and Phenanthrene-D10. The whole procedure (extraction, clean up and GC/MS analysis) was validated using the certified sediment sample RTC-CRM104-050 (LGC). PAH level results were classified according Long et al. (1995) and the detection limit is reported in Supplementary Table 4.

2.3. Resting cyst analyses

2.3.1. Resting cyst extraction

For resting cyst extraction, 1 g of wet sediment was re-suspended in Filtered Sea Water (FSW), sonicated (3 min) and then sieved through 100 and 20 μm mesh sieves. The fraction recovered on the 20 μm mesh sieve was centrifuged at 3000 rev·min^{-1} for 10 min at 4 °C, to recover the pellet containing dinocysts. Extraction was based on the gradient-density method by adding Polytungstate Solution (Bolch, 1997) and centrifuging the pellet again. The supernatant containing the cysts was then sieved through a 20 μm mesh and washed thoroughly to eliminate residues of PST.

2.3.2. Resting cyst identification and quantification

The taxonomic identification of resting cysts (RCs) is difficult and several steps such as isolation, germination and culture implementation are often required to confirm the identity of each species. Only the most abundant living dinocysts encountered within the sampled sediment were quantified and identified. The taxonomic identification of cysts was made according to the Matsuoka and Fukuyo (2000) method which is based on the photonic microscopic observation (Esselte Leitz GmbH, Germany) of morphological characteristics of RCs and also on germination experiments. Given the difficulty of identifying species belonging to the *Spinifera* group without using molecular tools, all related species were grouped together and identified collectively as *Spinifera* complex. *A. pseudogonyaulax* cysts were represented by different morphotypes which could lead to mis-identification; to avoid this, all related morphotypes were isolated and taxonomic identification was only confirmed after germination (Zmerli Triki et al., 2016). Cyst quantification was performed in duplicate according to the Uthermol method using a 3 ml sedimentation cell. Cyst densities were assessed per gram of dry sediment.

2.3.3. Germination experiments and culturing

To confirm the taxonomic identity of RCs, they were isolated individually with a micropipette and placed into wells onto a 96-culture plate (Nunc™ Delta surface) filled with.

Enriched Natural Sea Water (ENSW) culture medium (Harrison et al., 1980) and incubated at standard conditions (20 °C, Salinity 36, 100 μmol·m^{-2}·s^{-1} irradiance and 12 h:12 h light: dark). Cyst germination was examined daily and those exhibiting unsuccessful incubation were discarded after 30 days. Germling cell identification was done according to Drebes (1974), Delgado and Fortuno (1991), Steidinger and Tangen (1996). Cultures of the potentially toxic species *Alexandrium pseudogonyaulax* and of the abundant species *Scrippsiella rotunda* were established from excysted cells.

2.4. Statistical analyses

The degree of RC diversity was investigated using the Shannon-Wiener's index (H′ bits·ind^{-1}) (Shannon and Weaver, 1949) and Pielou's evenness (J) (Pielou, 1966), following these equations:

$$H'\left(\text{bits} \cdot \text{ind}^{-1}\right) = -\sum[(\text{ni}/\text{N}) * \ln(\text{ni}/\text{N})]$$

$$J' = H'/H' \max$$

where H'max = log S (S: total species number in the sample), ni: number of individuals of a species in the sample and N: total number of individuals in the sample.

The Principal Component Analysis (PCA) test was performed to investigate the importance of different environmental factors in determining the distribution of cyst abundance within the sampling area. Hierarchical Cluster analyses (HCA) was performed in order to group sampling stations on the basis of the average heavy metal pollution level they exhibit using the Ward aggregation method. A hypothesized organized spatial structure for resting cyst distribution was investigated using a spatial autocorrelation test, the Moran's I index which is based on a Delaunay triangulation. This index was computed using Moran.mc function from the spdep R-package. A Simultaneous Autoregressive (SAR) model was developed to analyze the predictive power of pollutant levels in the sediment for determining the resting cyst abundance. All statistical tests were performed using R software (available online at: http://www.r-project.org) and distribution maps for different pollutants and dinocysts were generated using the MapInfo professional 10.0 software.

3. Results

3.1. Sediment texture and contaminant levels

The texture results for benthic sediment from Bizerte lagoon are summarized in Supplementary Table 1. Sediment was mainly composed of the sandy-mud and muddy fractions. The fine fraction (<63 μm) was the prevailing one (Fig. 2), ranging between 50.48 and 97.6% of sample

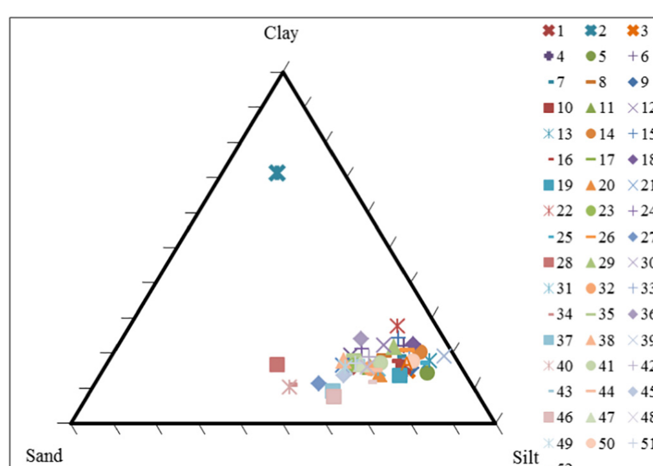

Fig. 2. Shepard and Folks representation of Bizerte lagoon sediment structure.

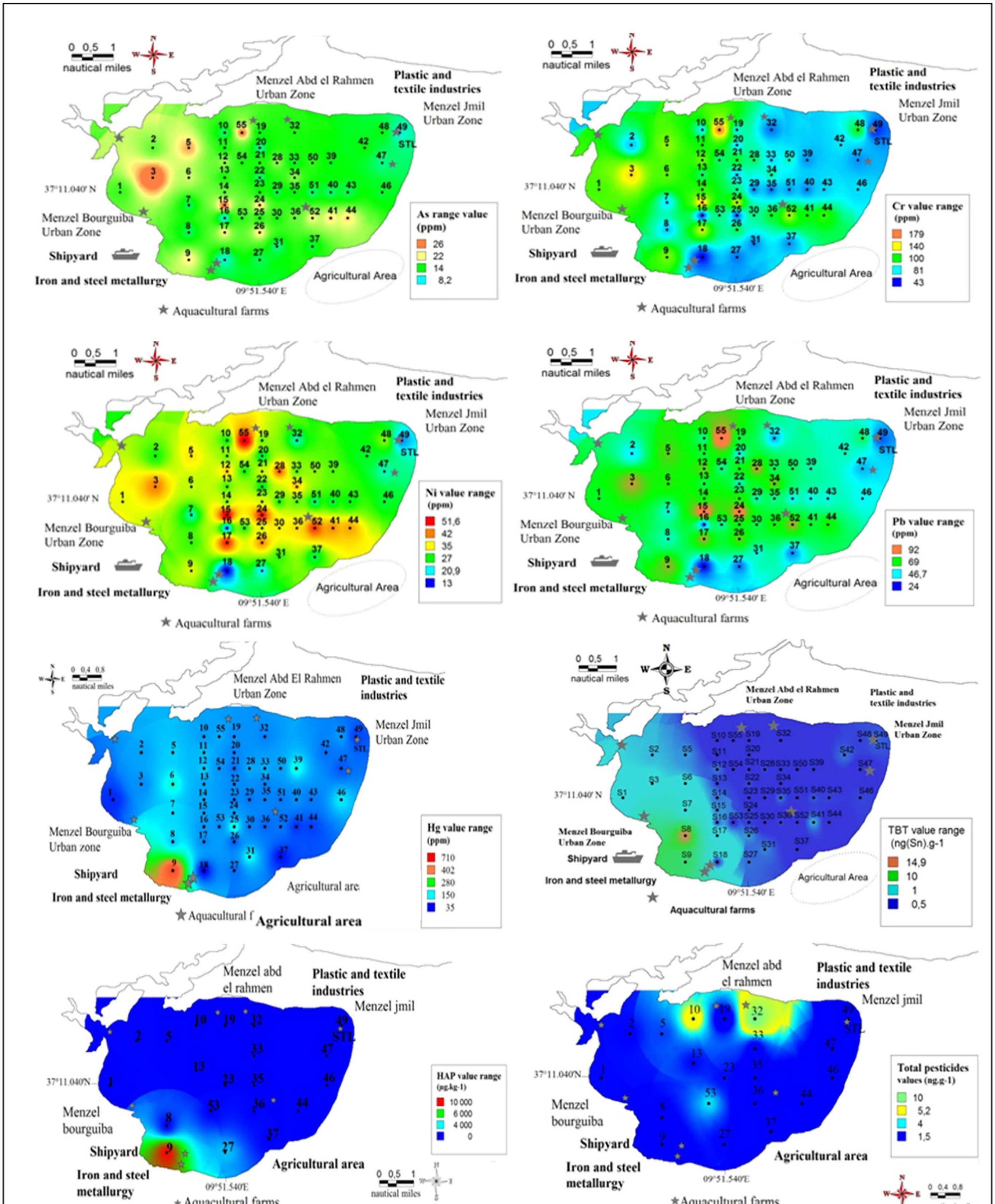

Fig. 3. Trace metals, TriButyltin, polycyclic aromatic hydrocarbon and polar pesticides levels and distribution within surface sediment of Bizerte lagoon.

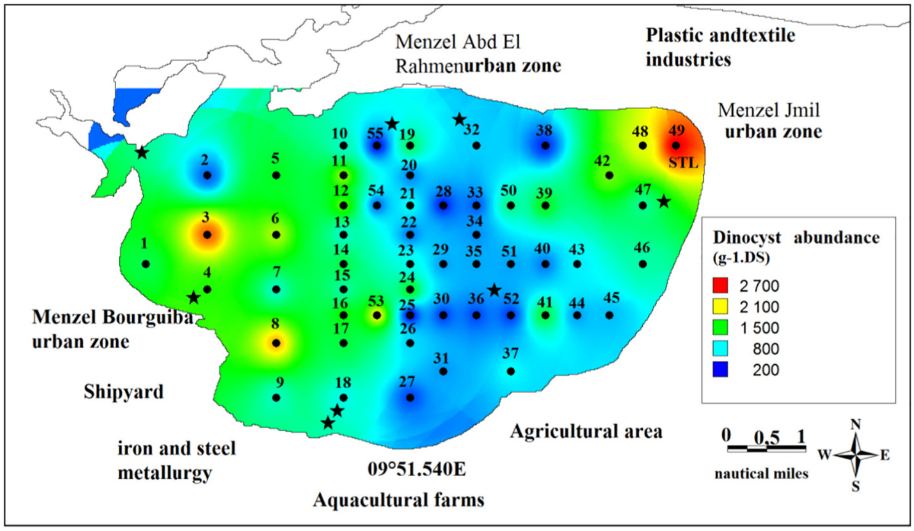

Fig. 4. Dinocyst (cysts·g^{-1} dry sediment) distribution in the sediment of Bizerte lagoon.

composition in terms of weight, and was mainly represented by silt (60.56 ± 3.5%), whereas the clay fraction was less abundant (17.99 ± 8.46% - Zmerli Triki et al., 2014).

Industrial contamination within Bizerte lagoon benthic sediments was mainly represented by trace metals and TBT. The southern sectors of the lagoon were the main areas impacted by Hg (171–524 ppm), total butyltin (23.9–39.76 ng(Sn)·g^{-1}) and PAH (3042–9948 ng·g^{-1}) contamination (Fig. 3, Supplementary Tables 2 and 3). All the trace metals investigated in this study were recorded within all the lagoon sampling sites. Cd (0.10–0.6 ppm), Cu (8.63–36.62 ppm) and Zn (93–408 ppm) levels detected in Bizerte lagoon benthic sediments (Supplementary Table 2) are unlikely to cause adverse biological effects. Recorded values for Cd and Zn were always below the ERL, at 1.2 ppm and 410 ppm, respectively. Conversely, high concentrations for Cu were registered and were above the ERL value of 34. Mercury contamination was lower than the ERL at most sampling stations along the northern side of the lagoon, whereas high values exceeding the ERL (150 ppm) were recorded (175.68 and 524 ppm) within the southern part. Four contaminants (As, Cr, Pb, Ni) were observed at most sampling stations, with contamination levels exceeding the ERL proposed by Long et al. (1995) (Fig. 3). Arsenic (As) levels ranged between 7.9 and 30.4 ppm (ERL = 8.2 ppm), Nickel (Ni) values ranged between 12.83 and 56.74 ppm (ERL = 20.9 ppm), Lead (Pb) levels from 23.87 to 123.92 ppm (ERL = 46.7 ppm) and finally Cr values ranged between 43.35 and 179.2 ppm (ERL = 81 ppm). High values for Ni were registered at four stations (15, 17, 24 and 54), with respective values of 56.48, 49.63, 52 and 56.74 ppm (>ERM = 51.6 ppm).

Moderate total butyltin levels ($\sum$BT) were recorded, with concentrations falling in the 4.76 - 39.76 ng(Sn)·g^{-1} range. MonoButyltin (MBT) levels ranged between 3.86 and 18 ngSn·g^{-1}, DBT values ranged between 0.17–6.61 ngSn·g^{-1} and finally the range of TBT levels was that of 0.14–14.91 ng(Sn)·g^{-1}. The full pollutant contamination levels recorded within the Bizerte lagoon benthic sediments are shown in Supplementary Tables 2, 3 and 4.

The total PAHs measure comprises the sum concentration of the 16 parent PAHs. PAHs contaminants were present at 45% of the sampled stations within Bizerte lagoon and were mainly localized along the southern part of the lagoon. Stations 2, 5, 8, 10, 13 and 47 were slightly

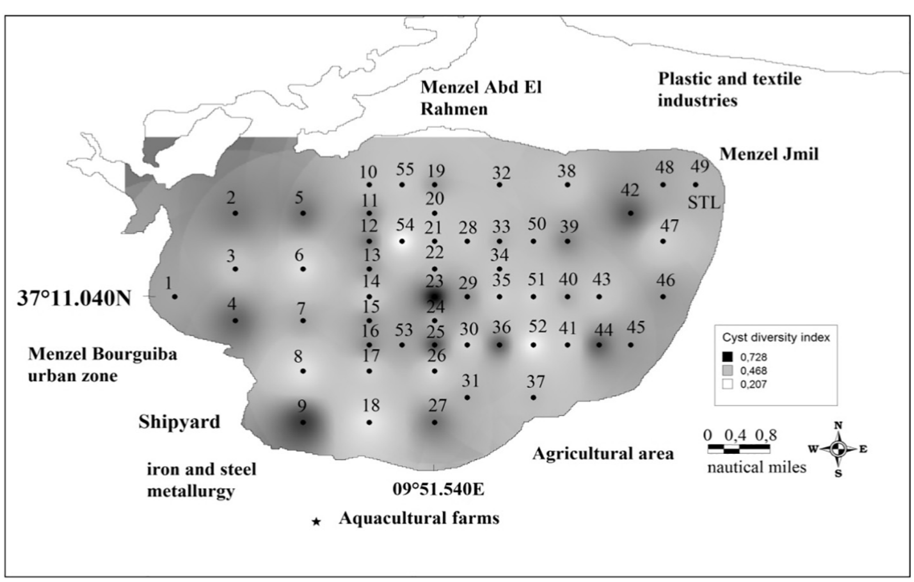

Fig. 5. Dinoflagellates resting cyst diversity index within Bizerte lagoon.

contaminated with $\sum$ PAHs values, with concentrations ranging from 2.3 to 49.75 ng·g^{-1} DS, whereas stations 9 and 27 were highly contaminated (9949 and 3043 ng·g^{-1} DS, respectively). PCB contamination was absent all over the lagoon and pesticides analysis revealed that alachlor and DCPU were the most abundant pesticides present within the sediment. DCPU ranged between 0.35 and 1.1 ng·g^{-1} DS and alachlor ranged between 0.67 and 4.95 ng·g^{-1} DS.

3.2. Dinocyst distribution and abundance

The spatial distribution of dinocysts in the benthic sediment of Bizerte lagoon is shown in Fig. 4. The highest cyst abundance was that of 2742 cysts·g^{-1} DS, recorded at station 49 and the lowest one was 203 cysts·g^{-1} DS recorded at station 25. Shannon-Wiener's diversity index values (H′) ranged between 0.21 and 0.73 bits·ind^{-1} (Fig. 5). H′ values <0.5 bits·ind^{-1} are normally associated with the dominance of biotic assemblages by a relatively small number of taxa. The low H′ index values recorded in our study can be attributed to high densities of *A. pseudogonyaulax* and *P. claudicans*. This was also confirmed by low values obtained for the Pielou equitability (evenness) index (Table 2).

In the laboratory, germination occurred mostly during the first three days of incubation. Successful excystment of most brown (the prevailing cyst colour) cysts frequently gave rise to motile cells belonging to the genus *Protoperidinium*, whereas excystment of rounded, colorless and walled cysts gave rise to motile stages of *Alexandrium* vegetative cells. Dinocyst assemblage was mainly represented by two groups: Peridiniales (33%) and Gonyaulacales (67%). A total of 22 cysts morphotypes representing 11 genera belonging to the following 4 orders were recorded within the entire Bizerte Lagoon sediment sampling area (Table 3): Peridiniales (Fig. 6), Gonyaulacales (Fig. 7), Gymnodiniales and Prorocentrales (Fig. 8). Two species dominated the dinoflagellate assemblage: *A. pseudogonyaulax* (29–89% of total RCs) and *P. claudicans* (5–38% of total RCs). *A. pseudogonyaulax*, the most abundant species, was present all over the lagoon and contributed significantly to the total cyst abundance.

Fig. 6. A–T: Peridiniales cysts isolated from surface sediments within Bizerte lagoon. **Organic Peridiniales**: Figs. A, B. *Protoperidinium claudicans* living cyst, Fig. C. *Protoperidinium claudicans* empty cyst, Figs. D–F. *Protoperidinium oblongum* living cyst, Figs. G, H. *Protoperidinium conicum* RCs, Fig. I. *Protoperidinium conicum* vegetative cell, Fig. J. *Protoperidinium compressum*, Figs. K, L. *Diplopsalis lenticula* living cyst and empty cyst, Fig. M. *Diplopsalis lenticula* vegetative cell, **Calcareous Peridiniales:** Figs. N, O. *Scrippsiella trochoidea* living cyst and vegetative cells, Fig. P. *Scrippsiella* cf. ramonii, Fig. Q. *Scrippsiella* cf. *precaria*, Fig. R. *Scrippsiella rotunda*, Fig. S. vegetative cells of *Scrippsiella rotunda*, Fig. T. *Ensiculifera* sp. Scale bar (10 μm).

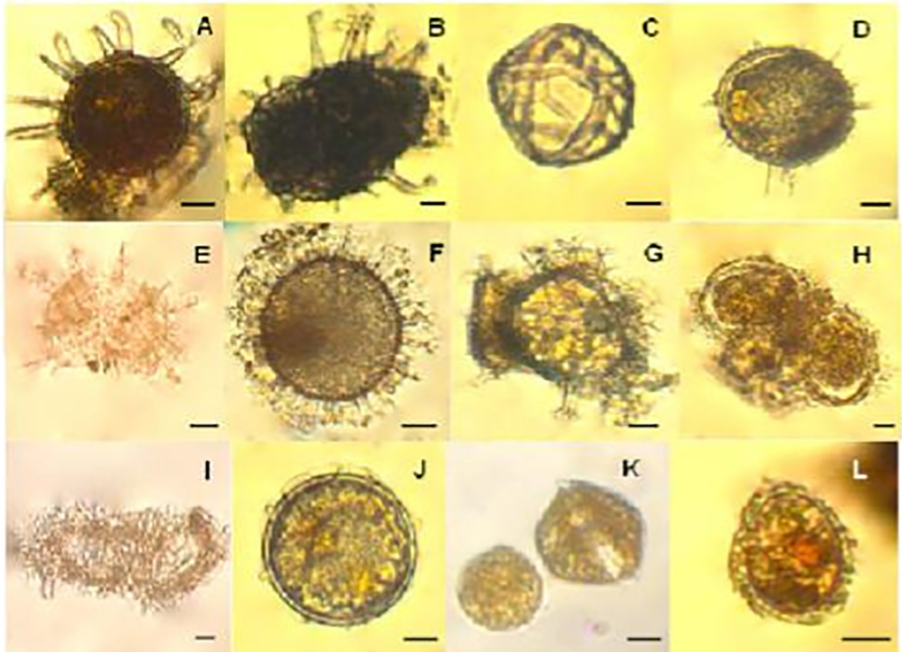

Fig. 7. A–L: Gonyaulacales cysts isolated from the surface sediment within Bizerte lagoon. Figs. A–C. *Lingulodinium polyedrum* living cysts and vegetative cell thequa, Figs. D, E. *Lingulodinium* sp., Fig. F. *Protoceratium reticulatum*, Fig. G. *Gonyaulax* cf. *spinifera* complex, Figs. H, I. *Alexandrium catenella/tamarense* complex resting cysts and empty cysts, Figs. J, K. *Alexandrium pseudogonyaulax* resting cyst and vegetative, Fig. L. *Alexandrium minutum* resting cyst. All scale bar (10 μm).

3.3. Correlations between chemical contamination and cyst abundance

The Pearson correlation analysis revealed a positive correlation between cyst abundance and sediment water content ($\alpha = 0.01$) and sediment fine silt content ($\alpha = 0.05$). Results are presented in Table 1. PCA was performed to investigate any correlation between the RC densities (considered as a supplementary factor), the sediment characteristics (texture, organic matter, H_2O percentage) and the contaminant concentrations (Fig. 9). Axes 1 and 2 accounted for 53.2% of this correlation. PC2 explained the highest part of the correlation (39.6%) and was mainly represented by trace metal concentrations. PC1 axis was mainly represented by sediment characteristics. Hierarchical clustering on stations based on RC abundance and contaminants revealed a correspondence between the highly polluted stations and the highest RC abundances (Fig. 10). The first cluster showed a mean abundance of 1114 cyst·DS^{-1} (Sd $\pm$ 660 cyst·DS^{-1}), the second cluster of 955 cyst·DS^{-1} (Sd $\pm$ 629 cyst·DS^{-1}) and the third one of 1015 cyst·DS^{-1} (Sd $\pm$ 559 cyst·DS^{-1}). Since Moran I index values revealed a strong degree of co-linearity between the stations, we applied Simultaneous Autoregressive Models (SAR) to remove this spatial autocorrelation in the residuals, and a backward stepwise method was performed to retain the most significant variables in explaining observed total cyst densities. The final SAR model was highly statistically significant ($\alpha = 0.05$, P-value $= 0.0335$) and revealed six explanatory environmental factors as shown in the following equation:

Total cyst density (cyst·g^{-1} DS) $= 13.43\,Cr - 2.941\,Hg - 2566\,Cd + 82.25\,Cu - 66.37\,Ni + 23.67\,H_2O$.

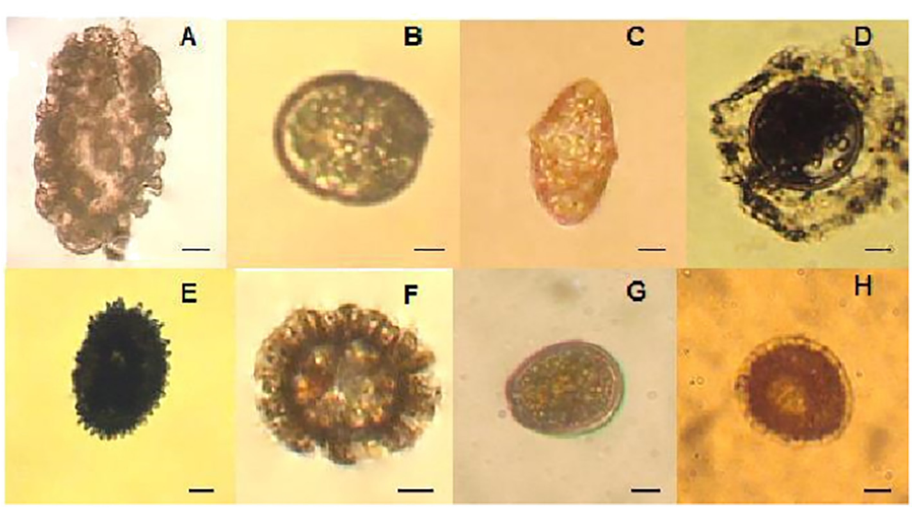

Fig. 8. A–H: Unidentified cysts, gymnodiniales and prorocentrales cysts isolated from the surface sediments within Bizerte lagoon. Fig. A. *Polykrikos schwartzii/kofoidii* complex, Figs. B, C, *Gymnodinium* spp. vegetative cells germinated from sediment, Fig. D. *Gymnodinium* sp., Figs. E, F, H. unidentified cysts, Fig. G. *Prorocentrum* cf. *micans*. Scale bar (10 μm).

Table 1
Correlation coefficient between resting cysts (RCs) abundance and sediment characteristics.

	Pearson correlation	Significance
RCs density	1	
Water content %	**0.414**[a]	**0.002**
Organic matter (OM%)	−0.130	0.343
Fine clay (0–2 μm)	−0.142	0.302
Coarse clay (2–4 μm)	−0.147	0.283
Fine silt (4–30 μm)	**0.269**[b]	**0.047**
Coarse silt (30–63 μm)	0.174	0.204
Fine sand (63–200 μm)	−0.027	0.844

Bold in the table highlight significance below 0.05 and their of corresponding pearson correlation.
[a] Significant correlation at 0.01 level.
[b] Significant correlation at 0.05 level.

4. Discussion

Cyst production occurs due to the occurrence of stressful environmental conditions such as turbulence, a decrease in the seawater temperature, nutrient deficiency or high densities of vegetative cells. This study is the first to simultaneously measure in the sediment levels of major pollutants (trace metals, butylin compounds, PAHs, polar pesticides, PCB) and dinocyst abundance and diversity in order to evaluate if there was any correlations between these contaminants and cyst abundance in recently-deposited sediment of a southern Mediterranean lagoon.

4.1. Contamination levels within Bizerte lagoon sediments

Inorganic contaminants within the Bizerte lagoon sediment were mainly represented by trace metals and Tributyltin. The south-western side of the lagoon, represented by stations 8, 9, and 27, was the most polluted area. The degree of trace metal contamination could be classified into three levels: rarely (<ERL), occasionally (ERL-ERM) and frequently (>ERM), in terms of its adverse biological effect (Long et al., 1995). Based on the effect-range classification, As, Cr, Pb, Ni and Hg were likely to pose environmental risks, with most values for their concentrations exceeding the ERL range. Trace metal levels recorded in this study were mostly similar to those registered in previous studies for most sampling stations (Barhoumi et al., 2014b; Pringault et al., 2015), whereas, the highest concentrations for Zn, As, Pb and Ni were recorded here for the first time. When comparing results from the current study to those of Yoshida et al. (2002), still conducted for Bizerte lagoon sediment, six trace metals (V, Co, Al, Cu, Cr and Pb) presented higher levels and three trace metals (Zn, As and Mo) presented lower levels than those previously recorded for the same benthic sediments. Compared with other Mediterranean ecosystems, Bizerte lagoon seems to be more polluted than Thau Lagoon (France), Venice lagoon (Italy) and the Turkey coastline, mainly in terms of Zn, Pb, Cr and Ni (Rigollet et al., 2004; Aydin et al., 2015).

Concerning butylin contamination, the spatial distribution of organotin was homogeneous throughout the lagoon sediments, with moderate levels (0.14–14.91 $ngSn \cdot g^{-1}$) generally being recorded, except for the two sampling sites 8 and 9 (39.76 and 23.9 $ngSn \cdot g^{-1}$, respectively), located near the industrial area and the shipyard of Menzel Bourguiba. Butylin levels in the sampled stations within the Bizerte lagoon are lower than those recorded from other comparable Mediterranean coastal systems, such as those in Spain (Barcelona port) 18.7 $ngSn \cdot g^{-1}$, France (Port-camargue) 10.73 $ngSn \cdot g^{-1}$ and Venice lagoon 39.3 $ngSn \cdot g^{-1}$. Whilst the degree of in situ TBT contamination is considered to be moderate, its impact on living organisms could still be considerable (Mzoughi et al., 2005). It has been shown that the Bizerte lagoon exhibited a high rate of imposex within mollusc populations, with an incidence of 28–100% for *Hexaplex trunculus* and 77–100% for *Bolinus brandaris*. (Lahbib et al., 2012; Abidli et al., 2013). PAHs contamination was mainly localized in the southern part of Bizerte lagoon, with the highest concentration recorded being that of 9948.84 $ng \cdot g^{-1}$. In general, recorded PAHs concentrations ranged from 2.3 $ng \cdot g^{-1}$ to 49.75 $ng \cdot g^{-1}$, being lower than those recorded previously in Bizerte lagoon (Trabelsi and Driss, 2005; Barhoumi et al., 2014b). When compared with other Mediterranean ecosystems, PAHs levels were lower than those recorded in the French Thau lagoon (59–76.79 $ng \cdot g^{-1}$) (Leaute, 2008) and in Pialassa Baiona (3.032–87.150 $ng \cdot g^{-1}$) in Italy (Guerra, 2012).

4.2. Dinocyst abundance and diversity

This study provides a database on dinocyst assemblage and abundance in the Bizerte lagoon. This could help to detect a potential future introduction of a non-indigenous harmful dinoflagellate species in this ecosystem through human-assisted dispersal. Along the southern Mediterranean shores, few studies were conducted on dinoflagellate cyst

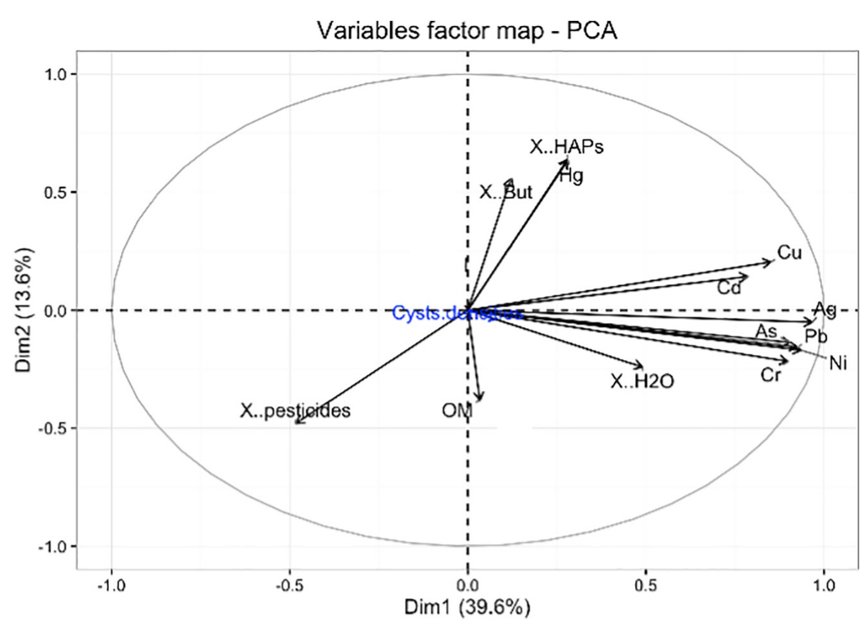

Fig. 9. Principal Component Analyses output results. Total resting cyst densities is included as supplementary factor.

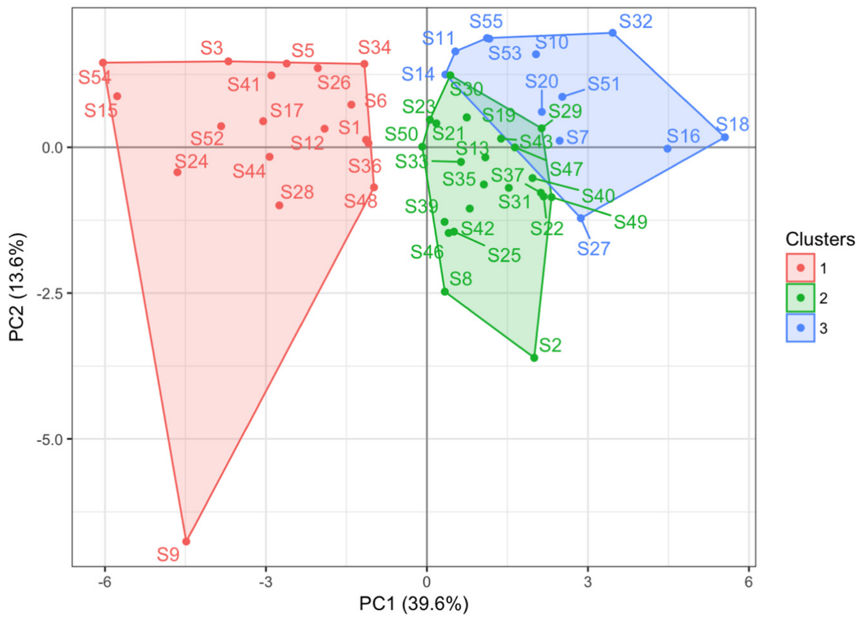

Fig. 10. Principal component analyses output results. Total resting cyst densities is included as supplementary factor.

distribution (Zmerli Triki et al., 2014, 2015b; Fertouna-Bellakhal et al., 2014). We considered Speniferites species as a single speniferites group morphotype so as to avoid potential mis-identification and, in addition, unidentifiable dinoflagellate cysts were not counted. Our data showed that all dinoflagellate cyst morphotypes encountered in the benthic sediment of Bizerte lagoon have been previously recorded in other Mediterranean ecosystems as in Spain and Italy, where they seem to be well-established. Within Bizerte Lagoon benthic sediment, we found high dinoflagellate cyst abundances (203–2742 cysts$\cdot$g^{-1}) as well as moderate cyst diversity (22 cyst morphotypes). Our results concerning dinocyst abundance contrasted with those from other Mediterranean ecosystems which showed a high diversity and a low cyst abundance. As an example, along the Ionian coasts of Sicily (Italy), 34 cyst morphotypes were recorded, with cyst densities ranging from 34 to 828 cysts$\cdot$g^{-1}, whilst in Alfacs and Fangar bays (Spain) 62 cyst morphotypes were recorded, and densities ranging from 21 to 322 cysts$\cdot$g^{-1} (Table 3 and references therein). The difference between the cyst morphotype richness values recorded in different studies could be explained in terms of several factors, such as differential dinoflagellate species colonization rates, the thickness of the investigated sediment layer and the inclusion or not of the unidentified morphotypes within the total count of cyst morphotypes and also in terms of the taxonomic identity of species belonging to the speniferites group (the latter is especially an issue when there is a high species richness for this group). In our study, potentially toxic dinoflagellate species represented 30% of the total morphotypes. *A. pseudogonyaulax* was the dominant morphotype, reaching a density of 1686 cysts$\cdot$g^{-1} DS, followed by *Lingulodinium polyedrum* and the *Alexandrium catenella/tamarense* complex with maximum values of 152 and 104 cysts$\cdot$g^{-1} DS, respectively. *Gonyaulax spinifera* and *Protoceratium reticulatum* were the least represented toxic/noxious species in the current study, whereas they are, together with *Lingulodinium polyedrum*, common in other Mediterranean coastal sediments (Montresor et al., 1998; Giannakourou et al., 2005; Rubino et al., 2010; Satta et al., 2010) and are known to cause toxic blooms, mainly in the northern Adriatic Sea (Honsell et al., 1992).

4.3. Correlation between the contaminants and dinocyst abundance and distribution

It's generally assumed that dinocyst distribution and abundance are greatly influenced by the sediment characteristics and the hydrodynamic features of a given area. Our results showed that the sediment in the Bizerte lagoon with the highest silt and water content was characterized by the highest RCs densities and that the hydrodynamics of this marine system influences the cyst dispersal patterns (Zmerli Triki et al., 2014). However, cyst accumulation remains a complex process which cannot be explained exclusively in terms of sediment characteristics and hydrodynamics. Our data suggest that contaminant levels in the sediment could be an additional parameter in explaining the cyst abundance and distribution patterns. In fact, hierarchical clustering on stations performed on RC abundance values and sediment contaminant levels revealed a relationship between the highly polluted stations and the highest RCs abundance. Moreover, the Simultaneous Autoregressive (SAR) model, computed in order to analyze the predictive power of contaminant levels for determining resting cyst abundance, showed that cysts densities in Bizerte lagoon were associated mainly with trace metal concentrations, including those for Hg, Cd, Cu, Ni and Cr, as well as with sediment water content, suggesting that concentrations for these pollutants could influence RC abundance in the sediment. These results are consistent with previous studies which discussed the effect of contaminants on cyst abundance and distribution. For instance, Horner et al. (2011) investigated the effect of the Cd concentration on *Alexandrium catenella* cyst distribution and they highlighted a significant positive correlation between the two. Aydin et al. (2015) showed that autotrophic cysts were dominant close to highly contaminated stations in Izmir bay in Turkey. In addition, significant positive correlations between the abundance and distribution of some dinoflagellate species, such as *Lingulodinium machaerophorum*, *Dubridinium caperatum* and *Polykrikos kofoidii*, and the sediment concentration of some metals as Cd, Pb, Cu and Zn were reported from Izmir bay. Liu et al. (2012) suggested that a high degree of sediment contamination by industrial pollutants might cause a decrease in cyst abundance or heterotrophic/autotrophic rate.

Within Bizerte lagoon, an anthropized ecosystem, high dinocyst densities were recorded in the surface sediment, whereas no dense blooms related to this phytoplankton group were observed in this ecosystem except for one caused by *Alexandrium catenella* in 2007. This apparent paradox could be partially explained by: a) the high cyst production rate and the low natural germination rate of the dominant assemblage species (*A. pseudogonyaulax*), b) the physiological stress induced on the vegetative cells by the remobilized pollutants in the water column could enhance cyst production and c) the inhibition of the

Table 2
Dinocyst species recorded within the surface sediment of Bizerte lagoon. The potential toxicity of these species as recorded within different Mediterranean case studies is indicated.

Groups	Dinocyst species	Abundance in Bizerte lagoon	Toxine/toxic effect	Presence in the Mediterranean Sea
Gonyaulacales	*Alexandrium pseudogonyaulax* (Biecheler)	***	Goniodomine A (Zmerli Triki et al., 2016)	Naple, Adriatic sea (Italy), Alfacs Bay (Spain) (Montresor, 1995; Bravo et al., 2006; Penna et al., 2010; Satta et al., 2013)
	Alexandrium minutum Halim	*	Paralytic shellfish toxins (Bravo et al., 2006)	Harbour of Alexandria (Egypt), Catalonia, Arenys del mar (Spain); Ionian sea, Adriatic sea, Tyrrhenian sea, Olbia (Italy), Aegean Sea (Turkey) (Ismael and khadr, 2003; Anglès et al., 2010; Rubino et al., 2010; Penna et al., 2010; Aydin et al., 2011)
	Alexandrium catenella/tamarense (Whedon & Kofoid) Balech	**	Paralytic shellfish toxins (Laabir et al., 2013)	Aegean Sea (Turkey), Ionian Sea, Olbia, (Italy), Thau lagoon (France), Tarragona and Barcelona Harbours (Spain) (Bravo et al., 2006; Satta et al., 2010; Rubino et al., 2010; Aydin et al., 2011)
	Lingulodinium polyedrum (Stein) Dodge *Lingulodinium sp*	**	Yessotoxin (Paz et al., 2004)	Ionian Sea, Adriatic sea (Italy), Catalan sea, Arenys del mar, Alfacs and Fangar bays (Spain) (Rubino et al., 2010; Penna et al., 2010; Satta et al., 2010, 2013)
	Gonyaulax cf. *spinifera* complex (Claparéde et Lachmann) Diesing	*	Yessotoxin (Rhodes et al., 2006)	Arenys del mar (Spain), Olbia (Italy) (Satta et al., 2010)
	Protoceratium reticulatum (Claparède et Lachmann) Butschli	*	Yessotoxin (Paz et al., 2004)	Adriatic sea (Italy), Alfacs and Fangar bays (Penna et al., 2010; Satta et al., 2013)
	Protoperidinium claudicans (Paulsen) Balec	**		Adriatic sea, Tyrrhenian sea, Olbia and Syracuse bay (Italy) Arenys harbour, Catalonia, Alfacs and Fangar bays (Spain) (Penna et al., 2010; Garcés et al., 2010; Satta et al., 2013)
	Protoperidinium leonis	*	Non toxic	Ionian sea (Rubino et al., 2010)
	Protoperidinium compressum (Abé) Balech	*	Non toxic	Arenys harbour, Alfacs and Fangar bays (Spain), Adriatic sea, Olbia and Syracuse bay (Italy) (Garcés et al., 2010; Penna et al., 2010; Satta et al., 2010, 2013)
Organic peridiniales	*Protoperidinium conicum* (Gran) Balech	**	Non toxic	Aegean Sea (Turkey), Arenys harbour, Alfacs and Fangar bays (Spain), Olbia, Adriatic sea, Syracuse bay and Ionian sea (Italy) (Garcés et al., 2010; Satta et al., 2010, 2013; Rubino et al., 2010; Penna et al., 2010; Aydin et al., 2011)
	Protoperidinium oblongum (Aurivillius) Parke et Dodge	**	Non toxic	Arenys harbour, Alfacs and Fangar bays (Spain), Olbia and Syracuse bay, Adriatic sea, Ionian sea (Italy) (Garcés et al., 2010; Penna et al., 2010; Satta et al., 2010, 2013)
	Zygabikodinium lenticulatum (Paulsen) Loeblich	*	Non toxic	Arenys harbour (Spain), Olbia and Syracuse bay (Italy) (Garcés et al., 2010; Satta et al., 2010)
	Diplopsalis lenticular	**	Non toxic	Ionian Sea (Rubino et al., 2010)
	Scrippsiella cf. *precaria* Montresor et Zingone	*	Non toxic	Arenys harbour (Spain), Olbia and Syracuse bay (Italy) (Garcés et al., 2010; Satta et al., 2010)
	Scrippsiella cf. *ramonii* Montresor	*	Non toxic	Arenys harbour, Alfacs bay (Spain) (Satta et al., 2010)
	Scrippsiella trochoidea (Stein)	**	Non toxic	Arenys harbour, Alfacs and Fangar bays (Spain), Olbia and Syracuse bay, Ionian Sea, Catalan sea, Tyrrhenian sea (Italy) (Garcés et al., 2010; Satta et al., 2010; Rubino et al., 2010)
	Ensiculifera cf. *acminata*	*	–	
	Scrippsiella rotunda	*	–	
Gymnodiniales	*Polykrikos kofoÏdi/schwartzii* complex	*	Non toxic	Aegean Sea (Turkey), Adriatic sea, Olbia (Italy) Alfacs and Fangar bays (Aydin et al., 2011; Penna et al., 2010)
	Gymnodinium spp	*	–	
Prorocentrales	*Prorocentrum micans*	*	Toxic	

The resting cyst abundance within the sediment of the Bizerte lagoon: 50 < * < 10^2 cysts·g^{-1} dry sediment (DS); 10^2 < ** < 10^3 cysts·g^{-1} DS; *** > 10^3 cyst·g^{-1} dry sediment.

excystement process by the contaminants within the sediment could lead to a considerable cyst accumulation in the sediment.

Liu et al. (2012) showed that toxic compounds in the sediment could induce a decrease of the proliferation of vegetative cells; hence, some cells might die whilst others perform sexual reproduction, resulting in large volumes of resistant cysts enhancing cyst banks. Several laboratory studies have been conducted to date to test the degree of stress posed by metal contamination on vegetative cells. Contaminants affect the physiology of vegetative cells by acting on the levels of oxidative stress in cells (Okamoto and Colepicolo, 1998; Pinto et al., 2003) or by reducing their light-harvesting capacity and by inhibiting cell growth (Miao and Wang, 2006; Herzi et al., 2013). Under high levels of iron, cell

Table 3
Comparison of the number of cyst morphotypes and the total cyst abundance (cysts·g^{-1}) in sediment of different coastal ecosystems. NR: not recorded.

Location	Sampling method	Number of stations number	Morphotypes number	Densities (cysts·g^{-1})	References
Gulf of Olbia and Arenys del Mar harbour	Cores (1 cm)	68	42	20–5484	Satta et al. (2010)
Alfacs and Fangar bays	Cores (1 cm)	16	62	21–322	Satta et al. (2010)
Ionian coasts of Sicily	Cores (3 cm)	4	34	43–828	Rubino et al. (2010)
Lisbon Bay, Portugal	Manual vacuum pump (0–2 cm)	23	58	212–574	Ribeiro and Amorim (2008)
Izmir Bay	Corer (0–2 cm)	13	41	3292	Aydin et al. (2011)
Thermaikos Gulf, Aegean Sea, Greece	Corer (0–10 cm)		36	385–5718	Giannakourou et al. (2005)
Sishili Bay, Yellow Sea, China	Core (0–5 cm)	22	35	122–1322	Liu et al. (2012)
Labrador fjords (canada)	Corer (5 mm)	13	16	1000–6000	Richerol et al. (2012)
Estuarine south Korea	Corer (2 cm)	23	47	1000–8900	Pospelova and Kim (2010)
Mexico	Birge Ekman style box sediment sampler	7	17	NR	Pena-Manjarrez et al. (2005)
Eastern coast of Russia	Corer (2 cm)	44	40	NR	Orlova et al. (2004)

mobility and protein production could be altered, so that vegetative cells can promptly undergo encystment so as to survive the induced high levels of pollutant stress (Lage et al., 1994; Okamoto et al., 1999; Liu et al., 2012). Wang et al. (2001) revealed that the intensity of the effect of pollutants on vegetative cells is largely dependent on the rates of nutrient availability.

It has been shown for instance that organic pollutant such as PAHs (phenanthrene, anthracene, pyrene and fluoranthene) could inhibit the cell growth of some phytoplankton species (Wang et al., 2008; Hong et al., 2008; Echeveste et al., 2010; Ben Othman et al., 2013). Mouhri et al. (1995) showed that the exposure of phytoplankton to TBT induced the intracellular accumulation of nitrogen and phosphorus without their assimilation, resulting in a decrease of the cellular biomass. da Leitao et al. (2003) showed a harmful effect of Arochlor 1254 and PCB derivative on phytoplankton cells, inducing the oxidative damage of proteins and causing cell growth inhibition.

Laboratory ecotoxicological studies on phytoplankton only assessed the effect of the pollutants on vegetative cells. Until now, no studies have been conducted to test the potential effect of the pollutants on resistant cysts of the dinoflagellate species responsible for the major Harmful Algal Blooms. This consideration should set the agenda for future research on coastal benthic sediments since such laboratory studies could help us better understand the effect of pollutants on the bloom dynamics of HAB-forming species.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.03.183.

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