Occurrence of three dominant epibenthic dinoflagellates (*Ostreopsis* spp., *Coolia monotis* and *Prorocentrum lima*) in relation to biotic substrates and environmental factors in a highly dynamic ecosystem, the Strait of Gibraltar (Southwestern Mediterranean)

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Abstract No studies have been carried out on the benthic harmful algal blooms (BHABs) along the Strait of Gibraltar in the Mediterranean, and little is known about the diversity of blooming species. Here, epibenthic dinoflagellates were monitored at least biweekly over 18 months (May 2019–November 2020) in Oued Lihoud, Cap Malabata and Dalia on the thalli of five dominant macrophytes and in the water column. This is the first report on the seasonal distribution of BHAB species hosted by natural biotic substrates in the Strait of Gibraltar, which is known for high hydrodynamics, major entry of Atlantic waters and important maritime traffic. Three BHAB dinoflagellates were observed in the surveyed areas: *Ostreopsis* spp., *Coolia monotis* and *Prorocentrum lima*. The analysis of all data at the three sites showed that *Dictyota dichotoma* was the most favourable macroalgae host for these benthic dinoflagellates. The highest cell densities were observed in Cap Malabata for *Ostreopsis* spp. (2.7×10^5 cells/g fresh weight in September 2020), *P. lima* (4.57×10^4 cells/g FW in September 2020) and *C. monotis* (4.07×10^4 cells/g FW in June 2019). Phosphate and temperature were positively correlated to the abundances of the studied thermophilic BHAB species. In contrast, negative correlations were recorded with salinity, ammonium, nitrite, nitrate, DIN, nitrogen/phosphate ratio and suspended material, attesting of the complex relationships between environmental factors and BHAB species dynamic in each marine ecosystem. Toxin analyses of the natural phytoplankton assemblage during BHABs showed the presence of only lipophilic toxins, namely okadaic acid and dinophysistoxins produced by *P. lima*. These BHABs species have to be isolated to establish monoclonal cultures for ribotyping and ecophysiological investigations.

Keywords Epibenthic dinoflagellates · Biotic substrate · Environmental factors · Toxins · Strait of Gibraltar

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Introduction

The frequency, intensity and geographical area of harmful algal blooms (HABs) have increased worldwide in recent decades (Burkholder, 1998; Hallegraeff, 1993; Smayda, 1990). This has been attributed to climate change, anthropogenic impacts and the implementation of monitoring programs (Berdalet & Tester, 2018; Tester et al., 2020). The growth, abundance and distribution of phytoplankton are controlled by chemical (nutrients, oligo-elements, vitamins and salinity), physical (temperature, irradiation and turbulence) and biological (competition and predation) environmental factors (Bužančić et al., 2016; Tester et al., 2020). Benthic harmful algal blooms (BHABs) are well known in tropical latitudes where they appear regularly. However, they are expanding to temperate ecosystems (Ben-Gharbia et al., 2016, 2019; Litaker et al., 2010; Parsons et al., 2012; Pérez-Arellano et al., 2005; Rhodes, 2011; Shears & Ross, 2009; Zingone et al., 2021), and their seasonal variation is often linked to temperature fluctuations (Cohu et al., 2013; Mangialajo et al., 2011). The highest abundances of epibenthic dinoflagellates, such as *Ostreopsis* and *Coolia* species, are often observed during the warm season (Aligizaki & Nikolaidis, 2006). According to IOC-UNESCO, among the 140 toxic species, 84 have been observed in the Mediterranean, of which 54 are dinoflagellates (Zingone et al., 2021). The Mediterranean waters of Morocco have experienced episodes of toxic microalgal blooms as well, such as with the planktonic dinoflagellate *Gymnodinium catenatum,* which has been associated with paralytic shellfish poisoning (PSP) events (Rijal Leblad et al., 2020; Taleb et al., 2001), and *Pseudo-nitzschia* spp., which can produce domoic acid (DA) (Rijal Leblad et al., 2013). However, studies on the temporal monitoring of the phytoplankton community along the Mediterranean waters of Morocco are rare (Daoudi et al., 2012; El Madani et al., 2011; Rijal Leblad et al., 2013, 2020), and there have been no reports on BHAB species until now. Elsewhere in the Mediterranean, several studies have been carried out to understand the relationships between epibenthic dinoflagellate assemblages and environmental factors (Abadie et al., 2018; Ben-Gharbia et al., 2019; Carnicer et al., 2015; Cohu et al., 2013; Giussani et al., 2017). The sporadic proliferation of toxin-producing phytoplankton species could cause human health problems and economic losses in the fish and mollusc farming industries (Hoagland et al., 2002; Kim, 2010; Park et al., 2013). The majority of these impacts occur once shellfish ingest toxic microalgae and accumulate toxins, which subsequently leads to increased levels of poisoning in humans or other consumers (Anderson, 2019). Direct contact with contaminated seawater or inhalation of aerosolized biotoxins can lead to intoxications considered a direct impact of HABs on human health (Berdalet et al., 2016; Durando et al., 2007). Many species belonging to the *Ostreopsis* and *Prorocentrum* genera are able to synthetize potent toxins such as palytoxins (PLTXs), ovatoxins (OVTXs) and okadaic acid (OA)/dinophysistoxins (DTXs) (Karafas et al., 2015; Lewis et al., 2018; Louzao et al., 2015; Nascimento et al., 2016; Scalco et al., 2012; Wakeman et al., 2015; Wang et al., 2015). Other toxins are synthesized by other dinoflagellates belonging to the *Coolia* and *Amphidinium* genera, but they have never been demonstrated as having caused human poisoning (Holmes et al., 1995; Pagliara & Caroppo, 2012; Rhodes & Thomas, 1997; Rhodes et al., 2014). *Ostreopsis* spp.*, Coolia* spp. and *Prorocentrum lima* have been observed in the Mediterranean Sea, on macrophytes and in the water column (Aligizaki & Nikolaidis, 2006; Ben-Gharbia et al., 2016, 2019; Cohu et al., 2013; Meroni et al., 2018; Nascimento et al., 2016; Yan et al., 2020). *Ostreopsis* species remain the most observed in the Mediterranean: in Greece (Aligizaki & Nikolaidis, 2006; Aligizaki et al., 2008), Cyprus (Accoroni et al., 2016), Croatia (Pfannkuchen et al., 2012), Lebanon (Açaf et al., 2020; Saab et al., 2013), France (Gémin et al., 2020; Pavaux et al., 2021), Monaco (Cohu et al., 2011), Italy (Accoroni et al., 2015; Casabianca et al., 2014; De Virgilio et al., 2021; Meroni et al., 2018), Catalonia in Spain (Carnicer et al., 2015; Mangialajo et al., 2011), the Andalusia coast (Cagide et al., 2009), Egypt (Hosny & Labib, 2019), Tunisia (Abdennadher et al., 2017; Ben-Gharbia et al., 2016, 2019; Hachani et al., 2018; Turki et al., 2006) and Algeria (Illoul et al., 2012). They have also been observed in the Atlantic Ocean near the Strait of Gibraltar in Portugal (David et al., 2013; Santos et al., 2019; Soliño et al., 2020). *Ostreopsis* cf. *ovata* is the most well-known species with a large distribution and increasing impact over time, showing a recent sudden upsurge. Ciguateric species have been found twice in the Mediterranean Sea, around the Balearic Islands in the western Mediterranean Sea and Crete in the eastern Mediterranean Sea. However, until now, it has been unknown whether *Gambierdiscus* species have proliferated in the Mediterranean Sea (Laza-Martínez et al., 2016).

The Strait of Gibraltar is the meeting of two opposite currents, a surface current entering the Mediterranean

and a deep current entering the Atlantic (Calero Quesada et al., 2014). The study area is the passage for ships and is characterized by the presence of two major ports in the region: the Moroccan Tanger Med and the Spanish Algeciras, which can lead to the appearance of exotic microalgal species introduced via ballast water discharges that remain uncontrolled.

The objectives of this study are: i) to assess the diversity and spatiotemporal distribution of BHAB dinoflagellates in three areas located in the Strait of Gibraltar, Oued Lihoud, Cap Malabata and Dalia, ii) to correlate the main environmental factors (temperature, salinity, nutrients), and biotic substrates (macrophytes) to the dynamics of epibenthic dinoflagellates in the sampling sites, and iii) to assess the toxins present in natural epibenthic microalgae during their development.

Materials and methods

Sampling sites

The study area is located in the north-western portion of the Moroccan Mediterranean and precisely in the Strait of Gibraltar (Fig. 1). Three sites, Oued Lihoud (Jews River) (35°47′45.45′′N – 5°50′12.34′′W), Cap Malabata (35°48′43.34′′N – 5°45′1.07′′W) and Dalia (35°54′34.05 N – 5°28′13.60′′W), were selected to investigate the presence of benthic harmful algal bloom (BHAB) species. The three surveyed sites are characterized by a generally gently sloping coastal topography, a rocky shoreline and sedimentary bottoms. On the Moroccan coast, the tide is semidiurnal. There is a slight diurnal inequality, with two successive tides differing slightly in amplitude (1 m).
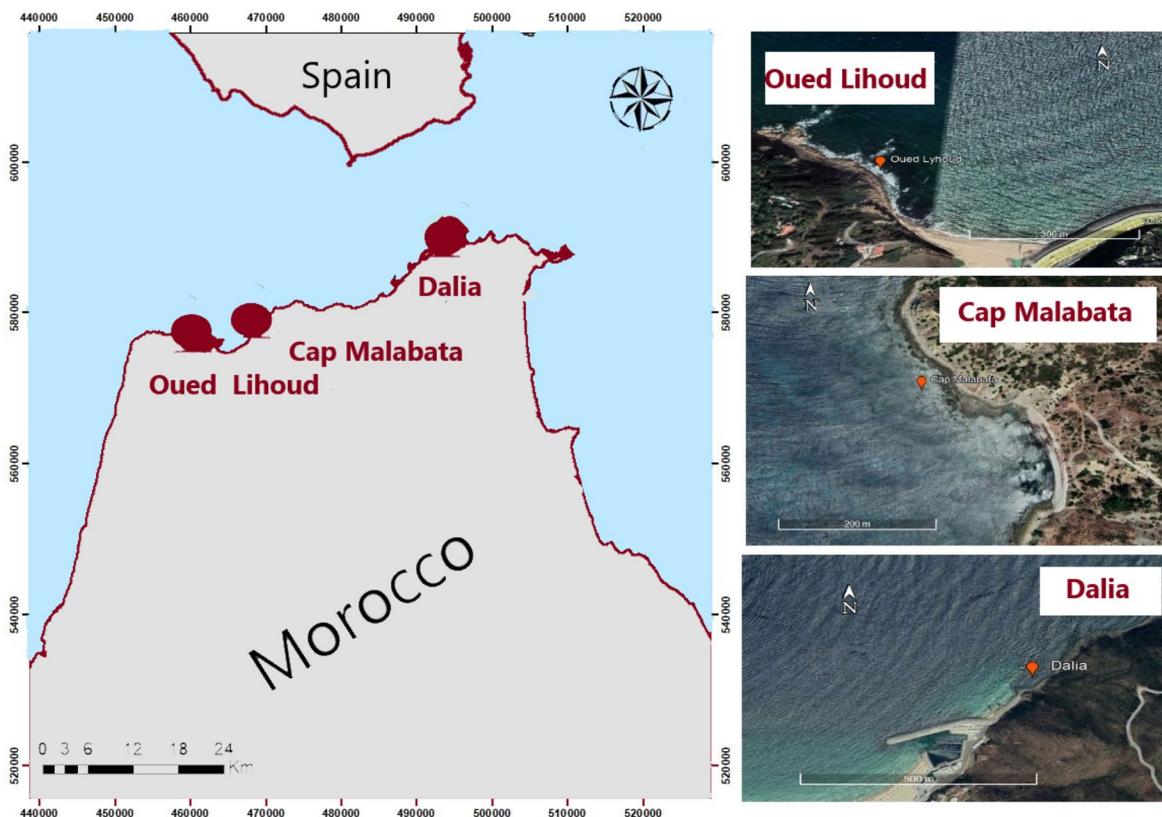

Fig. 1 Map of the sampled sites (Oued Lihoud, Cap Malabata and Dalia) in the strait of Gibraltar (Southwestern Mediterranean, Morocco)

Sampling was performed during low tide. In the Tangier region, two winds predominate: west winds of Atlantic origin often led to precipitation and are predominant from November to March in the northwest to southwest direction. The east wind of Mediterranean origin often blows in summer and spring from east to northeast. The sampled sites are located in the Strait of Gibraltar and influenced by entering Atlantic waters. Oued Lihoud sampling site is in the western portion of Tangier city (1,083,462 inhabitants) and receives water from small rivers during the rainy season, resulting in important discharges of chemical pollutants, bacteria and suspended matter (Blinda, 2007). Cap Malabata is located in the eastern part of Tangier Bay and could be considered as not anthropized. Dalia is located next to the large port of Tangier Med, which is characterized by an intense commercial exchange between Morocco and the five continents (transhipment of up to 1 million containers and 7 million passengers per year), leading to a potential transfer of exotic HAB species via ballast water discharges. The three stations are colonized by a variety of macroalgae present throughout the year (Kazzaz & Riadi, 2000).

Sampling and analysis

Sampling

During periods of low tides, the samples were taken from three sites once every 10 days from May to November 2019, monthly from December 2019 to March 2020 and biweekly from June to October 2020. Because of COVID-19 constraints, samples were not taken during the quarantine period from mid-March to 10 June 2020. The number of sampling dates was 31 for each station.

Five macroalgae, the Rhodophyceae *Asparagopsis armata* and *Plocamium coccineum* and the Phaeophyceae *Dictyota dichotoma*, *Halopteris scoparia* and *Cladostephus spongiosus* (Adama et al., 2021; Riadi & Kazzaz, 1998), were collected, taking 50 to 100 g fresh weight. Four seawater samples were collected from each site at depths between 0.5 and 1 m for the analyses of nutrients (0.5 L), chlorophyll *a* (1 L) and suspended material (0.5 L) and for the identification and counting of dinoflagellates present in the water column (1 L).

Dinoflagellate identification and cell abundance measurement

To detach the epiphytic cells, the macrophyte thalli were placed into bottles each containing a volume of 500 to 800 ml of seawater previously sieved (20 μm) and filtered (0.7 μm). Then, each sample was vigorously agitated and passed through a mesh sieve (1 mm) to remove large particles (thalli, sand, zooplankton) and washed with 250 ml of the same filtered seawater. Afterwards, the sample was passed through a filter vessel (20 μm) to recover the dinoflagellate cells bound to the macrophyte thalli. A total of 250 ml of the prepared sample and the same volume of the water column sample were fixed with Lugol iodine acid solution to identify and count the dinoflagellate species (Aligizaki & Nikolaidis, 2006; Mangialajo et al., 2011). After homogenization (30 to 60 s), the samples were stored in 10-ml chambers in the dark for 12 h (decantation) before being analysed. The identification and estimation of epiphytic dinoflagellate cells were performed under an inverted photonic microscope (Leica Microsystems model CMS GmbH, Germany) using the Uthermöl (1958) method. The macrophytes were weighed after water removal to determine their fresh weight to express the abundance of dinoflagellates on the thalli as follows: number of cells per gram of fresh weight (cells/g fresh weight).

Physicochemical parameter measurements

The seawater temperature (°C) and salinity values were measured using a probe (Cond 3310 SET 1, Germany). To determine the nutrient (nitrite NO_2^-, nitrate NO_3^-, ammonium NH_4^+, phosphate PO_4^{3-} and silicate $Si(OH)_4$) concentrations, a volume of 500 ml of seawater was taken in plastic bottles and analysed according to the protocol of Aminot and Chaussepied (1983). The second seawater sample (1000 ml) was used for chlorophyll analysis. Water was filtered (Whatman grade GF/F, pore size 0.7 μm) and kept frozen (-20 °C) before extraction in a 90% acetone solution. The chlorophyll *a* concentration was determined by fluorometry, according to the Lorenzen method (Lorenzen & Newton Downs, 1986).

To determine suspended material in surface seawater at the sampling sites, a representative 500-ml sample of seawater was collected and filtered through

Whatman GF/F 47 mm filters with a porosity of 0.7 μm previously dried. The material retained on the filter was dried at 109 °C and then weighed with the filter. The measurement of the suspended matter was obtained by calculating the difference between the mass of the filter including the dried residues and the initial mass of the filter using an analytical balance with a sensitivity of 0.1 mg while taking into account the filtered volume.

Meteorological data

The wind speed and solar irradiance are the meteorological parameters used in this study. The raw data were extracted by hour. We based parameter units on the sampling frequency to process and express the meteorological parameters as averages for 10, 15 or 30 days, and then the average of each parameter was calculated according to the period between two successive sampling dates. Oued Lihoud and Cap Malabata have identical meteorological values because they are located in the same area (distant by 27 km). The European Centre for Medium-Range Weather Forecasts (ECMWF) was the source used to download meteorological data related to the stations and study periods.

Analyses of cellular toxins of sampled natural epibenthic dinoflagellates

To access the diversity of toxins (okadaic acid, dinophysistoxins, pinnatoxins, portimines, ovatoxins, palytoxins and the ciguatoxins/maitotoxins group) which could be produced by epibenthic dinoflagellate species developing on the thalli of the macroalgae in the investigated ecosystems we used a highly sensitive LC–MS/MS method to analyse large spectra of toxins even if they are present in low amounts. This was done in parallel with counting the microalgae using photonic microscopy. During our field survey, when the abundances of *Ostreopsis* spp. were > 5000 cells/g FW of the macroalgae *Halopteris scoparia*, 40 ml of epibenthic dinoflagellate samples were taken for targeted analysis of known toxins produced by benthic species. Each sample was processed using a concentrate of 40 ml filtered through a GF/F filter. The filtrates were ultrafiltered (0.20 μm, Nanosep MF, Pall, Mexico) before LC–MS/MS analyses.

LC–MS/MS analysis of lipophilic toxins and pinnatoxins

The sample analyses were performed on a UFLC (model UFLC, Shimadzu) coupled to a triple-quadrupole mass spectrometer (4000Qtrap, ABSciex) equipped with a turboV® ESI source. Chromatographic separation was carried out on a C18 Kinetex column (100 Å, 2.6 μm, 100×2.1 mm, Phenomenex) with a C18 guard column (4×2.0 mm, 2.6 μm, Phenomenex). A binary mobile phase was used, phase A (100% aqueous) and phase B (95% aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. The flow rate was 0.4 ml min^{-1}, and the injection volume was 5 μL. The column and sample temperatures were 40 °C and 4 °C, respectively. A gradient elution was employed, starting with 20% B, rising to 95% B over 8 min, held for 3 min, then decreased to 20% B in 0.5 min and held for 3 min to equilibrate the system.

For quantitation, the mass spectrometer was operated in multiple reaction monitoring (MRM) acquisition mode, scanning two transitions for each toxin. Negative acquisition experiments were established using the following source settings: curtain gas set at 20 psi, ion spray at −4500 V, temperature of 550 °C, gas 1 and 2 set, respectively, at 40 and 55 psi, and an entrance potential of 13 V. In positive mode: curtain gas set at 30 psi, ion spray at 5500 V, temperature of 350 °C, gas 1 and 2 set, respectively, at 40 and 50 psi, and an entrance potential of 10 V. These parameters had been previously optimized using toxin standards. The mass spectrometer was operated in multiple reaction-monitoring (MRM) mode, analysing the two product ions per compound, for each toxin, the first transition, the most intense, was used for quantification. For ESI positivity, the transitions selected were [M^{+}NH$_4$]$^{+}$ ions: PnTX-A: 712.4 > 458.2/164.1; PnTX-G: 694.6 > 458.2/164.1. For ESI negative mode, the transition selected was [M–H]- ions: OA and DTX-2, m/z 803.4 > 255.1/113.1; DTX-1, m/z 817.5 > 254.9/112.9. Certified calibration solutions of OA, DTX-2, DTX-1, PnTX-A, and PnTX-G were obtained from the National Research Council Canada (NRCC, Halifax, NS, Canada).

LC–MS/MS analysis of toxins produced by *Ostreopsis* spp

Liquid chromatography was performed on a Poroshell 120 EC-C18 column (100×2.1 mm, 2.7 μm, Agilent,

France) equipped with a guard column (5×2.1 mm, 2.7 µm, same stationary phase) using a Nexera Ultra-Fast Liquid Chromatography system (Prominence UFLC-XR, Shimadzu, France). Gradients of water (A) and acetonitrile 95% (B), both containing 0.2% acetic acid, were used at a flow rate of 0.2 mL/min. The injection volume was 5 µL, and the column temperature was 25 °C. MS/MS analyses were performed with an API 4000QTRAP (AB Sciex, France) in positive ion mode and using MRM (multiple reaction monitoring) acquisition. UV detection at 220, 233, 263 and 220–360 nm.

Statistical analysis

was performed with a diode array detector (Prominence, SPD-M20A, Shimadzu, France). In total, two LC–MS/MS and one LC–MS/MS methods as described by (Chomérat et al., 2019) were used to detect palytoxin, 42-OH-palytoxin, 12 ovatoxins (-a to -k), ostreocins-B and -D, 3 mascarenotoxins (A to C) and ostreotoxins-1 and -3. Quantification was performed relative to the palytoxin standard (Wako Chemicals GmbH, Germany) with a 6-point calibration curve.

LC–MS/MS analysis of maitotoxins, gambierones *and Cooliatoxins*

A screening of several toxic compound families was performed using a system composed of an ultra-high-performance liquid chromatography (UHPLC) system (UFLC, Nexera, Shimadzu, Japan) coupled to a hybrid triple quadrupole-linear ion-trap mass spectrometer (API4000 QTRAP, Sciex, CA, USA) equipped with a TurboV source (ESI), according to the methods of Sibat et al. (2018) for detection of ciguatoxins and Yon et al. (2021) for the detection of maitotoxins and gambierones. The transitions monitored were either based on the literature (Nagai et al., 1992; Pisapia et al., 2017; Watanabe et al., 2013) or the result of optimization by infusion of available standards (only P-CTX3C and MTX1, Wako Chemicals).

Statistical analysis

Statistical analyses were performed using the software XLSTAT 2016.02.28430. We used Spearman correlation, principal component analyses (PCA) and factorial discriminant analyses (FDA) to investigate if there is any correlation between the abiotic factors (temperature, salinity, nutrients, N/P ratio, chlorophyll a, suspended material, wind speed, solar irradiance), biotic substrates (5 macrophyte species), the abundances of benthic dinoflagellates and the sampling stations along the Strait of Gibraltar. The data used come from all the sites (Oued Lihoud, Cap Malabata and Dalia). The Benthic microalgae abundance variable was ranked by macroalgae species (biotic substrate) for PCA and by sampling site for FDA.

Results

Physicochemical parameters

A summary of the physicochemical parameters is shown in Table 1. Figure 2 shows that the recorded maximum water temperature values were 23.3 °C at Oued Lihoud in July 2020, 23.7 °C at Cap Malabata in September 2020 and 19.9 °C at Dalia in September 2019. The minimum temperatures were 11.5, 15.1 and 16.02 °C recorded in Oued Lihoud in December 2019, in Cap Malabata in January 2020 and in Dalia in July 2019, respectively. The temperature was rather stable and relatively low, varying between 16.02 and 19.9 °C, at Dalia compared to those at Cap Malabata and Oued Lihoud (Fig. 2a). The highest values of salinity were registered in October 2020 at Oued Lihoud and Dalia (36.6) and at Cap Malabata (36.4). The lowest salinities were 35.3 at Oued Lihoud and Dalia in August 2019 and 36.6 at Malabata in July and August 2020 (Fig. 2b). The maximum suspended material values were observed during winter 2020 at Oued Lihoud (67.61 ± 36.47 mg/L) and Cap Malabata (85.49 ± 30.76 mg/L). The lowest averages were recorded in summer at Oued Lihoud (43.5 ± 17.44 mg/L) and in autumn at Cap Malabata (36.79 ± 14.1 mg/L). At Dalia, the suspended material was low in almost all of the samples in comparison with levels at Oued Lihoud and Cap Malabata (Fig. 2c).

Nutrients and chlorophyll a

Table 1 and Fig. 3 show that the maximum seasonal average nitrate concentrations were observed during winter,

Table 1 Summary of seasonal values of physicochemical parameters and climate data at Oued Lihoud, Cap Malabata and Dalia sampling sites

Site	Season	Stat	T (°C)	Salinity	Suspended solids (mg/L)	NH_4^+ (μmol/l)	NO_2^- (μmol/l)	NO_3^- (μmol/l)	PO_4^{3-} (μmol/l)	$Si(OH)_4$) (μmol/l)	Chlorophyll a (mg/m^3)	Wind speed (m/s)	Solar irradiance (W/m^2)
Oued Lihoud	Spring	Mean	21.22	35.12	47.76	4.94	0.10	1.72	2.12	4.28	0.04	0.81	3840
		SD	±0.14	±0.06	±22.59	±1.88	±0.07	±0.47	±1.54	±0.59	±0.03	±0.52	±234
		Min	21.00	75.20	20.00	2.52	0.02	0.92	0.46	3.68	0	0.1	3506
		Max	21.50	35.90	78.00	8.07	0.19	2.39	5.88	5.76	0.08	2.26	4175
	Summer	Mean	20.61	35.60	43.50	2.01	0.11	1.73	3.64	5.67	0.04	1.03	3446
		SD	±1.09	±0.16	±17.44	±0.77	±0.08	±0.20	±3.25	±2.33	±0.04	±0.52	±502
		Min	18.50	35.3	20	0.78	0.02	1.16	0.27	2.35	0	0.21	2110
		Max	23.30	36	100	3.85	0.36	1.72	9.03	9.35	0.19	2.28	4132
		Min	18.8	35.4	30.2	0.72	0.04	1.75	063	2.14	0	0.06	1358
		Max	20.4	36.6	68.4	3.75	0.23	2.77	17.52	6.22	0.06	1.10	2339
	Winter	Mean	14.9	35.8	67.62	2.06	0.24	3.83	1.78	6.83	0.007	0.62	1360
		SD	±2.25	±0.05	±36.47	±1.16	±0.11	±0.87	±1.9	±2.30	±0.001	±0.45	±571
		Min	11.5	35.7	28.3	0.41	0.06	2.77	0.44	2.47	0	0.22	670
		Max	17.8	35.9	131	3.75	0.44	5.17	5.58	10.12	0.003	1.51	1867
Cap Malabata	Spring	Mean	22.14	31.80	46.92	8.43	0.08	1.69	1.11	5.42	0.01	0.81	3840
		SD	±0.13	±0.04	±21.50	±2.62	±0.02	±0.02	±1.18	±0.62	±0.01	±0.52	±234
		Min	22.00	35.70	16.00	1.87	0.04	1.66	0.18	3.88	0	0.1	3506
		Max	22.40	35.90	81.50	11.23	0.10	1.72	4.06	5.93	0.03	2.26	4175
	Summer	Mean	22.20	35.76	42.14	1.5	0.09	1.69	3.91	4.81	0.03	1.03	3446
		SD	±0.96	±0.11	±22.64	±0.8	±0.02	±0.49	±3.60	±1.82	±0.02	±0.52	±502
		Min	20.20	35.40	10.33	0.1	0.02	0.16	0.13	2.54	0	0.21	2110
		Max	23.70	35.90	110	4.2	0.34	3.64	13.35	10.71	0.11	2.28	4132
	Autumn	Mean	20.99	35.83	36.79	1.77	0.22	1.78	6.13	5.74	0.03	0.49	1847
		SD	±0.64	±0.23	±14.1	±0.33	±0.25	±0.41	±4.74	±1.11	±0.01	±0.32	±328
		Min	19.9	35.5	18.8	1.03	0.01	0.16	0.29	3.18	0	0.06	1358
		Max	22.4	36.4	62.8	2.33	1.09	2.68	12.13	7.05	0.11	2.28	2339
	Winter	Mean	16.63	35.85	85.49	1.98	0.08	2.27	0.5	6.57	0.007	0.62	1360
		SD	±1.08	±0.05	±30.76	±0.55	±0.02	±0.43	±0.1	±1.36	±0.001	±0.45	±571
		Min	15.1	35.8	55.2	1.04	0.06	1.77	0.34	5.06	0	0.22	670
		Max	18.1	35.9	147	2.55	0.1	3.07	0.67	9.29	0.03	1.51	1867

Table 1 (continued)

|		T (°C)	Salinity	Suspended solids (mg/L)	NH$_4^+$ (µmol/l)	NO$_2^-$ (µmol/l)	NO$_3^-$ (µmol/l)	PO$_4^{3-}$ (µmol/l)	Si(OH)$_4$ (µmol/l)	Chlorophyll a (mg/m³)	Wind speed (m/s)	Solar irradiance (W/m²)
Dalia	Spring											
	Mean	17.82	35.76	51.00	2.51	0.06	2.32	0.44	6.46	0.01	0.83	3858
	SD	±0.62	±0.14	±10.80	±0.81	±0.01	±0.43	±0.31	±1.56	±0.01	±0.42	±193
	Min	11.20	35.60	24.00	1.68	0.04	1.62	0.18	3.36	0	0.36	3514
	Max	1.00	36.00	62.00	3.75	0.08	3.11	1.22	8.58	0.03	2.05	4104
	Summer											
	Mean	18.41	35.59	44.15	1.97	0.09	2.10	4.37	6.56	0.04	1.10	3400
	SD	±0.76	±0.18	±12.44	±0.93	±0.07	±0.61	±4.10	±2.62	±0.03	±0.5	±525
	Min	16.02	35.30	36.77	0.67	0.01	1.26	0.24	3.18	0	0.32	2054
	Max	19.90	36.00	67.60	5.62	0.6	4.47	19.56	14.92	0.11	2.36	4119
	Autumn											
	Mean	18.57	35.76	34.61	2.18	0.1	2.41	6.89	7.22	0.005	0.55	1834
	SD	±0.49	±0.28	±14.58	±0.78	±0.1	±0.61	±3.62	±1.47	±0.02	±0.4	±322
	Min	17.3	35.5	14	1.09	0.03	1.63	0.68	4.18	0	0.15	1363
	Max	19.5	36.6	60.4	3.75	0.45	3.06	14.53	9.09	0.02	1.56	2302
	Winter											
	Mean	16.95	36	45.83	3.29	0.06	2.66	2.58	5.87	0.04	0.81	1376
	(SD)	±0.2	±0.04	±6.89	±0.98	±0.03	±0.64	±2.52	±2.30	±0.03	±0.55	±549
	Min	16.7	35.9	37	1.45	0.02	1.86	0.2	3.53	0	0.34	722
	Max	17.3	36	59.6	4.53	0.1	3.53	7.62	10.47	0.08	1.92	1849
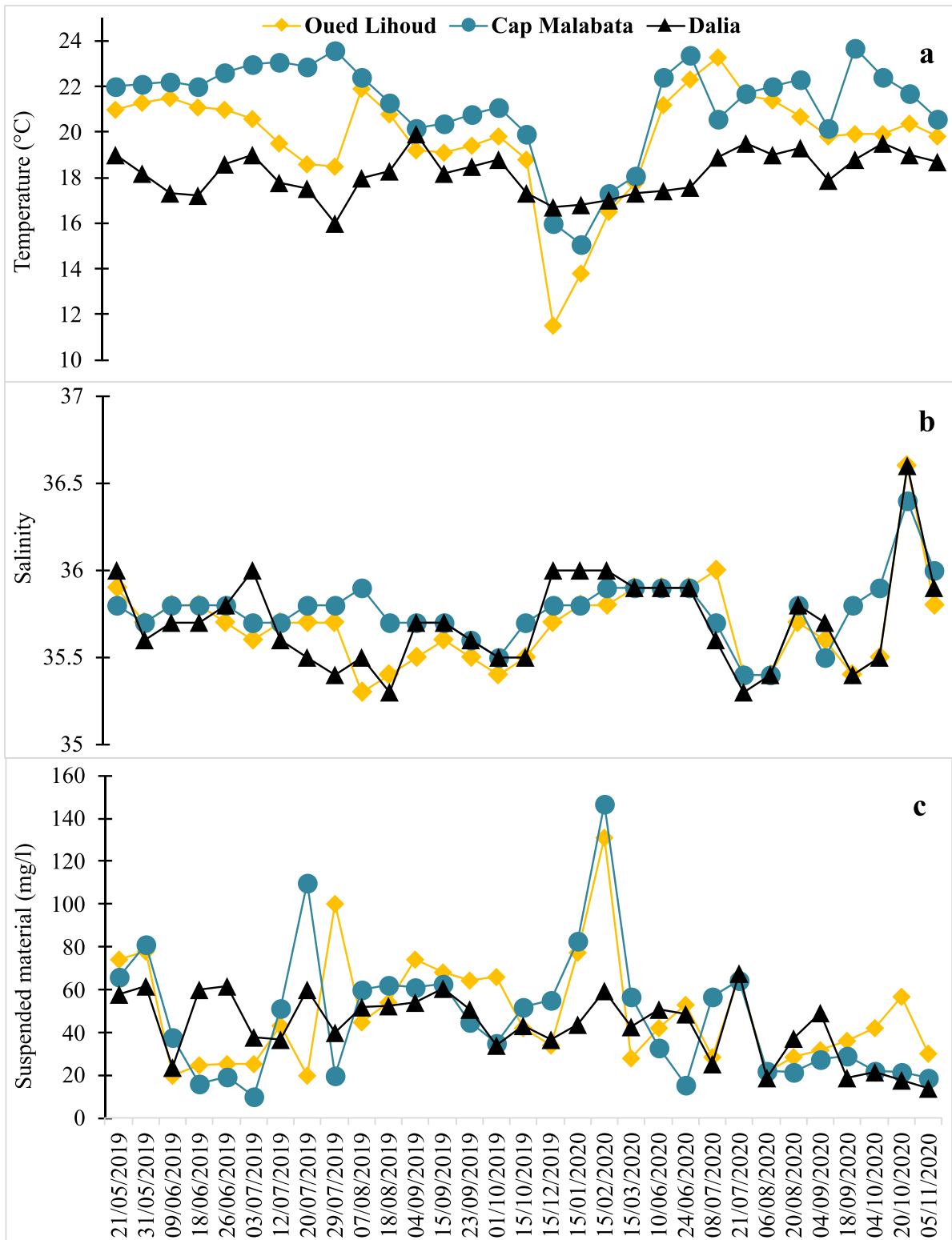
Fig. 2 Temporal variation of temperature (**a**), salinity (**b**) and suspended material (**c**) in Oued Lihoud, Cap Malabata and Dalia, Gibraltar Strait

with 3.83 ± 0.87, 2.27 ± 0.43 and 2.66 ± 0.03 μmol/L at Oued Lihoud, Cap Malabata and Dalia, respectively. The minimum average nitrate concentrations were 1.72 ± 0.47 in spring–summer, 1.69 ± 0.20 in spring–summer and 2.10 ± 0.61 μmol/L in summer at Oued Lihoud, Cap Malabata and Dalia, respectively (Fig. 3a). Nitrite concentrations were low at all three sites throughout the study period, ranging from 0.01 to 1.09 μmol/L (Fig. 3b). At the beginning of this study, ammonium was elevated in seawater at the three sampling sites; thereafter, the concentrations became stable and varied between 0.41 and 3.85 μmol/L for Oued Lihoud, between 0.1 and 4.21 μmol/L for Cap Malabata and then between 0.67 and 5.62 μmol/L for Dalia from the end of June 2019 to November 2020 (Fig. 3c). From May 2019, phosphate concentrations were very low at Oued Lihoud and Cap Malabata to low at Dalia. After mid-September 2019, the concentrations started to increase gradually to reach 17.52, 13.35 and 19.56 μmol/L at Oued Lihoud, Cap Malabata and Dalia, respectively (Fig. 3d). The silicate concentrations in seawater fluctuated at all sites, while the maximum seasonal average concentrations were recorded in winter for Oued Lihoud (6.83 ± 2.3 μmol/L) and Cap Malabata (6.57 ± 1.36 μmol/L) and in autumn for Dalia (7.22 ± 1.47 μmol/L). The minimum seasonal mean values of silicate were 4.26 ± 1.14 μmol/L in autumn, 4.81 ± 1.82 μmol/L in summer and 5.87 ± 2.3 μmol/L in winter at Oued Lihoud, Cap Malabata and Dalia, respectively (Fig. 3e).

The chlorophyll *a* concentrations ranged from 0 to 0.2 mg/m³ at Oued Lihoud and from 0 to 0.11 mg/m³ at Cap Malabata and Dalia (Fig. 4). The maximum seasonal average concentrations were observed in summer for Oued Lihoud and Cap Malabata at 0.04 ± 0.03 mg/m³ and 0.03 ± 0.02 mg/m³, respectively, and then in winter and summer with 0.04 ± 0.03 mg/m³ at Dalia (Table 1).

Meteorological data

Wind speed and solar radiation were almost identical for Oued Lihoud and Cap Malabata, with a slight difference for Dalia (Fig. 5). The maximum wind speed values were recorded in July 2020 with 2.28 m/s at Oued Lihoud-Cap Malabata and 2.36 m/s at Dalia,

while the minimum value (0.06 m/s) was observed in early October 2019 at Oued Lihoud-Cap Malabata and in mid-October for Dalia at 0.15 m/s (Fig. 5a, Table 1). When examining the seasonal averages, the wind speed was higher in summer (1.03 and 1.10 m/s at Oued Lihoud-Cap Malabata and Dalia, respectively) than in autumn (0.49 and 0.55 m/s) and winter (0.62 m/s at Oued Lihoud-Cap Malabata and 0.81 m/s at Dalia). The results also showed that the wind dynamics were stable in summer 2019 compared to summer 2020. Solar irradiance varied seasonally, with the highest values in summer. The maximum average values per day were 3558 W/m² at Oued Lihoud and Cap Malabata in May 2019 and 3531 W/m² at Dalia in July 2020. The minimum average values were recorded in winter (December 2019), with values of 670 W/m² at Oued Lihoud and Cap Malabata and 722 W/m² at Dalia (Fig. 5b).

BHAB dinoflagellate species diversity and distribution in the Strait of Gibraltar

The identification of *Ostreopsis* was limited to the genus. *Coolia monotis* and *Prorocentrum lima* were identified at the species level. During the period between 21 May 2019 and 05 November 2020 and at all study stations (Oued Lihoud, Cap Malabata and Dalia), the main benthic epiphytic dinoflagellates observed in the water column and on the thalli of the five macrophytes were *Ostreopsis* spp., *C. monotis* and *P. lima*. The highest densities of BHAB species were observed during the summer and early fall. In addition, other species of dinoflagellates, such as *Amphidinium* spp. and *Prorocentrum micans*, were observed during this study, with low abundances not exceeding 10 cells/g FW.

BHAB species abundances in the water column

The maximum densities detected in the water column for *Ostreopsis* spp. were 3.5×10^3 cells/L in September 2019 at Oued Lihoud, 9.78×10^3 cells/L in July 2020 at Cap Malabata and 3.61×10^3 cells/L in September 2019 at Dalia (Fig. 6). For *C. monotis,* the maximum values observed were 751 cells/L
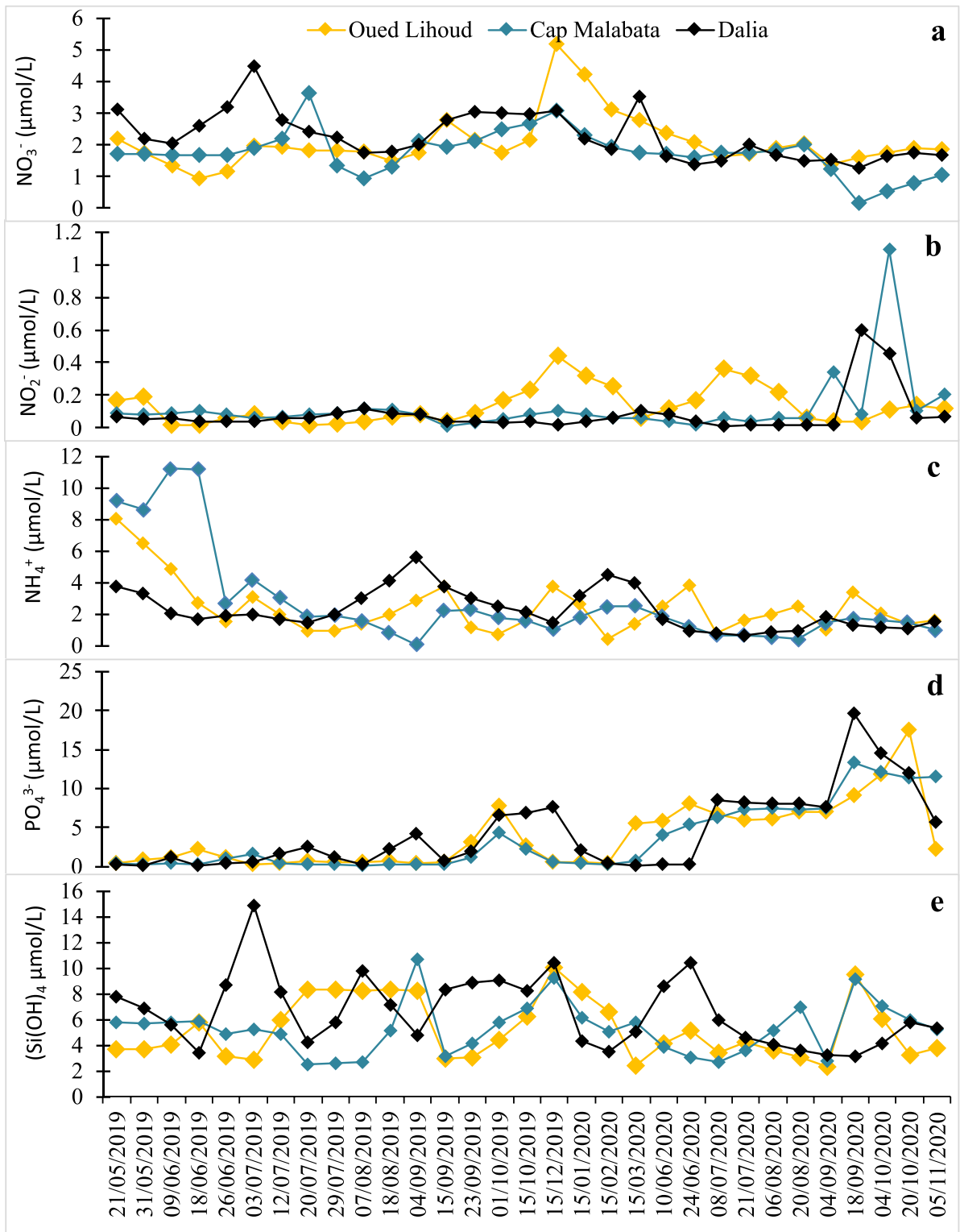
Fig. 3 Temporal variation of nitrates (NO_3^-), nitrites (NO_2^-), ammonium (NH_4^+), phosphorus (PO_4^{3-}) and silicate ($Si(OH)_4$) concentrations expressed in µmol/L at Oued Lihoud, Cap Malabata and Dalia
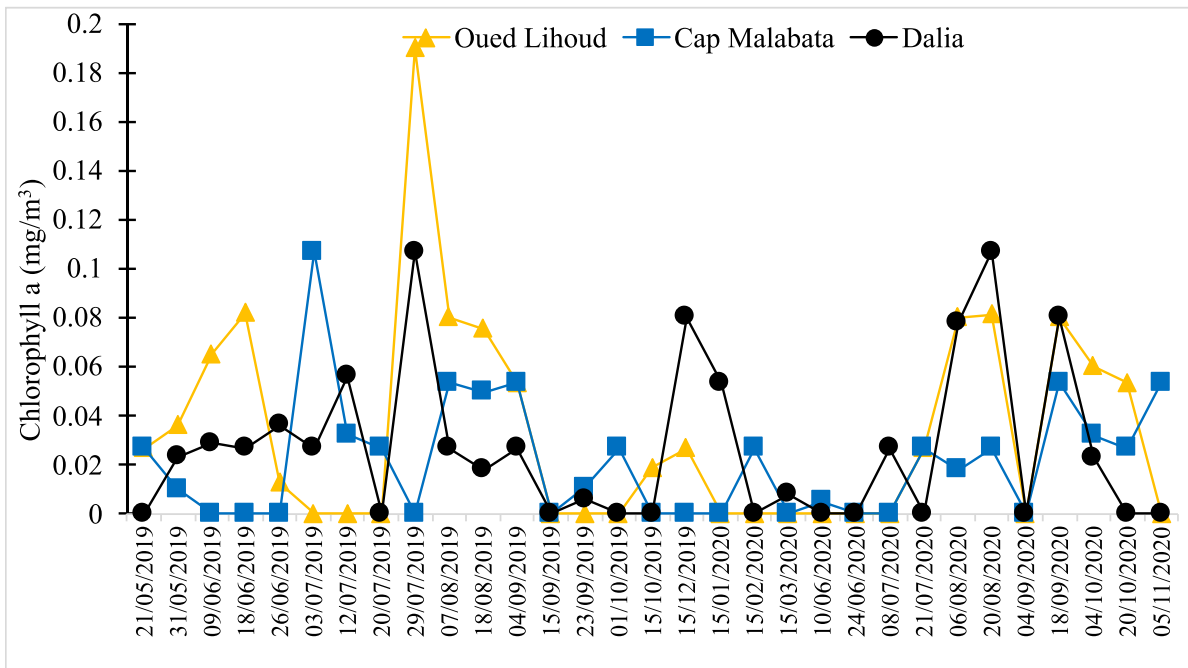
Fig. 4 Chlorophyll a (mg/m^3) concentrations in the water column at Oued Lihoud, Cap Malabata and Dalia

at Oued Lihoud in September 2019, 632 cells/L at Cap Malabata in June 2020 and 736 cells/L at Dalia in June 2019. The maximum cell abundances for *P. lima* a were 476 and 475 cells/L at Oued Lihoud and Cap Malabata in September and June 2020, respectively. For Dalia, *P. lima* was recorded only once on 18 September 2020, at a density of 245 cells/L (Fig. 6). *P. lima* and *C. monotis* were less abundant in the water column than *Ostreopsis* spp. at all of the sampled stations (Fig. 7).

BHAB species presence on the macrophytes

The dinoflagellate species were observed on macrophyte thalli throughout the year at all of the sampling stations, but at different densities (Figs. 7–10), and were abundant during the summer and early fall (Figs. 8–10). In general, dinoflagellate cell abundances in 2020 exceeded those observed in 2019, except that *P. lima* at Dalia and *Ostreopsis* spp., *C. monotis* and *P. lima* on *A. armata* and *C. monotis* at Cap Malabata, *P. lima* at Dalia on *P. coccineum* and *P. lima* at Cap Malabata on *C. spongiosus* were higher in 2019 than in 2020. We observed that the dinoflagellates appeared first at Oued Lihoud and thereafter at Cap Malabata and Dalia.

Ostreopsis spp.

During this study, *Ostreopsis* spp. was the most abundant dinoflagellate on the macrophytes. On *A. armata* thalli, the maximum densities were 3.26×10^4 and 3.75×10^4 cells/g FW at Oued Lihoud and Dalia, respectively, in September 2020 and 2.8×10^4 cells/g FW at Cap Malabata in June 2019 (Fig. 8a). On *P. coccineum*, *Ostreopsis* spp. maximum abundances were recorded in September 2020 for the three stations as follows: 1.48×10^4 cells/g FW at Oued Lihoud, 1.11×10^5 cells/g FW at Cap Malabata and 4.99×10^4 cells/g FW at Dalia (Fig. 8b). *Ostreopsis* spp. maximum densities recorded on *D. dichotoma* thalli were in 2020, 5.64×10^4 cells/g FW at Oued Lihoud in September, 7.341×10^4 cells/g FW at Cap Malabata in July and 4.65×10^4 cells/g FW at Dalia in 2020 (Fig. 8c). The highest cell density for *Ostreopsis* spp. recorded during our monitoring (2.72×10^5 cells/g FW) was on *H. scoparia* at Cap Malabata in September 2020. We observed maximum abundances on this macrophyte, 4.11×10^4 and 7.59×10^4 cells/g FW at Oued Lihoud and Dalia, respectively, in August 2020 (Fig. 8d). The maximum abundances of *Ostreopsis* spp. on *C. spongiosus* were 5.61×10^4 cells/g
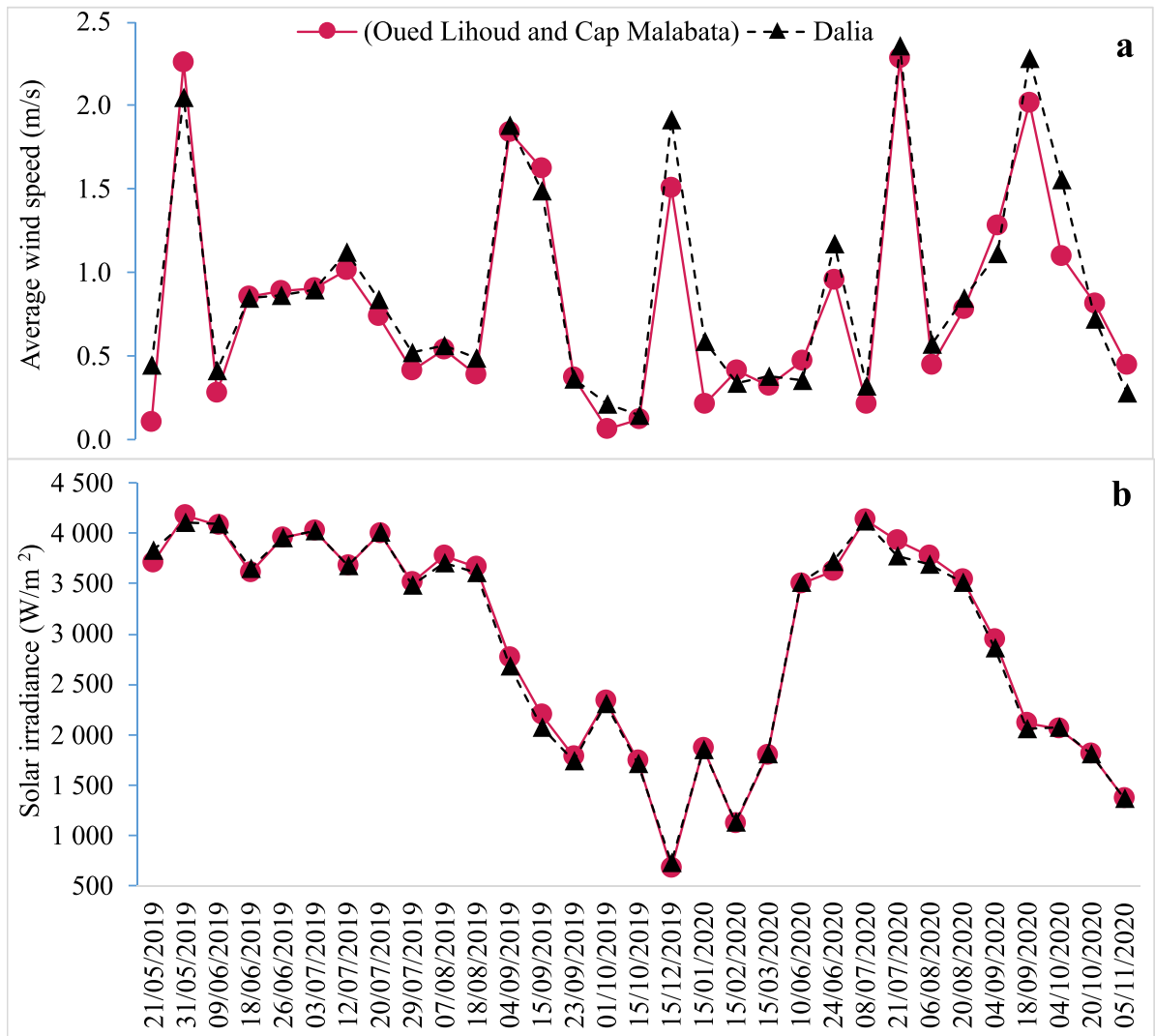
Fig. 5 Wind speed (**a**) and solar irradiance (**b**) at Oued Lihoud, Cap Malabata and Dalia

FW at Oued Lihoud, 1.13×10^5 cells/g FW at Cap Malabata in June 2020 and 8.69×10^4 cells/g FW in August 2020 at Dalia (Fig. 8e).

Coolia monotis

The occurrence periods of *C. monotis* corresponded to those of *Ostreopsis* spp. and *P. lima*. *C. monotis* densities were low on *A. armata* and *D. dichotoma* at Dalia when compared to those at Oued Lihoud and Cap Malabata (Fig. 9). The maximum cell abundances of *C. monotis* on *A. armata* were 6.49×10^3 cells/g FW

at Oued Lihoud in August 2020, 4.07×10^4 cells/g FW at Cap Malabata in June 2019 and 680 cells/g FW in July 2020 at Dalia (Fig. 9a). On *P. coccineum, C. monotis* reached 1.1×10^3 cells/g FW at Oued Lihoud in September 2020, 9.5×10^3 cells/g FW at Cap Malabata in September 2019 and 1.38×10^3 cells/g FW at Dalia in August 2020 (Fig. 9b). On *D. dichotoma*, the maximum cell abundances of *C. monotis* were 5.55×10^3 cells/g FW at Oued Lihoud, 3.94×10^3 cells/g FW at Cap Malabata and 850 cells/g FW at Dalia corresponding to June, July and August 2020, respectively (Fig. 9c). On *H. scoparia*, the maximum abundances of *C. monotis* were 3.93×10^3 cells/g FW
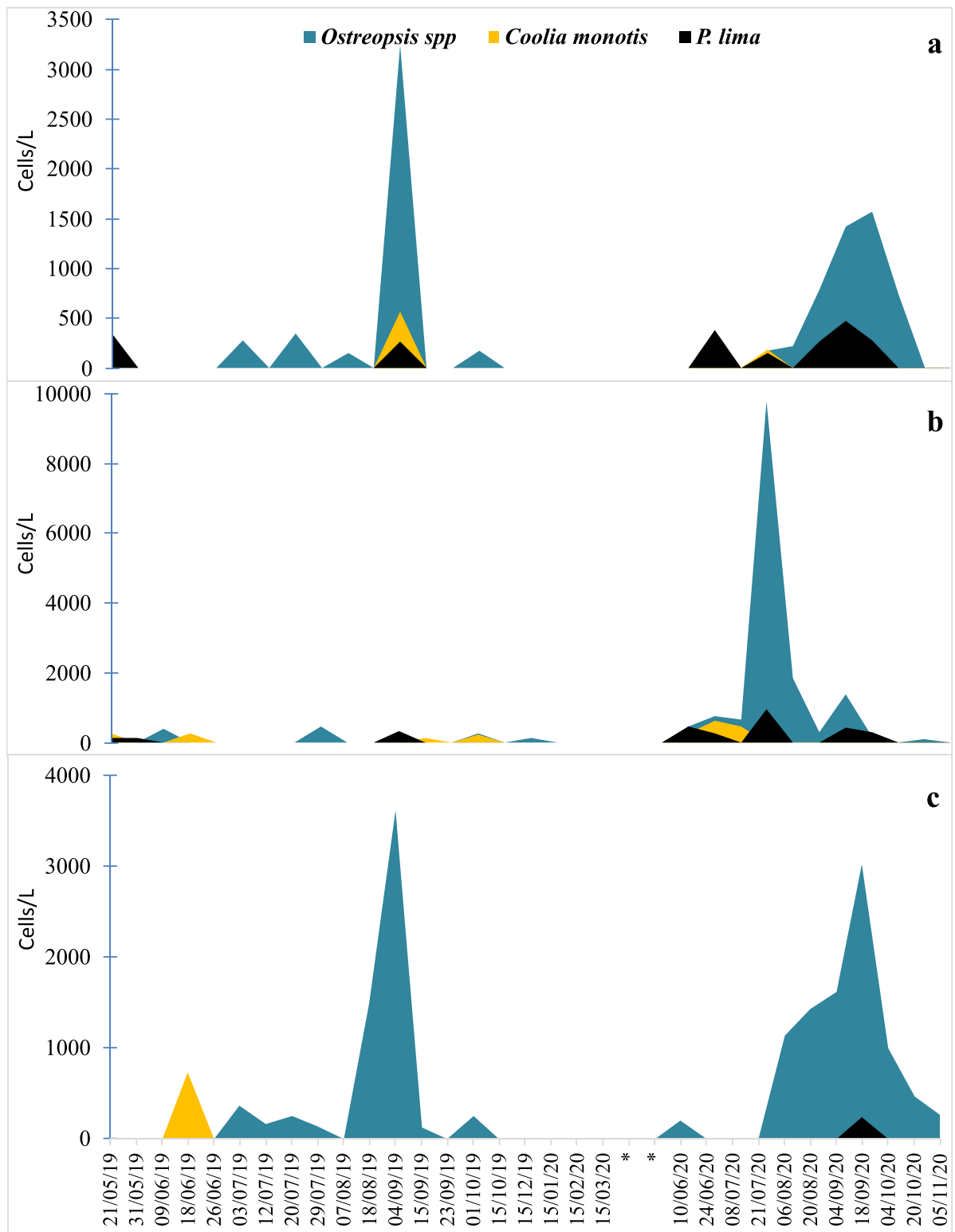
Fig. 6 Temporal variation of cell densities (Cells/L) of *Ostreopsis* spp., *Coolia monotis* and *Prorocentrum lima* in the water column at Oued Lihoud (**a**), Cap Malabata (**b**) and Dalia (**c**). (*) No sampling was made because of COVID-19 sanitary restrictions
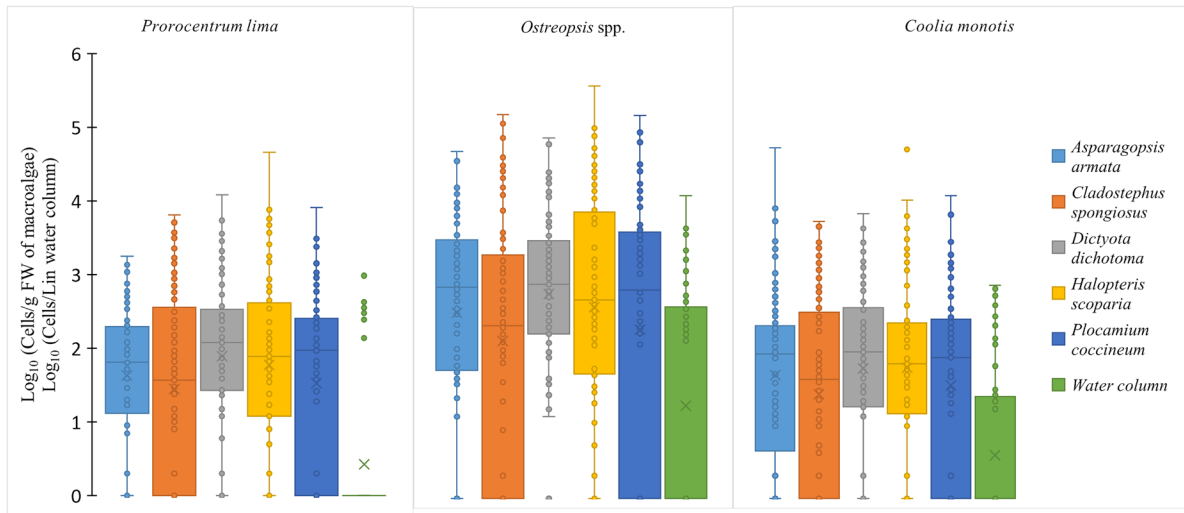
Fig. 7 *Prorocentrum lima, Ostreopsis* spp., and *Coolia monotis* abundances on the macroalgae thalli (Log$_{10}$ Cells/g Fresh Weight of the macroalgae) and in the water column (Log$_{10}$ Cells/L) considering the pooled data of Oued Lihoud, Cap Malabata and Dalia

at Oued Lihoud and 1.91×10^3 cells/g FW at Dalia in August 2020 and 3.86×10^4 cells/g FW at Cap Malabata in September 2020 (Fig. 9d). On *C. spongiosus*, the maximum abundances of *C. monotis* were all recorded in summer 2020, with 4.17×10^3 cells/g FW at Dalia in June, 2.5×10^3 cells/g FW at Cap Malabata in August and 4.38×10^3 cells/g FW at Oued Lihoud in September (Fig. 9e).

Prorocentrum lima

P. lima was more present on macrophytes at Oued Lihoud and Cap Malabata than at Dalia (Fig. 10). The highest cell densities of *P. lima* on *A. armata* were observed at Oued Lihoud and Cap Malabata in August 2020 and Dalia in October 2019, with 1.79×10^3, 596 and 476 cells/g FW, respectively (Fig. 10a). On the macrophyte *P. coccineum*, we recorded *P. lima* densities of 2.45×10^3 cells/g FW at Oued Lihoud in October 2019, 8.20×10^3 cells/g FW at Cap Malabata in September 2020 and 1.59×10^3 cells/g FW at Dalia in August 2020 (Fig. 10b). The maximum cell abundances on *D. dichotoma* were observed in June 2020 at Oued Lihoud, in September 2020 at Cap Malabata and at Dalia in August 2020 with 3.6×10^3, 1.22×10^4 and 734 cells/g FW, respectively (Fig. 10c). For

H. scoparia, the maximum cell abundances of *P. lima* were 4.71×10^3 cells/g FW at Oued Lihoud in August 2020, 4.57×10^4 cells/g FW at Cap Malabata in September 2020 and 1.94×10^3 cells/g FW at Dalia in August 2020 (Fig. 10d). On *C. spongiosus*, the maximum abundances of *P. lima* were 6.47×10^3 cells/g FW at Oued Lihoud in September 2020, 3.15×10^3 cells/g FW at Cap Malabata in September 2019 and 2.74×10^3 cells/g FW at Dalia in August 2020 (Fig. 10e).

Correlations between environmental parameters and epibenthic dinoflagellate densities

By using all the data, Spearman analysis (Table 2) showed significant positive correlations ($p < 0.05$) of *Ostreopsis* spp., *C. monotis* and *P. lima* with temperature, except for the case of *Ostreopsis* spp. on *A. armata* and *C. spongiosus*. Interestingly, the wind speed was positively correlated ($p < 0.05$) with *Ostreopsis* and *P. lima* densities in the water column. We note positive correlations for solar irradiance only with *C. monotis* on *A. armata*, *D. dichotoma* and *P. coccineum*. Phosphate was positively correlated with the three dinoflagellates living on almost all of the macrophytes. Significant negative correlations ($p < 0.05$) were observed between the BHAB species and salinity, ammonium, nitrite, nitrate, DIN, nitrogen/phosphate ratio and suspended material. In addition,
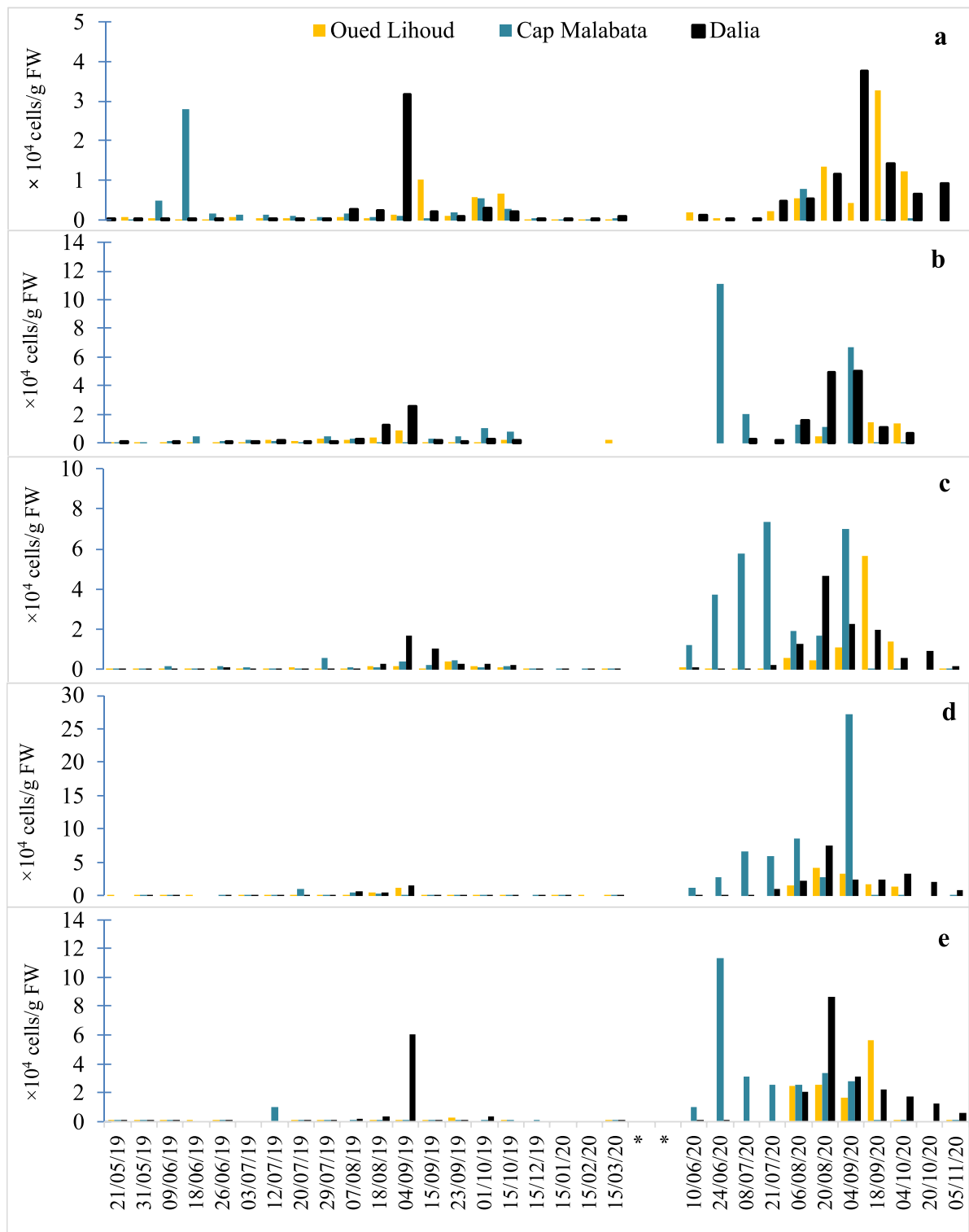
Fig. 8 *Ostreopsis* spp. densities (Cells/g fresh weight of the macroalgae) in Oued Lihoud, Malabata and Dalia in association with the five found macrophytes: *Asparagopsis armata* (**a**), *Plocamium coccineum* (**b**), *Dictyota dichotoma* (**c**), *Halopteris scoparia* (**d**) and *Cladostephus spongiosus* (**e**). (*) No sampling was made because of COVID-19 sanitary restrictions
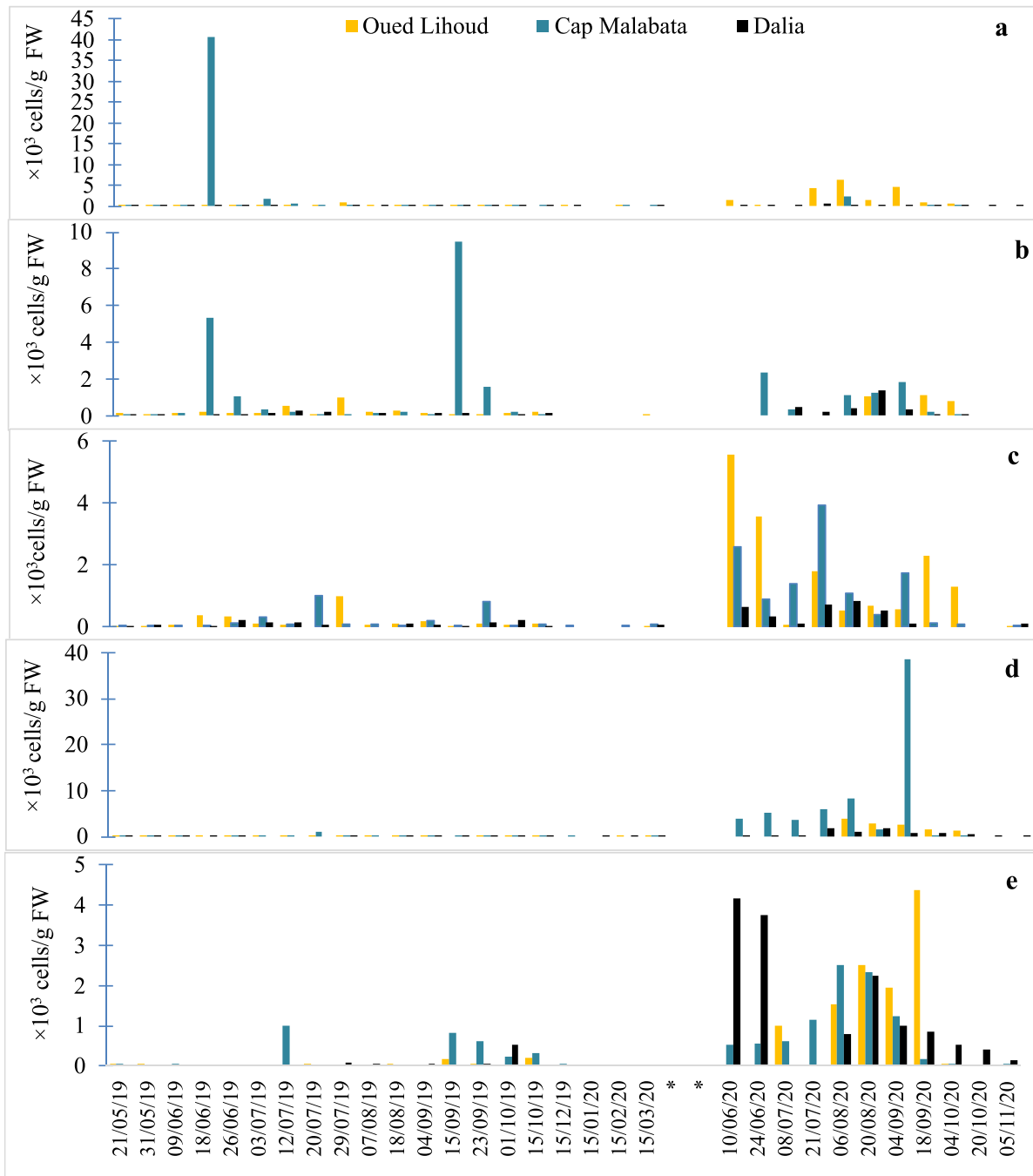
Fig. 9 *Coolia monotis* densities (Cells/g fresh weight of the macroalgae) at Oued Lihoud, Malabata and Dalia in association with the five found macrophytes, *Asparagopsis armata* (**a**), *Plocamium coccineum* (**b**), *Dictyota dichotoma* (**c**), *Halopteris scoparia* (**d**) and *Cladostephus spongiosus* (**e**). (*) No sampling was made because of COVID-19 sanitary restrictions

a significant negative correlation was found between *C. monotis* on *H. scoparia* and silicates (Table 2). PCA confirmed these results (Fig. 11). The percentages of

explained variance of the first 3 components (PCA1, PCA2 and PCA3) were 30.88, 10.44 and 7.02%, respectively. The first component (PCA1) showed that salinity,
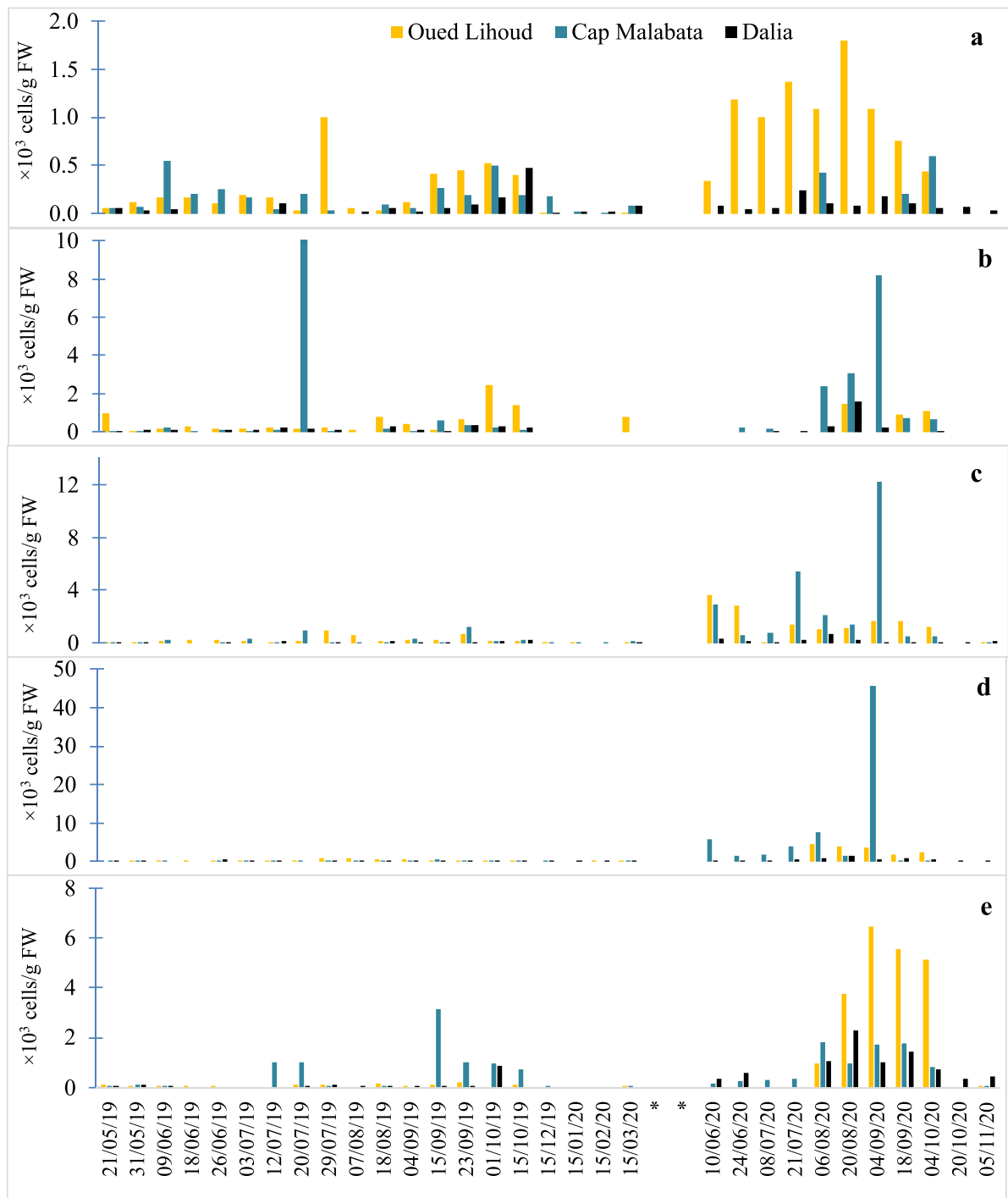
Fig. 10 *Prorocentrum lima* densities (Cells/g fresh weight of the macroalgae) at Oued Lihoud, Malabata and Dalia in association with the five found macrophytes, *Asparagopsis armata* (**a**), *Plocamium coccineum* (**b**), *Dictyota dichotoma* (**c**), *Halopteris scoparia* (**d**) and *Cladostephus spongiosus* (**e**). (*) No sampling was made because of COVID-19 sanitary restrictions

Table 2 Spearman correlations liking BHABs abundance (*Ostreopsis* spp, *Coolia monotis* and *Prorocentrum lima*) related to macrophytes and environmental factors using the entire data from Dalia, Oued Lihoud and Cap Malabata. Values in bold refer to significant correlations with p <0.05

		NH_4^+	NO_2^-	NO_3^-	DIN	PO_4^{3-}	N/P	$Si(OH)_4$	T°C	PSU	Suspended Material	Chl a	Wind	Solar irradiance
Ostreopsis spp	*A. armata*	0.122	-0.008	-0.085	0.073	0.160	-0.120	-0.017	0.142	**-0.485**	-0.119	0.047	0.133	-0.041
	C. spongiosus	-0.195	-0.165	**-0.309**	**-0.290**	**0.342**	**-0.383**	-0.192	0.221	**-0.282**	**-0.280**	0.042	0.134	-0.035
	D. dichotoma	**-0.211**	**-0.207**	**-0.238**	**-0.291**	**0.397**	**-0.427**	-0.111	**0.270**	**-0.470**	**-0.270**	0.018	**0.205**	-0.016
	H. scoparia	**-0.279**	**-0.257**	**-0.289**	**-0.352**	**0.382**	**-0.432**	-0.126	**0.261**	**-0.441**	**-0.253**	0.118	0.129	0.066
	P. Coccineum	-0.052	**-0.227**	**-0.223**	-0.122	**0.211**	**-0.212**	-0.081	**0.293**	**-0.476**	**-0.245**	0.179	0.064	0.161
	Water column	**-0.255**	-0.102	**-0.248**	**-0.288**	**0.369**	**-0.397**	-0.123	0.179	**-0.346**	**-0.289**	0.066	**0.257**	0.079
Prorocentrum lima	*A. armata*	**0.221**	0.011	-0.072	0.138	0.165	-0.098	-0.042	**0.304**	**-0.353**	-0.073	-0.036	0.078	0.038
	C. spongiosus	-0.179	-0.166	**-0.281**	**-0.290**	**0.354**	**-0.381**	-0.119	**0.231**	**-0.291**	**-0.248**	0.052	0.094	-0.063
	D. dichotoma	**-0.211**	-0.116	**-0.272**	**-0.333**	**0.361**	**-0.405**	-0.115	**0.451**	**-0.336**	**-0.210**	-0.020	0.092	0.109
	H. scoparia	**-0.254**	**-0.293**	**-0.290**	**-0.332**	**0.319**	**-0.370**	-0.111	**0.326**	**-0.451**	-0.199	0.085	0.134	0.074
	P. Coccineum	-0.056	**-0.251**	-0.055	-0.092	0.183	-0.178	0.012	**0.256**	**-0.431**	-0.082	0.125	-0.026	0.133
	Water column	0.011	0.081	-0.252	-0.079	0.178	-0.152	-0.124	**0.369**	-0.099	0.053	0.091	**0.332**	0.060
Coolia spp	*A. armata*	0.167	-0.120	-0.146	0.090	0.091	-0.051	0.005	**0.307**	**-0.361**	-0.140	0.186	0.098	**0.265**
	C. spongiosus	-0.184	-0.080	**-0.277**	**-0.264**	**0.294**	**-0.325**	-0.183	**0.238**	**-0.202**	**-0.217**	-0.001	0.009	-0.025
	D. dichotoma	-0.179	**-0.238**	**-0.247**	**-0.292**	**0.283**	**-0.341**	-0.105	**0.422**	**-0.248**	-0.180	0.022	0.144	**0.250**
	H. scoparia	**-0.273**	**-0.328**	**-0.340**	**-0.387**	**0.288**	**-0.352**	**-0.220**	**0.328**	**-0.361**	**-0.221**	0.128	0.083	0.162
	P. Coccineum	-0.051	**-0.302**	-0.193	-0.129	0.095	-0.104	-0.078	**0.407**	**-0.371**	**-0.200**	0.149	0.142	**0.295**
	Water column	0.024	-0.107	-0.079	0.041	0.151	-0.124	-0.086	-0.050	-0.090	-0.078	-0.061	0.118	0.041

suspended material, silicate, DIN, ammonium, nitrate and nitrite were opposite to BHABs abundance. This later was positively related to phosphorus, temperature, solar irradiance, wind and Chl *a*. The second axis (PCA2) showed that *P. lima*, *Ostreopsis* spp. and *Coolia monotis* presence on *A. armata*, *P. coccineum* and *D. dichotoma* was opposite to the abundances of these dinoflagellates in the water column and on the macroalgae *C. spongiosus*. The third axis (PCA3) showed that the abundance of *Ostreopsis* spp. in the water column and on the macroalgae *A. armata* and *D. dichotoma*, was positively related to temperature, solar irradiance and wind. The same tendency was observed for *Coolia monotis* living on the macroalgae species *D. dichotoma* and *A. armata*.

The relationship between BHABs species abundances and environmental factors was examined using FDA considering the three sampling stations separately (Fig. 12). The first two components of FDA showed 100% of the variance (75.30 and 24.70%). The sampled stations (Cap Malabata, Oued Lihoud and Dalia) were clearly different considering both environmental factors

and microalgae abundances (Fig. 12). FDA sample ordination plot showed that ammonia, salinity, N/P ratio and temperature were correlated positively to BHABs species abundances in Cap Malabata (Fig. 12).

Toxins analyses of the natural epibenthic microphytoplankton

Pinnatoxins (PnTX A, G), portimines A and B, ovatoxins, palytoxins and the ciguatoxins/maitotoxins group were analysed in all of the epibenthic phytoplankton assemblages attached to *H. scoparia* during the surveys in 2019 and 2020. None of these toxins were found (Table 3). In contrast, we note the presence of okadaic acid (OA) and its analogue dinophysistoxin 1 (DTX1), certainly produced by *P. lima* present on the sampled macrophytes at the three stations (Table 3). At Oued Lihoud, the highest concentration of OA (102.6 pg/cell) was registered in September 2019. The lowest concentration of OA (0.7 pg/cell) was measured in August 2020 at Oued Lihoud. At Cap Malabata, high
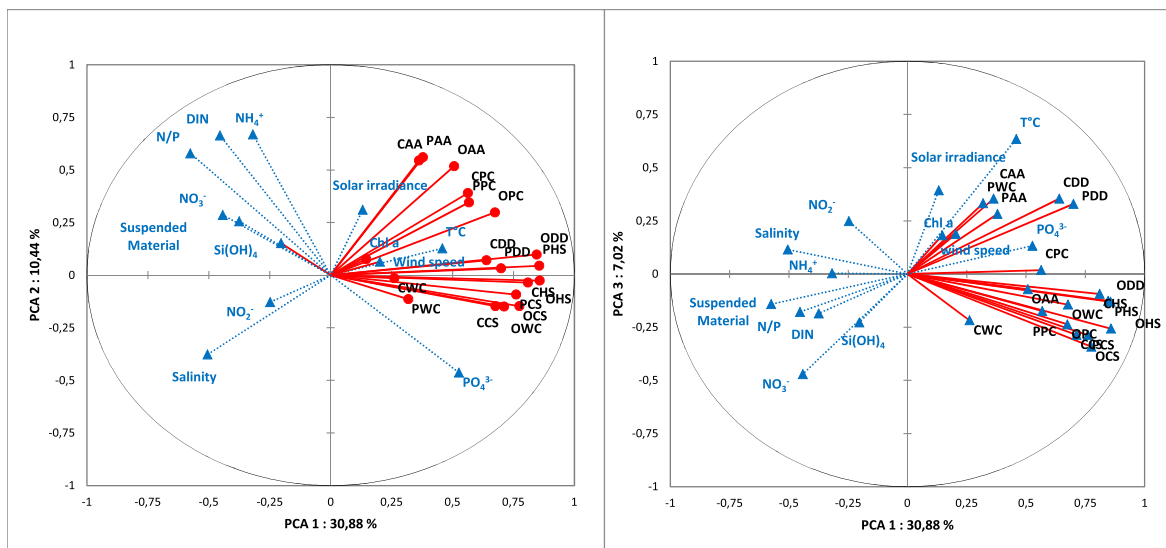

Fig. 11 Principal component analyses applied to all data from Oued Lihoud, Cap Malabata and Dalia and ranked by biotic substrate*s* OAA*: Ostreopsis* spp. on *Asparagopsis armata*, OPC*: Ostreopsis* spp. on *Plocamium coccineum*, ODD*: Ostreopsis* spp. on *Dictyota dichotoma*, OHS*: Ostreopsis* spp. on *Halopteris scoparia*, OCS*: Ostreopsis* spp. on *Cladostephus spongiosus*, OWC*: Ostreopsis* spp. in water column, CAA *: Coolia monotis* on *Asparagopsis armata*, CPC*: C. monotis* on *Plocamium coccineum*, CDD*: C. monotis* on *Dictyota dichotoma*, CHS*: C. monotis* on *Halopteris scoparia*, CCS*: C. monotis* on *Cladostephus spongiosus*, CWC*: C. monotis*

in water column, PAA*: Prorocentrum lima* on *Asparagopsis armata*, PPC*: P. lima* on *Plocamium coccineum*, PDD*: P. lima* on *Dictyota dichotoma*, PHS*: P. lima* on *Halopteris scoparia*, PCS*: P. lima* on *Cladostephus spongiosus*, PWC*: P. lima* in water column. The considered environmental variable are salinity, temperature (T°C), suspended material (mg/L), wind speed (m/s), solar irradiance (W/m²), Chl a (mg/m³), nitrates (NO_3^-) nitrites (NO_2^-), ammonium (NH_4^+), phosphorus (PO_4^{3-}), silicate ($Si(OH)_4$), dissolved inorganic nitrogen (DIN) in μmol/L, nitrogen/phosphate ratio (N/P)
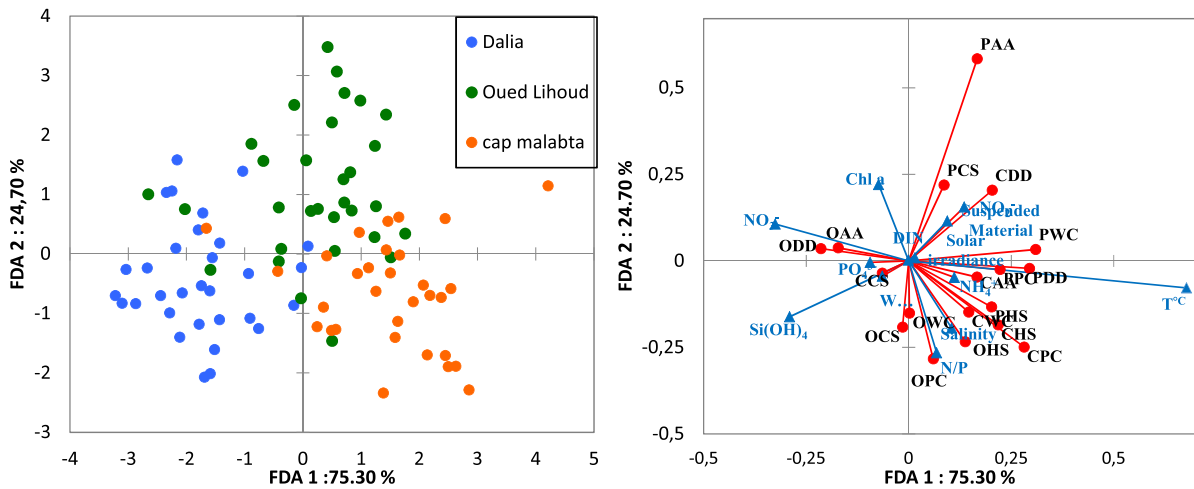
Fig. 12 Factorial discriminant analyses ranked by the stations (Oued Lihoud, Cap Malabata and Dalia) OAA: *Ostreopsis* spp. on *Asparagopsis armata*, OPC: *Ostreopsis* spp. on *Plocamium coccineum*, ODD: *Ostreopsis* spp. on *Dictyota dichotoma*, OHS: *Ostreopsis* spp. on *Halopteris scoparia*, OCS: *Ostreopsis* spp. on *Cladostephus spongiosus*, OWC: *Ostreopsis* spp. in water column, CAA: *Coolia monotis* on *Asparagopsis armata*, CPC: *C. monotis* on *Plocamium coccineum*, CDD: *C. monotis* on *Dictyota dichotoma*, CHS: *C. monotis* on *Halopteris scoparia*, CCS: *C. monotis* on *Cladostephus spongiosus*, CWC: *C.* *monotis* in water column, PAA: *Prorocentrum lima* on *Asparagopsis armata*, PPC: *P. lima* on *Plocamium coccineum*, PDD: *P. lima* on *Dictyota dichotoma*, PHS: *P. lima* on *Halopteris scoparia*, PCS: *P. lima* on *Cladostephus spongiosus*, PWC: *P. lima* in water column. The considered environmental variable are salinity, temperature (T°C), suspended material (mg/L), wind speed (m/s), solar irradiance (W/m^2), Chl a (mg/m^3), nitrates (NO_3^-), nitrites (NO_2^-), ammonium (NH_4^+), phosphorus (PO_4^{3-}), silicate ($Si(OH)_4$), dissolved inorganic nitrogen (DIN) in μmol/L, nitrogen/phosphate ratio (N/P)

concentrations of OA were registered in June 2019, with 192.4 pg/cell, and in June 2020, with 149.22 pg/cell. At Dalia, the OA concentrations were 2.09 pg/cell and 2.82 pg/cell in August and October 2020, respectively. OA concentrations were higher than those of DTX1. The DTX1 concentrations were 93.67 pg/cell and 5.38 pg/cell at Oued Lihoud in September 2019 and September 2020, respectively. Two high concentrations of DTX1 were observed at Cap Malabata in June 2019 (125.02 pg/cell) and in August 2020 (115.85 pg/cell). Lower concentrations were observed at Cap Malabata, with 3.38 pg/cell in July 2020. At Dalia, the DTX1 concentration was 4.38 pg/cell in August 2020 (Table 3).

Discussion

Ostreopsis spp., *C. monotis* and *P. lima in situ* dynamics

In our study, the dinoflagellates *Ostreopsis* spp., *P. lima* and *C. monotis* largely dominated the dinoflagellate assemblages on the macroalgae at the three investigated sites in the Strait of Gibraltar. However, we found *Amphidinium* and the planktonic dinoflagellate *P. micans* either

in the water column or on the substrate but at low densities not exceeding 10 cells/g FW of the macroalgae. *P. lima* has been shown to be responsible for diarrhetic shellfish poisoning (DSP) by synthesizing okadaic acid (OA) and dinophysistoxins (DTXs) (Lee et al., 1989; Zhou & Fritz, 1994). *Ostreopsis* cf. *ovata* and *Ostreopsis* cf. *siamensis* have been observed in the Mediterranean (Ben-Gharbia et al., 2016; Mangialajo et al., 2011; Pavaux et al., 2021; Turki et al., 2006). *O.* cf. *ovata* has been shown to be responsible for human respiratory distress both in the northern and southern Mediterranean Sea (Mangialajo et al., 2011) because of the emission of volatile toxic compounds such as palytoxins and ovatoxins (Amzil et al., 2012; Ciminiello et al., 2006; Vila et al., 2016). There has been some confusion about the taxonomy and toxicity of *C. monotis*, but until now no human health problems have been associated with this species (Rhodes & Thomas, 1997; Pagliara & Caroppo, 2012; Mohammad-Noor et al., 2013; Rhodes et al., 2014; Ben-Gharbia et al., 2016).

Our results corroborated many works carried out in the Mediterranean, where *Ostreopsis* spp., *P. lima* and *C. monotis* have been found in association with almost all of the biotopes (Aligizaki & Nikolaidis, 2006; Ben-Gharbia

Table 3 Okadaic acid (OA) and dinophysistoxins 1 (DTX1) concentrations in sampled natural microphytoplankton present On the macrophyte *Halopteris scoparia* at Oued Lihoud, Cap Malabata and Dalia. (n.m) No sampling for toxins analysis was made, (n.d) Not detected

	Oued Lihoud				Cap Malabata				Dalia			
	Cell density (cell/g)	OA (pg/cell)	DTX1 (pg/cell)	OA/DTX1 Ratio	Cell density (cell/g)	OA (pg/cell)	DTX1 (pg/cell)	OA/DTX1 Ratio	Cell density (cell/g)	OA (pg/cell)	DTX1 (pg/cell)	OA/DTX1 Ratio
18-Jun-19	12	n.m	n.m	-	56	192.40	125.02	1.54	00	n.m	n.m	-
18-Aug-19	0.5×10^3	n.m	n.m	-	00	n.m	n.m	-	1.1×10^3	n.d	n.d	-
04-Sep-19	0.6×10^3	102.60	93.67	1.09	25	n.m	n.m	-	56	n.d	n.d	-
24-Jun-20	00	n.m	n.m	-	48	149.23	115.85	1.29	69	n.m	n.m	-
08-Jul-20	00	n.m	n.m	-	1.8×10^3	4.19	3.26	1.29	118	n.m	n.m	-
21-Jul-20	00	n.m	n.m	-	4×10^3	3.64	3.38	1.08	5.1×10^2	n.m	n.m	-
20-Aug-20	3.9×10^3	0.7	n.d	-	1.5×10^3	83.26	59.55	1.40	2×10^2	2.09	4.4	0.48
04-Sep-20	2.6×10^3	7.42	5.38	1.38	1.7×10^3	2.77	n.d	-	6.3×10^3	0.6	n.d	-
20-Oct-20	00	n.m	n.m	-	00	n.m	n.m	-	3.4×10^2	2.82	n.d	-

et al., 2019; Blanfuné et al., 2015), with *O.* cf. *ovata* being the most abundant (Carnicer et al., 2015). BHAB species have been extensively studied in northern Mediterranean waters (Abadie et al., 2018; Cohu et al., 2013; De Virgilio et al., 2021; Dolapsakis et al., 2006; Mangialajo et al., 2008, 2011; Pagliara & Caroppo, 2012; Pavaux et al., 2021; Penna et al., 2006; Vila et al., 2001). In the southern Mediterranean, studies on BHABs species need to be strengthened (Zingone et al., 2021). *O.* cf. *ovata* and *C. monotis* have been reported in Egyptian waters (Hosny & Labib, 2019; Ismael & Halim, 2012). *Ostreopsis* spp. was reported in Alger Bay (Algeria) and was associated with the hospitalization of over 300 people in 2009 (Illoul et al., 2012). However, most of the studies on the quantification of epibenthic dinoflagellates in the southwestern Mediterranean have been conducted in Tunisia (Ben-Gharbia et al., 2016, 2019; Moncer et al., 2017). In our study, the maximum density of *Ostreopsis* spp. was recorded at Cap Malabata with 2.7×10^5 cells/g FW on *H. scoparia* thalli in September 2020. This cell density exceeded the majority of those recorded in the southern Mediterranean, where the highest cell densities of *O. siamensis* on macrophytes were reported by Turki (2005), with 3.6×10^3 cells/g FW on *Posidonia oceanica* thalli in Marsa Bay (Tunisia) in summer/autumn 2001. For *O.* cf. *ovata*, the maximum cell concentrations were observed by Moncer et al. (2017) at Oued Lafrann, Chabba, Tunisia, in February and September with 1.85×10^3 cells/g FW on *Posidonia oceanica* and with 0.5×10^3 cells/g FW on *Cymodocea nodosa* and by Hosny and Labib (2019) in Abu Qir Bay, Alexandria, Egypt, in July on *Ulva compressa*. The highest abundances of *Ostreopsis* spp., with up to 10^4 cells/g FW, were recorded in August 2014 by Hachani et al. (2018). Our results corroborated other studies that have reported close densities of *O.* cf. *ovata*: 5.1×10^5 cells/g FW in the French Mediterranean in the period July 2007–2009 (Mangialajo et al., 2011), 5.28×10^5 cells/g FW along the Trabocchi coast, Abruzzo, Italy, in September 2013 (Ingarao & Pagliani, 2014), and 3.34×10^5 cells/g FW along coastal areas in Croatia (Pfannkuchen et al., 2012). A density of 4.05×10^5 cells/g FW of *O.* cf. *ovata* and *O.* cf. *siamensis* was found in September 2004 in the North Aegean Sea, Greece (Aligizaki & Nikolaidis, 2006), and 8.8×10^5 and 2.64×10^5 cells/g FW of *O. fattorussoi* were found along the coast of Lebanon (Açaf et al., 2020). Other works have been carried out in the northern Mediterranean showing abundances of *Ostreopsis* (up to 10^6 cells/g FW) higher than those we have observed (Accoroni

et al., 2011, 2015; Brissard et al., 2014; Carnicer et al., 2015; Cohu et al., 2011, 2013; Mangialajo et al., 2008, 2011; Totti et al., 2010). In Mediterranean waters, very often the highest densities of *Ostreopsis* have been observed in summer and autumn (Ben-Gharbia et al., 2019). This is in agreement with our observations which showed that the maximum cell abundance of *Ostreopsis* spp. occurred in the summer and autumnal seasons in the Moroccan Strait of Gibraltar. Along the coast of Portugal (northeast Atlantic), both *O.* cf. *ovata* and *O.* cf. *siamensis* have been found (Santos et al., 2019). Our data suggest the thermophilic character of *Ostreopsis* populations developing in the Strait of Gibraltar.

For *C. monotis,* the densities observed at Cap Malabata were among the highest ones reported in the southern Mediterranean Sea, with 4.07×10^4 cells/g FW on *A. armata* thalli in June. The *C. monotis* densities at our study sites approached those reported by Vila et al. (2001) with 3.38×10^4 cells/g FW in spring in Palmós, Costa Brava and Majorca (Spain) and by Algizaki and Nkolaidis (2006) in August in the North Aegean waters (Greece) on the macrophyte *Padina pavonica* with 1.6×10^4 cells/g FW. In the southern Mediterranean, the densities of *C. monotis* have been found to be low on various macrophytes: the maximum was 8.4×10^2 cells/g FW in autumn 2001 on *C. nodosa* in Marsa Bay in Tunisia (Turki, 2005), with 24.8×10^3 cells/g FW on *P. oceanica* in March 2013 at Oued Lafrann, Chebba, Tunisia (Moncer et al., 2017), 3.1×10^2 cells/g FW on *P. oceanica* in July 2008 and January 2009 at Mahdia, Tunisia (Mabrouk et al., 2011), 4.54×10^2 cells/g FW in March and autumn (2005–2010) along the Alexandria coast, Egypt (Ismael, 2014), 8.65×10^2 cells/g FW in 2014 in the Gulf of Tunis, Tunisia (Hachani et al., 2018), and 1.1×10^3 cells/g FW on *C. nodosa* in May in Bizerte Bay, Tunisia (Ben-Gharbia et al., 2019). *C. monotis* has been shown to be able to survive in a broad range of temperatures (Rhodes et al., 2000), but it proliferates massively in warm waters (from spring to midautumn) (Armi et al., 2010). In our study, *C. monotis* proliferated between June and the end of October, suggesting its preference for warm temperatures.

Maximum *P. lima* cell densities were as high as 4.5×10^4 cells/g FW in August and September 2020 at Cap Malabata on *D. dichotoma* thalli. Our results corroborated those of Turki (2005), who reported densities of *P. lima* of 2.2×10^4 cells/g FW on *C. nodosa* in June and July in the Bay of Marsa, Tunisia.

Blanfuné et al., (2015) showed that this dinoflagellate appeared at densities of 4.57×10^4 cells/g FW on the macrophyte *Junia rubens* in August along the French Mediterranean coast. Giussani et al. (2017) reported values of 7.42×10^4 cells/g FW *H. scoparia* in July in Quarto dei Mille, Genoa, Italy. Hachani et al. (2018) found that *P. lima* showed densities of 7.42×10^4 cells/g FW in August in the Gulf of Tunis, Tunisia. The maximum recorded abundance of *P. lima* was 1.33×10^5 cells/g FW on *C. nodosa* in Greek coastal waters (North Aegean Sea) during summer and autumn (Aligizaki et al., 2009).

Correlations with environmental parameters

Abiotic parameters

We showed a significant positive correlation between *Ostreopsis* spp. abundance and temperature. The data showed that the maximum abundance of *Ostreopsis* spp. was observed in September when the water temperature was 23.7 °C. In laboratory experiments, *Ostreopsis* spp. (Tanimoto et al., 2013) and *O.* cf. *ovata* (Ben-Gharbia et al., 2016) exhibited high growth rates at 25 °C. In addition, it has been shown that the highest cell densities of *O.* cf. *ovata* occurred at 25 °C (Ciminiello et al., 2006; Ingarao & Pagliani, 2014; Meroni et al., 2018), with cell development occurring between 18 and 30 °C. In the same way, we found significant positive correlations between *C. monotis, P. lima* and temperature. The highest abundances of these BHAB species were observed during the summer and autumnal periods at our study sites along the Strait of Gibraltar. It has been suggested that seawater temperature is the most important environmental factor affecting the physiology of phytoplankton (Penna et al., 2006). The three BHAB species could be considered thermophilic and therefore could proliferate in a more intense and regular manner as a result of the observed water warming in the Mediterranean (Armi et al., 2010; Cohu et al., 2011; Rhodes et al., 2000).

We found significant positive correlations between the solar irradiance and the abundances of *C. monotis* on thalli of *A. armata*, *P. coccineum* and *D. dichotoma*. Light intensity is an important factor to consider for the growth and proliferation of dinoflagellates (Morton et al., 1992). Significant positive correlations were observed between wind speed and abundances

of *Ostreopsis* spp. and *P. lima* in the water column. Strong wind contributes to the mixing of water masses and to the detachment of microalgae from macrophytes (Hachani et al., 2018; Telesh, 2004). Significant negative correlations were noted between abundances of the three BHAB species and suspended material. The maximum suspended material was observed in winter, when the rainfall reached the maximum and seawater temperatures were minimal. The difference in suspended material quantity among the study sites can be explained by the location of Oued Lihoud and Cap Malabata near an important urban area (Tangier), bringing pollutants and discharges in winter.

Significant negative correlations were found between abundances of the studied dinoflagellates and salinity (Table 2). Morton et al. (1992) suggested that the optimal salinity for *Ostreopsis* spp. and *C. monotis* is approximately 34, and for *P. lima,* it is almost 31 to 32. The maximum growth of *Ostreopsis* spp. and *O. ovata* has been recorded for salinities between 30 and 35 (Tawong et al., 2015). For nutrients, we found significant positive correlations of abundances of dinoflagellate species (*Ostreopsis* spp.*, P. lima* and *C. monotis*) with phosphate, which is characterized by relatively high concentrations (up to 6.32 μmol/L) in the study zone (Table 2). This corroborated the work of Parsons and Preskitt (2007) on *Ostreopsis* spp. In contrast, our study highlighted significant negative correlations between abundances of the study dinoflagellate species and nitrite, nitrate, ammonia, DIN and the nitrogen/phosphate (N/P) ratio. A single significant negative correlation was observed between *C. monotis* abundance and silicate for the macroalgae *H. scoparia*. Several previous studies have reported negative or nonsignificant correlation between epiphytic microalgal abundances and nutrients (Accoroni et al., 2011; Carnicer et al., 2015; Cohu et al., 2011; Dhib et al., 2013; Parsons & Preskitt, 2007; Sahraoui et al., 2013; Shears & Ross, 2009; Ungaro et al., 2010; Vila et al., 2001). In contrast, other studies have shown positive correlations between abundances of *Ostreopsis* spp., *P. lima* and *C. monotis* with various nutrients (Armi et al., 2010; Hachani et al., 2018; Parsons & Preskitt, 2007). *O.* cf. *ovata* developing in Mediterranean waters was shown to be affected by the availability and concentrations of organic nutrients (Accoroni et al., 2017; Elwood et al., 2020; Jauzein et al., 2017). The effects of nutrients on dinoflagellates remain unclear, and each species or strain could react differently in the colonized ecosystem to nutrient variation.

Biotic substrates and differences in BHABs among the three sites

The analysis of all data at the three sites (Fig. 7) showed that *D. dichotoma* was the most favourable macrophyte host for *Ostreopsis* spp., *P. lima* and *C. monotis*. The data analyses by site and by macrophyte showed that *D. dichotoma* is the most favourable macrophyte for *Ostreopsis* spp. at all three sites. *D. dichotoma* at Oued Lihoud and Cap Malabata and *H. scoparia* at Dalia were the most favourable macrophytes for *P. lima*. For *C. monotis,* the most favourable macrophyte host was *D. dichotoma* at Oued Lihoud and Cap Malabata, followed by *H. scoparia* at Cap Malabata and Dalia. It has been suggested that the specificities of the host macroalgae, such as thallus architecture and allelopathic compounds produced by some macrophytes, could influence the development of phytoplankton, and epibenthic dinoflagellates (Accoroni et al., 2015; Laabir et al., 2013; Parsons & Preskitt, 2007). Allelochemicals produced by many macrophytes could inhibit or stimulate epibionts (Ben-Gharbia et al., 2019; Berdalet et al., 2017; Ianora et al., 2011; Laabir et al., 2013; Morton & Faust, 1997; Pavaux et al., 2021) and in turn structure the epibenthic community. Competition for nutrients between the macrophytes and microalgae must also be considered in such interactions.

Sampling stations

Using FDA and considering the three sampling stations separately, we found that ammonia, salinity, N/P ratio and temperature were correlated positively to BHABs species abundances in Cap Malabata (Fig. 12). This site holds the highest cell densities of *Ostreopsis* spp., *P. lima* and *C. monotis*. Interestingly, in Cap Malabata, ammonia concentration, temperature and salinity values were relatively high when compared to those observed in Oued Lihoud and Dalia (Table 1). Our results corroborated those of Jauzein et al. (2017) who showed a high affinity of *O.* cf. *ovata* cells for ammonia. Benthic microalgae were shown to be thermophilic (Ciminiello et al., 2006; Ingarao & Pagliani, 2014; Meroni et al., 2018).

Table 4 Summary for *Prorocentrum lima* toxicity from various marine ecosystem

Location	Strain ID / Natural	Temperature (°C)	Salinity	Medium culture / Natural	Light intensity with μmol photons/m²/s (light: darkness)	Toxins maximum concentration (pg/cell) OA	DTX1	OA/DTX1 Ratio	Reference
Pacific waters (Japan)	MIO12P	25	32–33	metals mix SWII + GJE1	90–100 (12 h: 12 h)	28.5	23.6	1.21	Hashimoto et al. (2021)
	OMI029P					39.4	7.8	3.76	
	AOF55P					18.5	11.5	1.61	
	P. lima complex subclade 1 a-i	25	33	metals mix SWII medium+GJE10 and IMK/4	100 (12 h: 12 h)	55.27	70.33	0.78	Nishimura et al. (2020)
Pacific waters (Taiwan)	TIO32	25	-	f/2 & L1	90 (12 h: 12 h L: D)	10.26	1.81	5.67	Luo et al. (2017)
Mediterranean waters (Tunisia)	PLBZT14	25	36	ENSW	80 (12 h 12 h L: D)	28.33	7.4	3.83	Ben-Gharbia et al. (2016)
Atlantic waters (Brazil)	UNR-01	24±2	35	L2	60 (12 h 12 h L:D)	45.6	1.12	40.61	Nascimento et al. (2016)
Mediterranean waters (Italy)	Not mentioned	20	25	F/2	90 (16 h: 8 h L:D)	15.8	0.39	40.51	Vanucci et al. (2010)
Atlantic waters (Portugal)	IO66-01	19±1	35	F/2—Si	40 (14 h: 10 h L: D)	41.0	12.0	3.41	Vale et al. (2009)
Atlantic waters (UK)	Naturel samples					1.5	5.4	0.28	Foden et al. (2005)
Atlantic waters (UK)	Not mentioned	15	-	Modified L2	70 (12 h: 12 h L: D)	17.13	11.29	1.52	Nascimento et al. (2005)
Cuba	Not mentioned	22±1	-	K	40 W (12 h: 12 h L:D)	ND	7.15	-	Delgado et al. (2005)
	Natural sample	-			-	ND	4.2	-	
Spain	PL16V	19±1	-	F/2 and K	60–70 (14 h: 10 h L:D)	12.87	4.60	2.8	Bravo et al. (2001)
	PL12V					5.11	12.39	0.04	
Mediterranean waters (France)	MARS1	20	-	F/2	40 (12 h: 12 h L: D)	1.9	0.8	2.38	Barbier et al. (1999)
Pacific waters (Japan)	-	25, 20 and 25	-	T1	170 (14 h: 10 h L: D)	1.3	ND	-	Koike et al. (1998)
Gibraltar Strait waters(Morocco)	Natural sample	19.2	35.5		-	102.6	93.7	1.09	This study
	Natural sample	22	35.8		-	192.4	125.02	1.54	
	Natural sample	19.3	35.8		n.m	2.09	4.38	0.48	
	Natural sample	18.7	36.6		n.m	2.82	NA	-	
Atlantic waters (Spain)	Strain 02	n.m				n.m	7.2	-	Lee et al. (1989)
	Strain 03	n.m	n.m			8.0	11.0	0.73	
Pacific waters (Japan)	Strain 06				n.m	26.0	13.0	2	Lee et al. (1989)

Toxins produced by epibenthic dinoflagellates developing in the Strait of Gibraltar

Analyses were performed for pinnatoxins (PnTX A, G), portimines A and B, ovatoxins, palytoxins and the ciguatoxins/maitotoxins group in the natural epibenthic phytoplankton assemblage attached to the macrophyte *H. scoparia* at different sampling dates, but none of these toxins were found (Table 3). These data suggest that blooms of *Ostreopsis* spp. developing in the Strait of Gibraltar, at least at Oued Lihoud and Cap Malabata, are not toxic. Here, toxins analyses confirmed that *C. monotis* is not toxic and that V*ulcanodinium rugosum* and *Gambierdiscus* sp. were not present among the epibenthic microalgae attached to the macrophytes. In contrast, we detected the presence of okadaic acid (OA) and dinophysistoxin-1 (DTX1) (Table 3). The maximum concentrations were 192.4 and 125.02 pg/cell of OA and DTX1, respectively, on 18 June 2019 at Cap Malabata. Although the cell densities at Oued Lihoud of *P. lima,* the dinoflagellate species that produces these lipophilic toxins, were low in summer 2019, the concentrations of OA and DTX1 were 102.6 and 93.7 pg/cell, respectively. We also recorded other high concentrations in August 2020 at Cap Malabata, with 83 and 59.5 pg/cell OA and DTX1, respectively. In comparison with previous works on laboratory cultures of *P. lima* strains isolated from various marine ecosystems (Table 4), it seems that the obtained maximum concentrations in this study were among the highest ever measured.

Conclusions

This is the first study highlighting the presence of world widely distributed BHAB species (*Ostreopsis* spp*., P. lima* and *C. monotis*) on five macrophytes at three distinct sites in the Strait of Gibraltar, southwestern Mediterranean. The densities of these dinoflagellates were not different from those registered in other ecosystems located in the northern and southern Mediterranean (Cohu et al., 2011, 2013; Mangialajo et al., 2011; Pavaux et al., 2021). OA and DTX toxins were found in the cells of natural BHAB dinoflagellate assemblage, suggesting that the *P. lima* strains developing in the investigated ecosystems are toxic and could be vectors of DSP intoxication. To protect marine ecosystems and human health from potential hazards, regular monitoring of these benthic dinoflagellates and their emerging biotoxins must be implemented. This study demonstrated the presence of

epiphytic dinoflagellates almost year round but with the highest cell densities in summer and autumn, suggesting the thermophilic character of these BHAB species. In further studies, the observed strains will be isolated, and monoclonal cultures will be established to determine their toxin profiles and genetic identities. Ecophysiological experiments in controlled laboratory conditions will provide information on the effects of environmental factors on the growth and toxicity of the species of interest.

Declarations

Conflict of interest   The authors declare no competing interests.

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