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Consequences of a contaminant mixture of bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP), two plastic-derived chemicals, on the diversity of coastal phytoplankton

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Abstract

To assess the impact of two plastic derived chemicals: bisphenol A (BPA) and the di-2-ethylhexyl phthalate (DEHP), on phytoplankton biomass and community structure, microcosm incubations were performed during spring and summer, with offshore and lagoon waters of a south-western Mediterranean ecosystem. Phytoplankton were exposed to an artificial mixture of BPA and DEHP and to marine water previously enriched with plastic-derivative compounds, originated from in situ water incubations of plastic debris for 30 days. After 96h of incubation, changes were observed in phytoplankton biomass in the contaminated microcosms, with a net decrease (up to 50% of the control) in the concentration of Chlorophyll a in offshore waters. Concomitantly, plastic-derivative contamination provoked structural changes, especially for offshore waters. This suggests a relative tolerance of the lagoon communities to BPA and DEHP contamination, related to the dominance of *Chaetoceros* spp., which could potentially be used as a bioindicator in bioassessment studies.

Keywords: bisphenol A; di-2-ethylhexyl phthalate; coastal ecosystems; spring; summer; phytoplankton.

Highlights:

BPA and DEHP release from plastic debris was higher in spring than in summer.

BPA and DEHP contaminations strongly impact phytoplankton biomass and structure.

Impacts of BPA and DEHP were more marked in offshore, relative to lagoon, waters.

Diatoms were more tolerant to BPA and DEHP than the other phytoplankton groups.

1. Introduction

Plastic marine pollution is a major environmental concern in response to the damage to organisms observed, such as the accumulation of debris in invertebrates (Thushari *et al*., 2017), the ingestion of plastic particles, the entanglement of aquatic organisms (GESAMP, 2015) and the transfer of this components along the trophic webs (Staples *et al*., 1997b; Possatto *et al*., 2011; Cole *et al*., 2013). Unfortunately, in the marine environment, plastic debris is persistent and durable. It accumulates in the ecosystem and degrades to smaller micro-plastics (Bergmann *et al*., 2015b; Causey and Padula, 2015). These latter items could, in turn, release plastic-derived chemicals, including endocrine disrupting chemicals (EDCs) such as bisphenol A (BPA) and di-2-ethylhexyl phthalate (DEHP). BPA and DEHP are two man-made chemicals used extensively in commercial and industrial applications, such as in additives and plasticizers. In the environment, plastic items are persistent and take a long time to disappear: from hundreds to thousands of years (Mansui *et al*., 2015). Furthermore, they are able to release other ester molecules, which impact marine food webs, leading to the bio-accumulation and bio-transport to higher organisms in the trophic levels (Ying and Kookana, 2003; Turki *et al*., 2014; Hahladakis *et al*., 2017). BPA and DEHP are hazardous components. They occur in aquatic areas at different levels: hundreds of ng/L to tens of µg/L in rivers and estuaries (Careghini *et al*., 2014), in coastal seawaters (Preston and Al-Omran, 1986; Huang *et al*., 2012; Paluselli *et al*., 2017) and the open ocean (Giam *et al*., 1978).

Being at the base of the food webs and as primary producers, phytoplankton have a crucial position in maintaining the equilibrium of the aquatic ecosystem (Cloern, 1996; Li *et al*., 2009). Obviously, they are crucial in the transfer of organic compounds between all matrixes of the marine environment: water, sediments and organisms (Staniszewska *et al*., 2015b). In fact, several previous studies have reported the significant ability of phytoplankton to accumulate organic pollutants, such as polychlorinated biphenyls (PCBs) (Lynn *et al*., 2007),

polycyclic aromatic hydrocarbons (PAHs) (Echeveste *et al*., 2010) and endocrine disrupting chemicals (for example, bisphenol A (BPA), di-(2-ethylhexyl) phthalate (DEHP) and di-butyl phthalate (DBP) (Huang *et al.*, 1999; Chi *et al*., 2007; Kang *et al*., 2007; Liu *et al*., 2010a). Species composition of phytoplankton is highly diverse and expresses different sensitivity responses to environmental changes. Evaluation of the toxicological profile of a pollutant towards phytoplankton, being the lowest trophic level, could define the entry point of contamination to the aquatic ecosystem. This is why phytoplankton are often studied to assess the environmental risk and evaluate the impact of toxic chemicals and other environmental factors. This evidently leads to a prediction of eco-toxicological consequences and, importantly, to the implementation of the necessary measures of prophylaxis to prevent any anomaly occurring in the aquatic system. Also, some sharp increases in phytoplankton biomass are associated with pollution occurrences (Leboulanger *et al*., 2011; Pandey *et al*., 2015). This has been interpreted as an adaptive state to minimize the impact of the toxicant (Söderström *et al*., 2000; Nizzetto *et al*., 2012). The exposure of phytoplankton to a sediment resuspension results in an enhancement of photosynthetic performance, a stimulation of growth and a change in the community structure in favor of toxic species tolerant to the toxicant (Lafabrie *et al*., 2013 a, 2013b; Ben Othman *et al*., 2017). Here, the amounts of phytoplankton may lead to disposal problems, such as the outbreak of harmful algal blooms (HABs) in the case of stimulation of toxic algae. Yet other studies have reported a decline in phytoplankton abundance, a decrease of Chlorophyll *a* and a disruption of photosynthetic performance when exposed to pollutants (Dorigo *et al*., 2004; Leboulanger *et al*., 2011; Pandey *et al*., 2015).

In the context of plastic-derived pollution, Causey and Padula (2015) showed that following the degradation of these items, micro-plastics, considered as hazardous pollutants (Cole *et al*., 2011), could be mistaken as food by small organisms. Furthermore, emphasizing the interaction between plastic-derived chemicals, such as BPA and DEHP, and primary producers, little is known about the eco-toxicological response of phytoplankton to those components at approximately in situ concentrations (Castañeda and Avlijas, 2014; Lusher *et al*., 2014). Previous studies have mainly been performed to assess the eco-toxicological response of some phytoplankton species exposed to high concentrations of BPA (up to 3 mg/L) compared to what is observed in situ (Li *et al*., 2009; Liu *et al*., 2010a; Ebenezer and Ki, 2012) and to evaluate the capacity of these phytoplankton to accumulate and to degrade these components (Kang *et al*., 2006; Chi *et al*., 2007; Staniszewska *et al*., 2015b). Considering the lack of information regarding the impact of BPA and DEHP on the base of the aquatic trophic

web, this study aimed to investigate the toxicological answer of phytoplankton exposed to these plastic-derived chemicals. We targeted two different marine coastal ecosystems: a lagoon and a bay in the South-Western Mediterranean Sea and during two different seasons: spring and summer. The assessment was based on two strategies: i) mimicking an environment polluted with plastic debris by stimulating the release of BPA and DEHP from plastic items previously incubated in marine water and ii) simulating critical cases of chronic BPA and DEHP contamination in coastal marine ecosystems. For these purposes, monitoring was conducted on the biomass, abundance and the functional diversity (Franklin *et al*., 2001; Bellemare *et al*., 2006) of phytoplankton from the marine coast of Bizerte. The whole system (phytoplankton and contaminant) was incubated in microcosms under natural sunlight and temperature to mimic natural conditions.

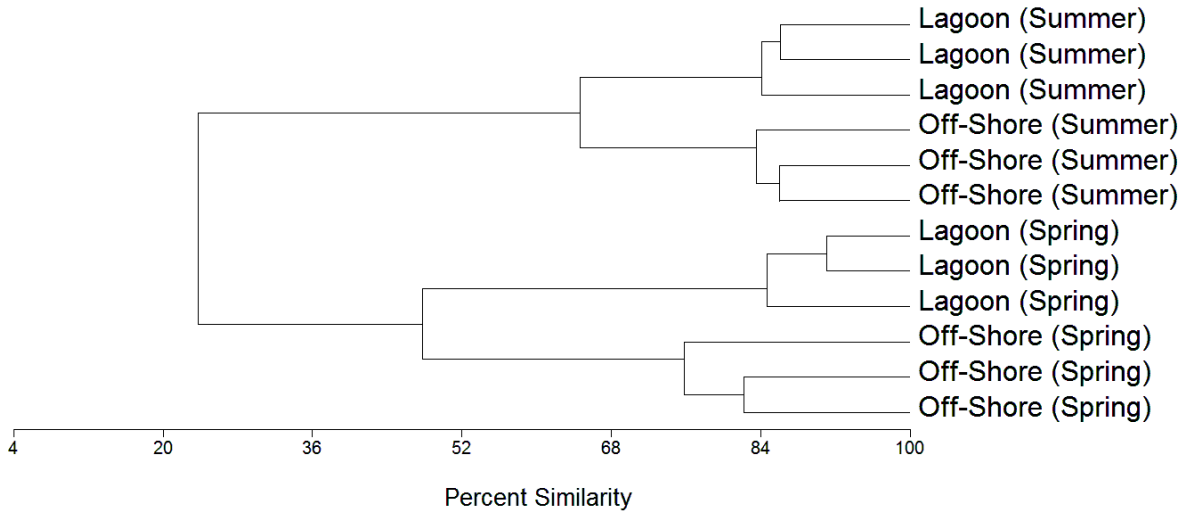
2. Materials and methods

2.1. Study area and plastic release preparation

The study was conducted in South-Western Mediterranean ecosystem: the lagoon and the bay of Bizerte (Tunisia), in April–May 2016 (~20 °C) and August–September 2016 (~28 °C). Bizerte lagoon is an important ecosystem. It is subject to the influence of several anthropogenic pressures, such as agriculture, fishing and farming activities, urbanization, industrialization (a cement factory, metal treatment, a dye-works, metallurgy, a steelworks, a military arsenal, a naval port, a commercial harbor, maritime traffic, etc.) (Sakka *et al*., 2008; Ben Said *et al*., 2010). As a consequence, this human intervention might alter and modify the quality, diversity and structure of this ecosystem by discharging pollutants and contaminants such as PCBs, herbicides and metals, etc., into the sea (Barhoumi *et al*., 2013b; Pringault *et al*., 2016; Goni-urriza *et al*., 2018). In keeping with what currently happens in the lagoon, Bizerte bay is also considered a contaminated ecosystem. In fact, oil-polluted seawater has been recorded due to discharges from a refinery unit located on the shore, creating petroleum stress (Zrafi-Nouira *et al*., 2009; Boufahja *et al*., 2010; Jaafar Kefi *et al*., 2016). To our knowledge, BPA and DEHP have until now never been extracted and quantified in the aquatic biota of Bizerte. Water sampling was carried out in a lagoon station (Lagoon station (L): 37° 12' 43.96" N 9° 50' 79.78" E) and a bay station (Offshore station (O): 37° 16' 46.46" N 9° 53' 50.98" E) (Fig. S1, Annex 5) as described by Pringault (2016). Collected water was immediately filtered through 200 µm mesh to remove large organisms and to curtail grazing impacts during the incubation time.

2.2. Incubation procedure

One month prior to water sampling, contaminated seawater was prepared using manufactured plastic items incubated in natural seawater using glass microcosms. Several plastic materials were incubated: two plastic bags, one Plexiglas plate, one PVC tube and one blood bag were incubated in glass containers. These microcosms were filled with natural seawater sampled from the Bizerte channel (filtered on a 0.22 µm capsule filter). The plastic incubation was prepared in triplicate. More details about the plastic items (composition, size, weight) are shown in Table S1 of the Supplementary Material. To choose the plastic items to incubate, we followed previous studies on the occurrence and fate of plastic in aquatic ecosystems (Morét-Ferguson *et al*., 2010; Zhou *et al*., 2011; Sadri and Thompson, 2014) and on the potential release of BPA and DEHP from plastic fragments into the marine environment (Sajiki and Yonekubo, 2003; Takehisa *et al*., 2005; Kastner *et al*., 2012). The whole system was kept under natural sunlight for 30 days to create water enriched with plastic-derived chemicals through release by the incubated plastic items (Ishihara and Nakajima, 2003; Kang *et al*., 2006). At the end of the incubation period, concentrations of BPA and of DEHP were measured in each microcosm. Seawater was incubated in 10 L covered glass microcosms as described in Pringault (2016). Three groups of microcosms were filled as follows: the first series with in situ water (control lagoon water: C-L and control offshore water: C-O). A second series of microcosms was filled with in situ water (75%) and mixed with plastic-contaminated seawater (25%) as described above (enriched plastic contaminated water microcosms for lagoon experiment: P-L and for offshore experiment: P-O). A final series of microcosms was filled with lagoon and offshore waters, spiked with a mixture of pure solution of 20 µg/L of BPA (Sigma Aldrich) and 10 µg/L of DEHP (Sigma Aldrich), previously dissolved in DMSO (BD-L for lagoon experiment and BD-O for offshore experiment). All treatments (C-L, C-O, P-L, P-O, BD-L and BD-O) were performed in triplicate and were incubated under natural sunlight in an open–circulating seawater system to mimic natural environmental conditions (in situ water temperature and natural sunlight). The monitoring was carried out for 96 h to evaluate the possible changes in phytoplankton diversity (Ben Othman *et al*., 2017; Lafabrie *et al*., 2013b). Phytoplankton abundance and diversity were measured at the beginning (t = 0 h) and the end of the experiment (t = 96 h) and phytoplankton biomass (Chl *a*) was measured every day.



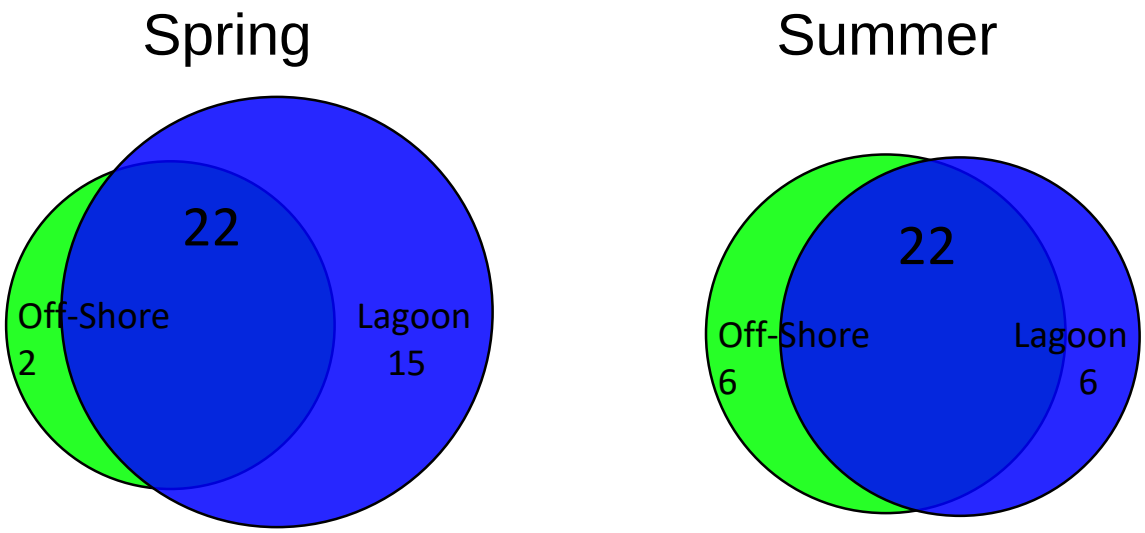


Figure 1: Venn diagram and dendrogram performed with the in situ offshore and lagoon waters communities. Numbers indicate the numbers of phytoplankton species observed.

2.3. BPA and DEHP analysis

DEHP was analyzed using Solid-Phase Micro-Extraction (SPME) coupled with Gas Chromatography/Mass Spectrometry (GC/MS). For the extraction of water samples, 100 µm fiber SPME-PFMS (Supelco) was used. The quality parameters of the SPME were: i) an immersion of the fiber in liquid phase (15 mL of the sample), ii) an incubation temperature of 65 °C, iii) an incubation time of 5 min, iv) agitation 250 rpm and v) 30 min for extraction and 3 min for desorption. DEHP analysis was performed with GC/MS working in electro-ionization impact mode (GC-7890 A; MSD-5975C, Agilent Technologies). An HP5MS-UI column (5% phenyl methyl siloxane, 30 m × 0.25 mm ID × 0.25 µm phase thickness, Agilent Technologies)

was used. BPA analysis was performed using Liquid Chromatography/tandem Mass Spectrometry (LC/MSMS) in a negative ionization mode (UPLC Acquity; MSMS-Quattro Premier XE, Waters). The cartridge used was an Acquity UPLC BEH C18 (50 mm × 2.1 mm ID × 1.7 µm granulometry, Waters). Direct injection volume was set at 40 µL. The quality parameters of chromatography were as follows: i) solvent tank A: milliQ-water with 0.5 mM ammonium acetate, ii) solvent tank B: methanol, iii) a mobile phase flow rate of 0.5 mL min^{-1}. BPA and DEHP were analyzed following the analytical protocols of (Dévier *et al*., 2013) for DEHP and of (Gagnaire *et al*., 2009) for BPA (more details about the validation parameters in Table 2 of the Supplementary Material).

2.4. Determination of phytoplankton biomass (Chlorophyll *a*)

Chl *a* was determined by spectrophotometer (UV 2650). Subsamples of 500 mL were filtered onto glass fiber filters (GF/F, Whatman) then stocked until analysis (detection limit 0.02µg/L) (Aminot and Chaussepied, 1983). Chl *a* was extracted using 10 mL of 90% acetone after cooling for 24 h at 4 °C according to the protocol of Lorenzen (1965).

2.5. Phytoplankton identification and abundance

Phytoplankton identification and count were made according to the method of Utermöhl (1958). Subsamples were fixed and then phytoplankton cells were counted on an Utermöhl chamber of 25 mL, using an inverted microscope (Leica 521234). At least 400 cells were counted in each sample to get a reliable representation of the community.

2.6. Diversity indexes

The specific richness index: Shannon and Wiener index-H', the diversity index: Simpson index-S and the equitability index: Pielou's evenness index-J' were calculated using Multi-Variate Statistical Package (MVSP 3.22, Kovach Computing) software to study the effect of treatments on the functional diversity structure of phytoplankton communities (Shannon, 1948; Pielou, 1966).

2.7. Statistical analysis

Differences in variables between treatments and time incubation samplings were investigated by two-factor analysis of variance (ANOVA). Prior to ANOVA, normality and homoscedasticity (Shapiro test and Leven's test, respectively) were calculated. For statistical analysis of diversity of phytoplankton, the relative abundance was transformed with arcsin ($x^{0.5}$)

to obtain a normal data distribution (Legendre and Legendre, 1998). These analyses were performed using Stat-Graphics software (Centurion XVI.II). Multidimensional analyses were performed to detect variation of phytoplankton structure between the treatments using Multi-Variate Statistical Package (MVSP 3.22, Kovach Computing) software. Simper test was conducted to highlight the phytoplankton species contributing to the dissimilarity between contaminated communities and control communities using R Core Team v.3.3.3 and vegan package v.2.0–10, (Oksanen *et al*., 2013).

3. Results

3.1. In situ phytoplankton structure

A total of 48 phytoplankton species were identified in all samples. The phytoplankton structure was markedly different (only 24% similarity) between spring and summer (Figure 1) and, to a lesser extent, between offshore and lagoon. Similarity between the two stations was about 48% in spring and 65% in summer, despite the fact that 22 species were common to both ecosystems for both seasons (Figure 1). Phytoplankton diversity (H' index) (Figure 1) and richness (Table 1) for in situ waters was significantly greater ($p < 0.05$) in summerthan in spring for both stations. Species common to stations were observed, i.e. *Gyrodinium lachryma* and *Nitzschia longissima* for spring and *Heterocapsa minima* and *Thalassiosira levanderi* for summer (Figure 2); nevertheless, these species were not dominant (Table 4).

Diatoms were the most dominant group in in situ waters in both seasons and for the studied stations. In spring, as in summer, dominant species were different between offshore and lagoon stations. In spring, offshore waters were dominated by *Nitzschia cf. acicularis*, *Leptocylindrus minimus*and *Dactyliosolen blavyanus*, representing 49% of the total relative abundance (Figure 2) while lagoon waters were dominated by *Chaetoceros laciniosus* and *L. minimus* (26% of the total relative abundance) (Figure 2). In summer, *C. costatus* and *C. constrictus* represented 23% of the total relative abundance in offshore waters, and *C. curvisetus*, *C.tortissimus*, *C. constrictus* and *L. minimus* represented 56% of the total relative abundance in lagoon waters (Figure 2).

Table 1: Diversity indexes calculated for the functional diversity assessed by phytoplankton identification and cell count in the lagoon and offshore experiments during spring and summer. In situ: inoculum diversity; Control: control microcosm, Plastic: plastic

Season	Station	Treatment	Shannon-Wienner index (H')	Pielou's eveness index (J)	Simpson diversity (S)
Spring	Off-Shore	In situ	2.77 ±0.16	0.96 ±0.01	19.50±1.50
		Control	3.10 ±0.09	0.96±0.00	24.50 ±1.50
		Plastic	3.14 ±0.09	0.97 ±0.00	27.50±0.50
		BPA & DEHP	3.23 ±0.01	0.95 ±0.00	31.50±1.50
	Lagoon	In situ	2.76 ±0.10	0.91 ±0.01	19.50±0.50
		Control	2.83 ±0.03	0.91 ±0.01	22.33±0.58
		Plastic	2.90 ±0.14	0.89 ±0.01	27.50±0.50
		BPA & DEHP	3.08 ±0.07	0.94 ±0.00	25.50±0.50
Summer	Off-Shore	In situ	3.05±0.03	0.96±0.00	23.67 ±0.58
		Control	3.09±0.05	0.95±0.01	27.00 ±1.00
		Plastic	3.29±0.03	0.95±0.01	32.00±1.00
		BPA & DEHP	2.93 ±0.14	0.92±0.01	26.00 ±2.00
	Lagoon	In situ	3.06±0.09	0.94±0.00	26.33±2.52
		Control	3.33±0.01	0.95±0.00	33.00±0.00
		Plastic	3.37±0.01	0.94±0.00	35.67±0.58
		BPA & DEHP	3.28±0.03	0.94±0.00	33.67±1.53

3.2. BPA and DEHP contamination levels

Natural levels of DEHP contamination were higher in summer than in spring for both stations. Statically significant difference was observed for the offshore station during both seasons ($P < 0.05$). DEHP concentration in summer (0.5 µg/L) was over three times higher than in spring (0.15 µg/L). However, no statistically significant differences was observed between seasons in lagoon waters. DEHP was about 0.24 µg/L in spring and 0.3 µg/L in summer ($P > 0.05$). BPA concentrations measured in situ for both stations were below the limit of detection (0.3 µg/L) for the two studied seasons (Table 2). The potential release of plastic-derived chemicals from the incubation of plastic items in natural seawater for 30 days was more important for BPA (2.4 µg/L) than DEHP (0.41 µg/L) in spring, while in summer, similar concentrations of BPA and DEHP were released; about 0.3µg/L (Table 2). For the pure mixture of BPA and DEHP used during the present study, analysis supported that the nominal concentrations added were close to the measured ones (Table 2).

3.3. Impact of treatments on phytoplankton biomass

Phytoplankton biomass was impacted with plastic derivative contaminants with respect to the corresponding control. Significant changes (Two factor Anova, $p < 0.05$) were observed for Chl*a* concentrations at both seasons (spring and summer) and more particularly for the offshore station (Figure 3). These changes in Chl *a* concentrations were also followed with significant changes ($p < 0.05$) in diversity of phytoplankton groups (Figure 4). In the offshore station, similar patterns of Chl *a* were observed, at a level about two-fold higher in spring than in summer. The Chl *a* of control experiments increased and reached a peak at 72 h of incubation during the two seasons (6.9 µg/L and 4.4 µg/L in summer and spring, respectively). Similarly, a strong increase in phytoplankton abundance (up to 2.7×10^5 cell/L and 44×10^5 cell/L in spring and summer, respectively) was observed in control microcosms. Bacillariophyceae (80% and 95% in spring and summer, respectively), chlorophyceae (10% and 0.8% in spring and summer, respectively), cryptophyceae (6.6% and 0.3% in spring and summer, respectively) and dinophyceae (2.8% and 3.5% in spring and summer, respectively) were the most abundant groups. For contaminated microcosms, significant impact ($p < 0.05$) was observed on total phytoplankton biomass, irrespective of the season (Figure 3A and 3C and Figure 4A and 4C). During spring, Chl *a* concentration in contaminated treatments decreased significantly ($p < 0.05$) in offshore microcosms, especially for the plastic enriched (P-O) microcosm. Nevertheless, this decrease in Chl *a* concentration was concomitant with a significant increase

($p < 0.05$) in phytoplankton abundance, particularly in bacillariophyceae and dinophyceae, despite a significant decrease ($P < 0.05$) in chlorophyceae and cryptophyceae (Figure 4A). During summer (Figure 3C and Figure 4C), Chl *a* concentrations and phytoplankton abundance in contaminated microcosms were lower than those observed in the control, with a significantly more marked impact for offshore waters incubated with a pure mixture of BPA and DEHP (BD-O microcosm). These decreases in abundance were concomitant with the disappearance of two main groups, chlorophyceae and cryptophyceae. In contrast, no significant impact (Two factor Anova, $P > 0.05$) of plastic derivatives (P and BD microcosms) on Chl *a* relative to control was observed for lagoon waters, neither in spring nor in summer (Figure 3B and 3D). A slight increase in Chl *a* was observed in spring, while in summer Chl *a* decreased slightly during incubation, irrespective of the treatment. Phytoplankton abundance did not follow the patterns observed for Chl *a* concentration. During spring, a significant increase ($P < 0.05$) phytoplankton abundance was observed for P-L (up to 16.2×10^5 cell/L) relative to C-L and BD-L microcosms; in contrast, during summer higher abundance was observed for BD-L (up to 75×10^5 cell/L) relative to C-L and P-L. For both seasons, bacillariophyceae was the most abundant group (more than 80% of the relative abundance).

3.4. Impact of treatments on phytoplankton structure and diversity

As observed for biomass, treatments with plastic-enriched water and with pure mixture of BPA and DEHP impacted significantly the phytoplankton structure ($P < 0.05$) irrespective of the season, but with a more pronounced effect for offshore waters. In spring, treatments with plastic-enriched water and with pure mixture of BPA and DEHP provoked a significant increase of diversity indexes (S and Shannon), relative to C, for lagoon and offshore waters. In summer, the impact of plastic contamination on phytoplankton diversity was less pronounced in both stations (Table 3), no significant difference ($P > 0.05$) was observed between treatments for all the diversity indexes (H', J and S indexes). Nevertheless, , correspondence analysis (CA) performed with the normalized relative abundance of the offshore and lagoon phytoplankton community during spring (Figure 5A) and summer (Figure 5B) showed that BPA and DEHP can have an impact on the phytoplankton structures. The two first axes of the CA explained 45% of the observed variance in spring (Figure 5A) and 46% of that in summer (Figure 5B).

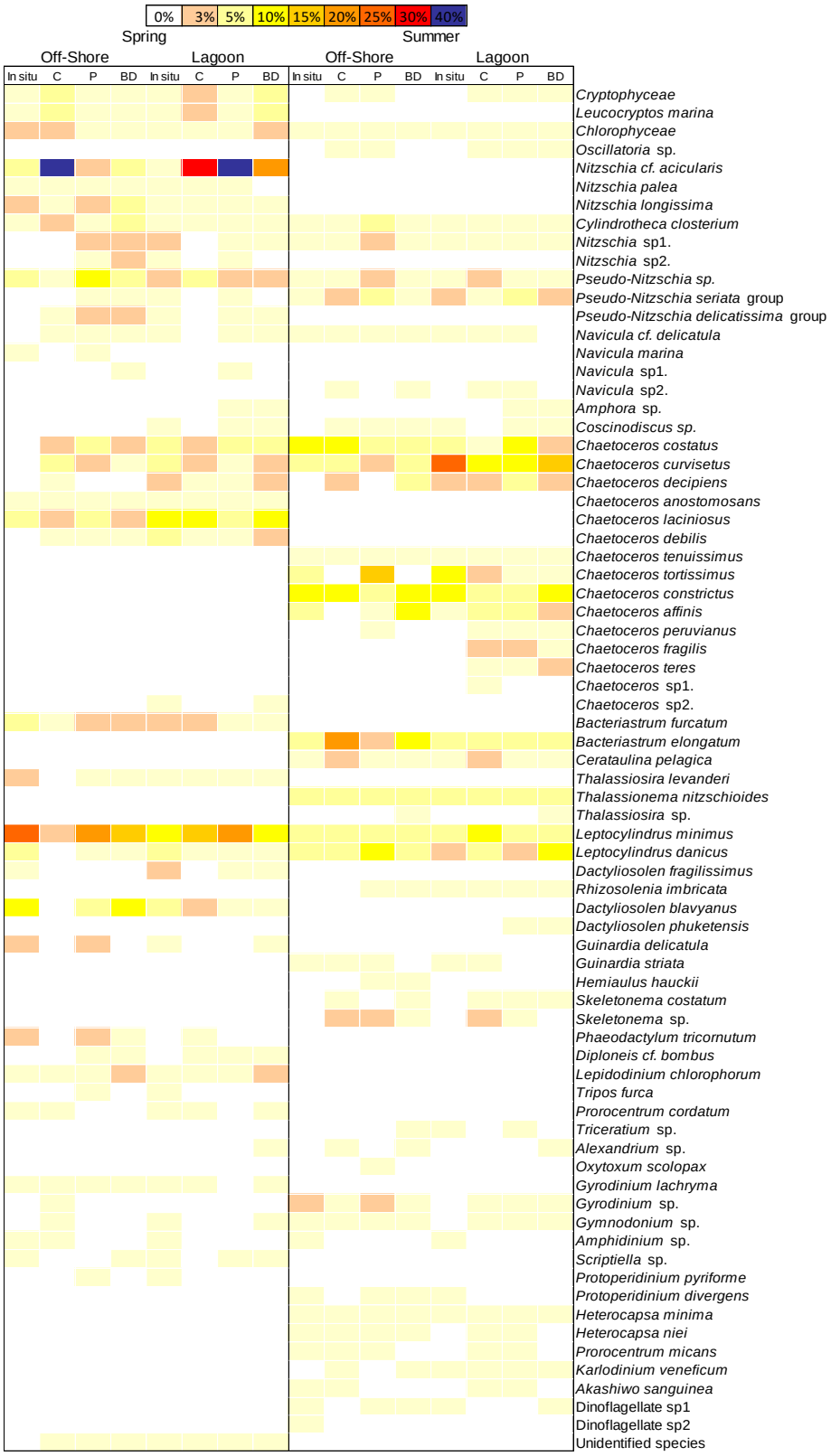


Figure 1: Heatmap of the functional diversity assessed by phytoplankton identification and cell count as a function of the microcosm treatment. In situ: inoculum, C: control microcosm, P: plastic enriched microcosm, BD: pure mixture of BPA and DEHP microcosm.

In both seasons, stations were separated along the horizontal axis, while treatments were distributed along the vertical axis. The CA indicated structural changes between offshore and lagoon, with more distinct separation between treatments in offshore waters than that observed for lagoon waters, irrespective of the season. Three distinct clusters were observed during spring; a first cluster with BD-O, a second with P-O and C-O, and the third cluster grouping all the community structure of lagoon waters. The differences observed between control and plastic microcosms (P and BD microcosms) were mainly explained by changes in relative abundance of *N. cf. acicularis* and *L. minimus* (Table 3). The same cluster pattern was observed during summer (Figure 5B), irrespective of the treatments: a first cluster with P-O; a second with BD-O and C-O; the third grouped all the community structure of lagoon waters, as observed in spring. *B. elongatum* and *C. tortissimus*were the species most responsible for the differences observed between control and treatments for offshore waters (Table 3), whereas the differences observed for lagoon water were mainly explained by *L. minimus* and *C. costatus* (Table 3). When focusing on a particular station and season (Figure S2, Annex 5) significant differences could be observed between treatments and control experiments. In offshore waters, The CA, which accounted for 55% and 73% of the variance observed in spring and summer, highlighted clear structural changes in spring and in summer, with distinct separation between treatments; three distinct clusters were observed relative to the treatments (Figures S2 A and S2 B, Annex 5). Similarly, for lagoon waters, the two first axes of the CA explained 59% and 74% of the variance observed in spring and summer, respectively. As observed for offshore waters, clear separation between treatments was observed (Figures S2 C and S2 D, Annex 5). Nevertheless, these differences observed at the station level were masked when offshore and lagoon stations were pooled in the analysis (Figures 5A and 5B), suggesting that the impacts of season and station were more pronounced than those of the plastic treatments.

4. Discussion

4.1. In situ condition

The diversity of in situ waters was greater in summer than in spring. Despite a strong similarity of phytoplankton richness observed between offshore and lagoon stations (22 species in common out of 28–30 species in total, depending on the season), the phytoplankton structure exhibited important dissimilarities. Phytoplankton diversity and richness in the lagoon and offshore waters were similar to those previously observed in the same area (Sakka *et al*., 2007; Lafabrie *et al*., 2013b) and in similar coastal ecosystems (Ben Othman et al., 2017; Huang et

al., 2011; Ramdani et al., 2009), with the dominance of bacillariophyceae (i.e: *L. minimus* and *C. laciniosus*); dinophyceae (i.e: *Gyrodinium sp*. and *Heterocapsa minima*, in summer) and chlorophyceae. In contrast, Goni-Urriza *et al* (2018) and Hlaili *et al* (2006) reported different diversity trends from the same area during the spring phytoplankton bloom, where micro-flagellates and dinoflagellates dominated in situ waters. These results could suggest that: i) the spring bloom process is not limited to the same phytoplankton group for a particular station and ii) the timing of the spring phytoplankton bloom is variable and related to external factors that affect the climate system (Townsend *et al*., 1994).

Table 3: Natural contamination in Bizerte lagoon and offshore waters (µg/L), the release level of plastic items incubated after 30 days and the measured concentration of BPA and DEHP from the pure mixture.

Station/Sample		DEHP (µg/L)		BPA (µg/L)	
		Spring	Summer	Spring	Summer
Natural contamination	*In situ* Lagoon	0.24±0.08	0.3±0.06	<0.3	<0.3
	In situ Off-Shore	0.15±0.01	0.5±0.25	<0.3	<0.3
Natural seawater incubated with plastic items		0.41±0.02	0.3±0.06	2.4±0.45	<0.3
Pure mixture of BPA and DEHP		12.2±0.73	7.64±1.9	22.83±2.00	17.42±0.8

4.2. Chemical situation

Our results showed that in situ concentration levels of DEHP were higher in summer (average temperature ≈ 28 °C) relative to spring (average temperature ≈ 20 °C), which is contrary to the findings of Chi *et al* (2003). These authors reported higher DEHP concentrations in an inland lake concomitant with low temperature (~20 °C), whereas low DEPH concentration was observed during summer. These results might indicate that for a particular season the DEHP levels could be different between one aquatic ecosystem and another. Natural in situ contamination of the offshore and the lagoon waters presented higher DEHP levels compared

to similar coastal ecosystems, where DEHPconcentrations did not exceed hundreds of ng/L (between 5 and 617 ng/L), such as in the bay of Marseille (Paluselli *et al*., 2017) or the Spanish coast (Brossa *et al*., 2005; Ji *et al*., 2014). In contrast, DEHP levels in river and inland lake waters are significantly greater, in the range of hundreds of µg/L (Chi et al., 2003; Dargnat et al., 2009; Fromme et al., 2002). Unfortunately, in the present study, the detection limit of BPA was high 0.3 µg/L, compared to the natural BPA concentrations, which prevented us from having exact values of this compound. The presence of BPA in surface waters was detected in different aquatic ecosystems, with a large range of concentrations depending on the environment, from 0.001 µg/L in the Venice lagoon to 92 µg/L in the Elbe river of Germany (Careghini et al., 2014; Huang et al., 2012).

When plastic items were incubated in seawater, the release of BPA and DEHP into the surrounding water was strongly affected by the season; higher BPA and DEHP release was observed during spring relative to summer (Table 3). Temperature is known to enhance the release into marine waters of plastic derivatives such as BPA, with rates 20 times higher at 37 °C relative to 20 °C (Sajiki and Yonekubo, 2003). Similarly, the release of DEHP was reported to be more than 44 times higher at 47 °C relative to 18 °C (Clausen *et al*., 2012; Bakir *et al*., 2014). Different hypotheses might explain the discrepancy observed between our results and the expected accumulation according to the literature. Firstly, in our study, plastic items were incubated under natural sunlight for 30 days; it is likely that these environmental conditions promote the photodegradation of BPA and DEHP (Zhan *et al*., 2006; Diepens and Gijsman, 2007; Hahladakis *et al*., 2017). Secondly, the plastic items were not disinfected before incubation; consequently the risk of bacterial contamination cannot be excluded, so favoring bacterial biodegradation of BPA and DEHP molecules (Zhang *et al*., 2007). Lastly, it is likely that the plastic items incubated for 30 days may have released other compounds, such as hydrocarbons (Teuten *et al*., 2009; Cole *et al*., 2011) that were not analyzed; compounds that could promote BPA and DEHP binding, so decreasing their accumulation in the dissolved phase. Unfortunately, we did not measure the kinetics of BPA and DEHP release during the incubation. Plastic items were incubated for 30 days, the incubation time required to measure a significant accumulation in the surrounding water according to the laboratory study of Sajiki and Yonekubo (2003). Nevertheless, the long incubation might also promote photodegradation and/or biodegradation, so decreasing the potential accumulation of BPA and DEHP measured after 30-day incubation.

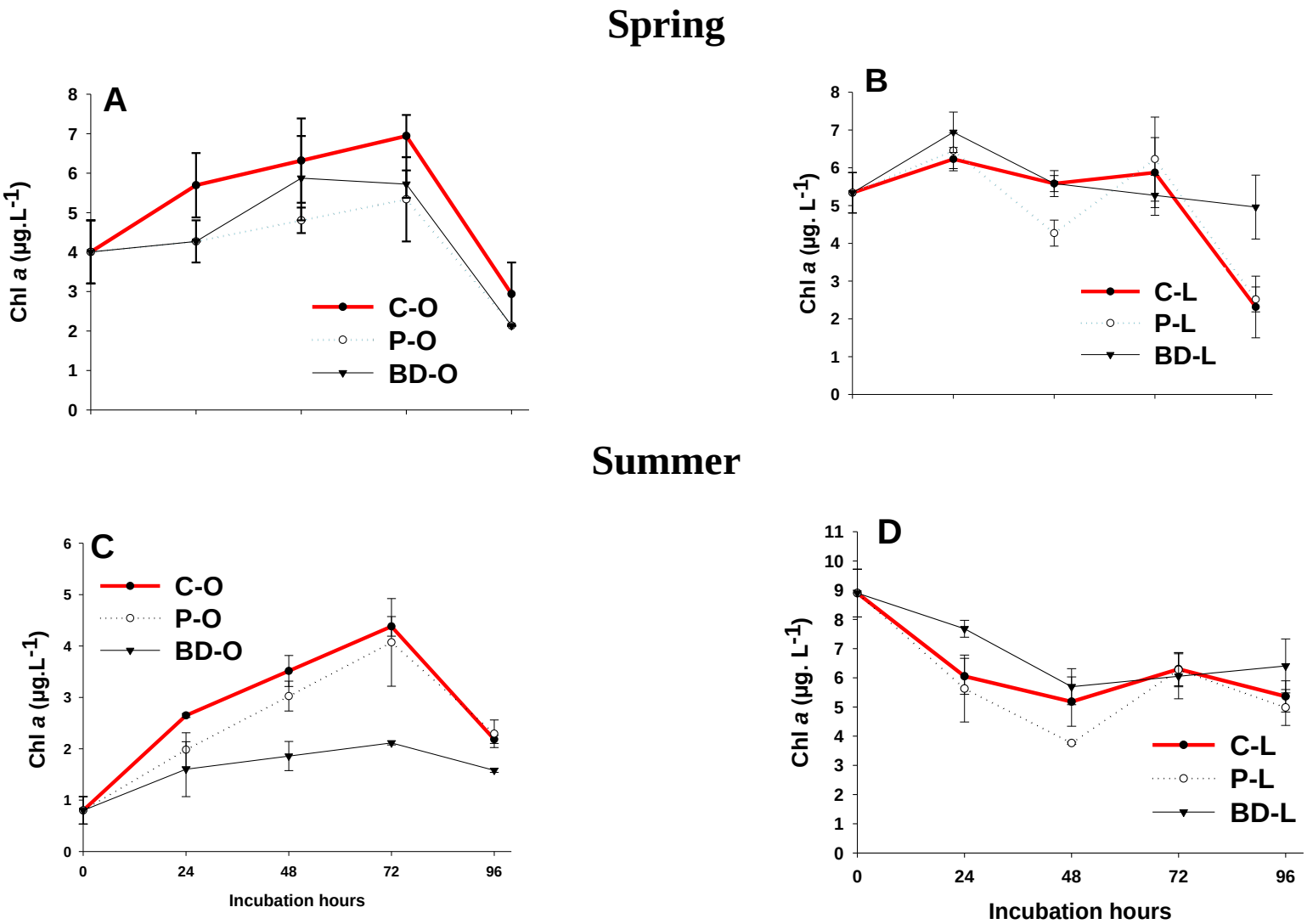


Figure 3: Evolution of Chlorophyll a concentrations in the microcosms for the offshore experiment in spring (A) and in summer (C) and for the lagoon experiment in spring (B) and in summer (D). (Mean of three replicates ± standard deviation (SD)).

4.3. Impact of treatments on phytoplankton biomass

The interaction between plastic-derived chemicals and primary producers has so far been poorly studied; consequently, information about the impacts of those molecules on phytoplankton is scarce (Staples *et al*., 2011; Lee *et al*., 2015). Studies focused mainly on the ability of phytoplankton species to bio-accumulate and/or to biodegrade plastic-derived molecules (Chi *et al*., 2007; Liu *et al*., 2010a). In our study, both contaminant treatments, plastic enriched water (P) and pure mixture of BPA and DEHP (BD), impacted Chl *a* concentrations during spring and summer. The effects on Chl *a* concentration were also accompanied by changes in phytoplankton groups for both seasons and stations. Nevertheless, the changes were more pronounced for offshore waters, with Chl *a* concentration representing only 50% of that observed in the control. These results are in agreement with previous studies performed with monospecific phytoplankton cultures exposed to similar contaminants (Ebenezer and Ki, 2012; M'RABET *et al*., 2018). The growth of dinoflagellates was strongly inhibited by BPA (Ebenezer and Ki, 2012) and also by DEHP or BPA–DEHP mixtures (M'RABET *et al*., 2018). In contrast, Liu *et al* (2010) observed tolerance of a marine diatom belonging to the group bacillariophyceae upon contamination with BPA, ranging from 1 to 5 mg/L. This suggests that the sensitivity to these two plastic derivatives is phytoplankton-group-dependent.

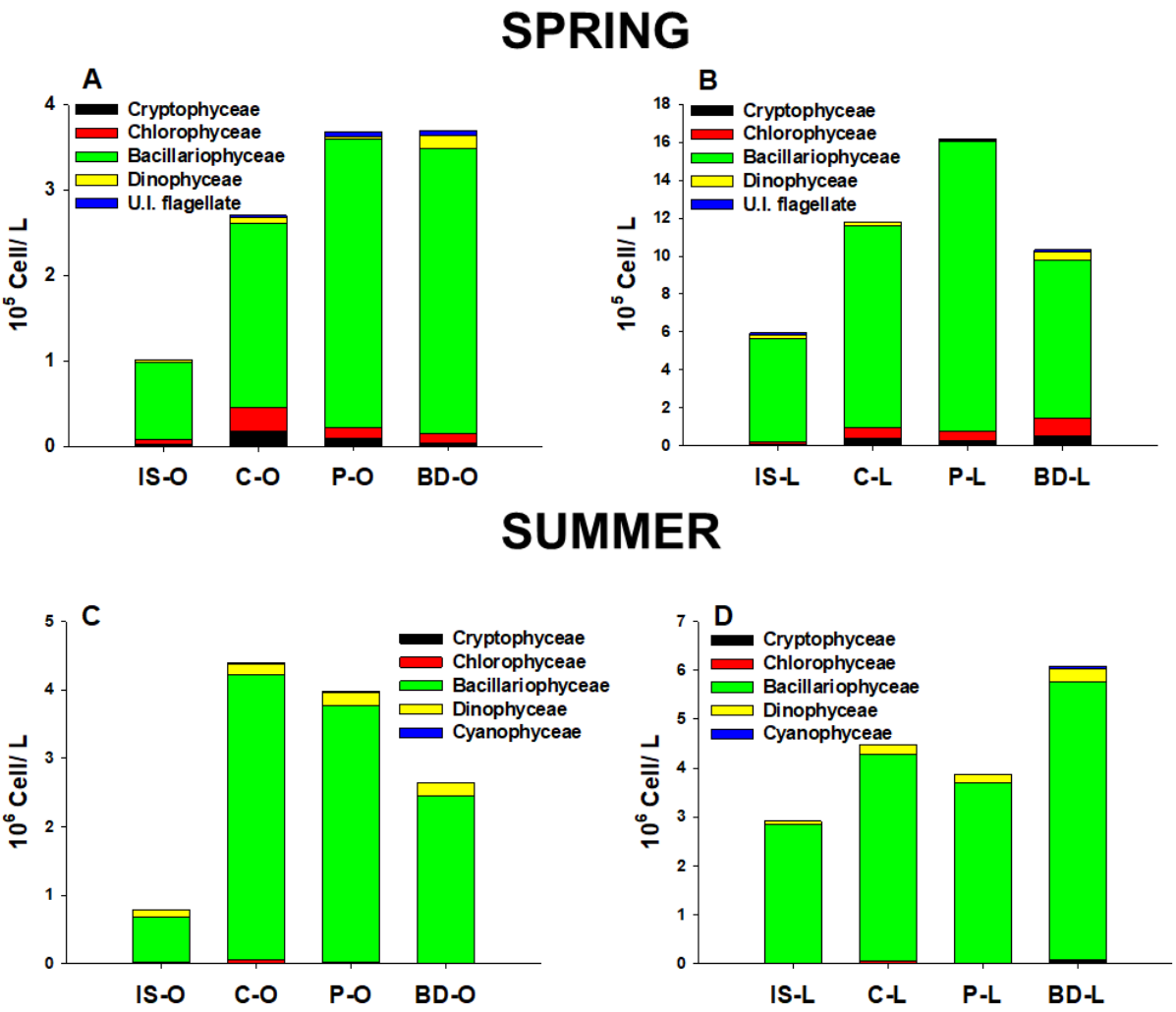


Figure 4: Contribution of the various taxonomic groups to the total phytoplankton abundances in the inoculum community (in situ offshore: IS-O and in situ lagoon: IS-L) and after 96 h in the control (C-O and C-L), plastic-enriched water (P-O and P-L), and pure mixture of BPA and DEHP (BD-O and BD-L) for the offshore experiment in spring (A) and summer (C) and for the lagoon experiment in spring (B) and summer (D).

The weak sensitivity of phytoplankton biomass to plastic derivatives observed for lagoon waters suggest a relative tolerance of the primary producers in this ecosystem, as has been observed for other pollutants (such as metals and pesticides) in similar coastal environments (Leboulanger *et al*., 2011; Pandey *et al*., 2015). Obviously, phytoplankton may adapt changes to accommodate stress conditions by: i) enhancing their cell division, increasing biomass in order to reduce the concentration of a toxicant with a higher number of cells (Tikoo *et al*., 1997), ii) varying their chlorophyll content and cell size to cope with environmental changes (Finkel *et al*., 2010;

Leboulanger *et al*., 2011), and iii) modifying their biological, chemical and cellular composition to compensate for the toxicity (Huang *et al*., 2011; Leboulanger *et al*., 2011). The environmental conditions observed in lagoon waters with significant pollution by different chemical compounds (Barhoumi *et al*., 2013a) can promote adaptive mechanisms by phytoplankton according to the concept of Blanck *et al*. (1988).

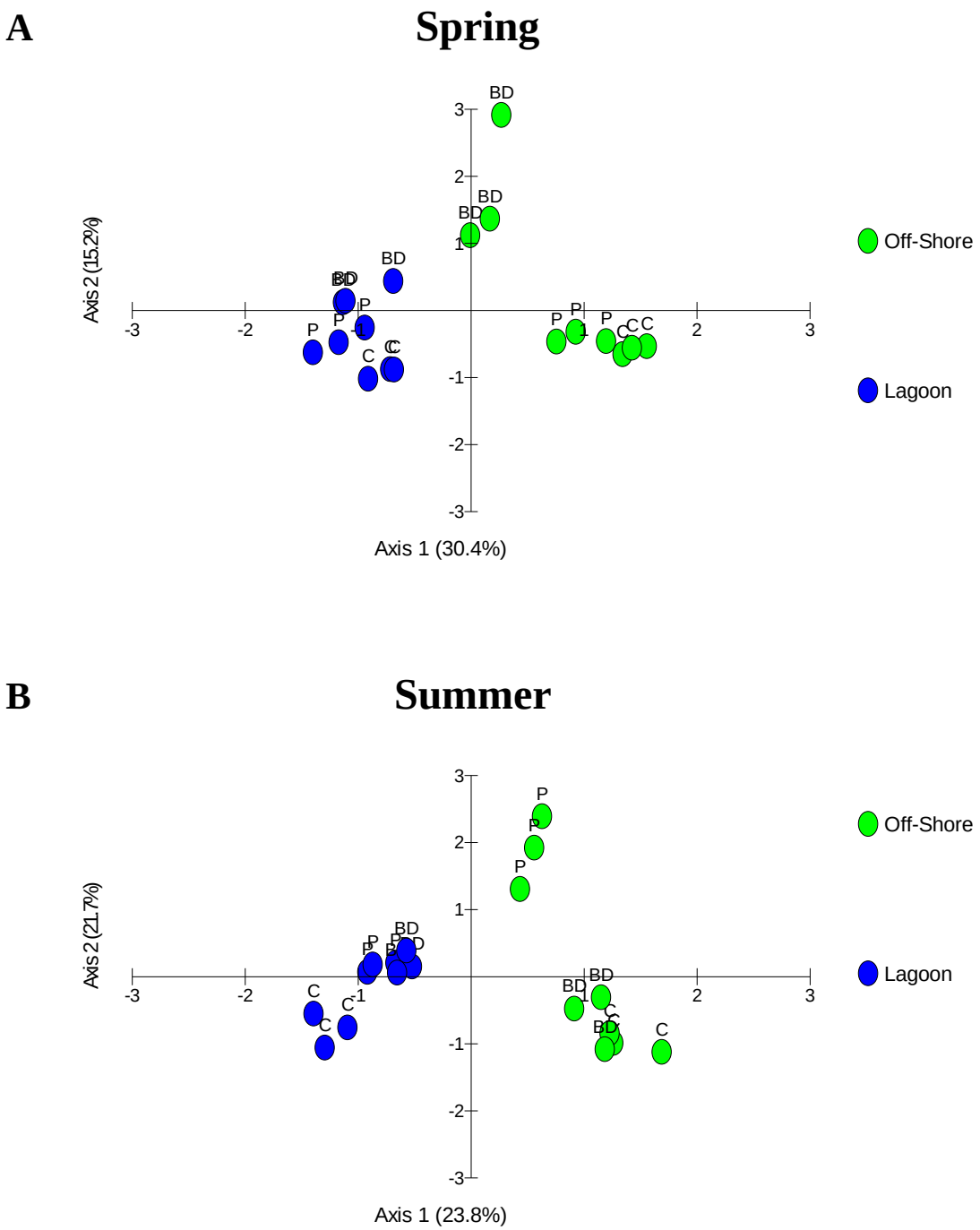


Figure 5: Correspondence analysis scatter plot of the contaminated phytoplankton communities (P and BD) and of the corresponding control (C) obtained at the end of microcosm incubation for the offshore (green circle) and lagoon (blue circle) experiments in spring (panel A) and summer (panel B).

4.4. Impact of plastic derivatives on phytoplankton structure

As observed for biomass, the phytoplankton structure was strongly impacted by the plastic-derived chemicals, irrespective of the season and the station. Nevertheless, the changes in phytoplankton community structure were more marked for offshore waters than for lagoon waters during spring and summer (Figure 5). For offshore waters, structural changes were observed in the contaminated microcosms (P and BD) relative to control. These marked changes were season dependent. The most pronounced dissimilarity was observed in spring for P microcosm, which was concomitant with the important release of BPA and DEHP from plastic items. Interestingly, the communities observed in P and BD microcosms were roughly similar to those observed in lagoon waters, with the dominance of *Leptocylindrus spp.* and *Chaetoceros spp.* The dominance of these species was concomitant with the disappearance of *Nitzschia sp*. and *B. elongatum*. In contrast, in lagoon waters changes were less pronounced, suggesting that lagoon phytoplankton structure exhibited a tolerance to plastic derivatives, as already pointed out for phytoplankton biomass (see above). The apparent tolerance observed for lagoon phytoplankton structure could be due to the possible resistance promoted by species structuring lagoon waters, such as *Chaetoceros spp*., species belonging to the group bacillariophyceae. Indeed, this phytoplankton group was shown to be tolerant to high levels of BPA (Liu *et al*., 2010a). These results were similar to Staniszewska *et al* (2015) findings. They showed that an increase of diatom biomass was in concomitant with BPA rise, in the Gulf of Gdansk. Diatoms produce amounts of EPS (Haugl and Myklestad, 1976; Staats *et al*., 1999) that have the capacity to adsorb phenolic derivatives, which might limits their accumulation in the cells and inhibits their effects. Nevertheless, when focusing on a particular season, few structural changes could be observed between treatments and control experiments for lagoon waters (Figure S2, Annex 5). These slight structural shifts were due mainly to changes in relative abundance of *N. acicularis, L. minimus* and *C. costatus* relative to the control (Table 3).

Table 4 : Relative abundance (means of three replicates ± SD) and percentage contribution of the top five species, indicating which species contributed more to the dissimilarities in abundance (SIMPER analysis) between control (C) and contaminated communities (Plastic and BPA & DEHP) in the two stations (Offshore and Lagoon) and the two seasons, with cumulative contributions of ~50% in spring and ~40% in summer. (Rel. Ab. (%) percentage of relative abundance, Contrib. (%) contribution of phytoplankton species to the dissimilarity).

Season	Station	Species	Control	Plastic		BPA & DEHP	
			Rel. Ab. (%)	Rel. Ab. (%)	Contrib. (%)	Rel. Ab. (%)	Contrib. (%)
Spring	Off-Shore	*Nitzschia cf. acicularis*	53.6±5.8	4.27±1.2	28.4	8.71±0.2	27.0
		Leptocylindrus minimus	7.4±0.9	23.3±6.0	14.8	16.6±1.9	11.1
		Pseudo-nitzschia sp.	1.3±0.4	11.3±0.7	8.9	7.5±0.9	9.7
		Dactyliosolen blavyanus	0.0±0.0	8.5±1.6	7.1	11.1±2.6	6.0
		Chaetoceros laciniosus	4.1±1.5	6.83±2.1	4.0	-	-
		Cylindrotheca closterium	2.9±1.6	-	-	8.4±3.2	5.3
	Lagoon	*Nitzschia cf. acicularis*	34.6±3.5	41.7±1.9	31.4	23.2±1.1	26.3
		Leptocylindrus minimus	15.3±1.7	20.6±1.7	17.7	10.4±0.7	11.4
		Chaetoceros costatus	4.3±0.1	7.6±0.3	8.4		-
		Chaetoceros laciniosus	14.2±1.2	7.1±0.6	6.3	11.9±0.2	7.1
		Dactyliosolen blavyanus	5.0±0.9	0.6±0.0	5.1	2.2±0.1	6.2
		Chaetoceros decipiens	0.5±0.1	-	-	4.2±0.3	6.0

Table 4 (following): Relative abundance (means of three replicates ± SD) and percentage contribution of the top five species, indicating which species contributed more to the dissimilarities in abundance (SIMPER analysis) between control (C) and contaminated communities (Plastic and BPA & DEHP) in the two stations (Offshore and Lagoon) and the two seasons, with cumulative contributions of ~50% in spring and ~40% in summer. (Rel. Ab. (%) percentage of relative abundance, Contrib. (%) contribution of phytoplankton species to the dissimilarity).

Season	Station	Species	Control	Plastic		BPA & DEHP	
			Rel. Ab. (%)	Rel. Ab. (%)	Contrib. (%)	Rel. Ab. (%)	Contrib. (%)
Summer	Off-Shore	*Bacteriastrum elongatum*	21.4±2.4	3.3±1.1	20.4	11.2±1.8	21.2
		Chaetoceros tortissimus	0.0±0.0	15.7±3.4	16.0	-	-
		Leptocylindrus danicus	5.2±0.9	13.2±1.6	7.4	-	-
		Cylindrotheca closterium	0.6±0.3	6.6±0.5	6.0	-	-
		Chaetoceros costatus	11.3±1.5	7.7±1.1	5.6	5.1±1.0	12.0
		Chaetoceros affinis	0.0±0.0	-	-	11.5±2.0	10.2
		Leptocylindrus minimus	9.6±1.5	-	-	6.0±1.8	6.2
		Chaetoceros constrictus	10.6±1.6	-	-	11.3±1.4	5.9
	Lagoon	*Leptocylindrus minimus*	14.5±1.7	6.1±0.5	12.7	6.7±0.7	5.3
		Chaetoceros costatus	2.2±0.7	10.1±1.4	12.1	4.2±0.9	4.2
		Chaetoceros affinis	9.0±2.5	7.2±1.1	7.4	-	-
		Pseudo-nitzschia sp.	4.3±1.0	2.1±0.5	4.6	-	-
		Pseudo-nitzschia "seriata"	2.1±0.5	5.4±1.1	4.3	-	-
		Chaetoceros curvisetus	11.0±2.4	-	-	17.4±3.0	16.5
		Chaetoceros constrictus	8.2±1.1	-	-	14.5±0.6	14.2
		Leptocylindrus danicus	5.0±0.7	-	-	10.0±0.1	10.4

Structural changes could be observed for both stations and at both seasons upon contamination with plastic derivatives; those structural changes were less pronounced for lagoon waters. These changes were provoked either by the station or by the season. In other words, the impact of the external anthropic factor (artificial chemical contamination) was less important than that of the abiotic factors (season and site) that usually govern phytoplankton composition in coastal environments. Such differences between natural and anthropogenic factors have previously been reported by Louati *et al*. (2013), who observed a greater effect of a biotic factor (nematode predation) relative to chemical contamination by PAH in the bacterial community structure in coastal sediments.

As mentioned for phytoplankton biomass, data in the literature regarding the impacts of plastic derivatives on phytoplankton structure are scarce. Nevertheless, similar studies performed with other chemical compounds have also reported phytoplankton structural changes (Pérez *et al*., 2006; Pandey *et al*., 2015; Ben Othman *et al*., 2017). *N. longissima*, *N. palea* and *L. danicus* were reported to be sensitive to sediment resuspension and to heavy metals at low levels of contamination (Pérez *et al*., 2006; Pandey *et al*., 2015; Ben Othman *et al*., 2017). Interestingly, in our study we also observed that the relative abundance of *Nitzschia spp.* and *Leptocylindrus spp*. decreased upon plastic-derivative contamination, suggesting a low tolerance of these species to BPA and DEHP.

In addition, previous studies performed on monospecific phytoplankton species showed that the growth of *Alexandrium pacificum* and *Cochlodinium polykrikoides* was significantly sensitive to BPA and DEHP contamination (Ebenezer and Ki, 2012; Couet *et al*., 2018; M'RABET *et al*., 2018). The dominance of *Chaetoceros spp.* upon plastic-derivative contamination is in agreement with the tolerance of this species to PAHs (Hallare *et al*., 2011; Ben Othman *et al*., 2012); tolerance that might be explained by the size of the cells (Ben Othman *et al*., 2012). Diatoms have a low surface/volume ratio compared to smaller species such as picoplankton, so favoring their tolerance to toxic chemical compounds.

5. Conclusion

The present study showed that the offshore and lagoon waters in the Bizerte area were more contaminated DEHP relative to similar coastal areas. BPA and DEHP impacted strongly the

phytoplankton biomass and structure, with a more pronounced effect offshore relative to lagoon, suggesting a possible phytoplankton resistance in this ecosystem. Nevertheless, the structural changes observed upon contamination were smaller than those induced by the effect of season. Our study has identified few species that might be proposed as bioindicators of anthropogenic impacts on coastal ecosystems, such as *Chaetocerosspp.*, belonging to bacillariophyceae, a phytoplankton group already used in pollution assessment (Pandey *et al*., 2014). Further studies are required to understand better the mechanisms developed by phytoplankton to face these anthropogenic compounds, considering their expected increase in occurrence in coastal environments as a result of the huge growth in world plastic production.

Acknowledgment

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Supplementary material

Table S1: Nature and size of manufactured plastic items used for contaminated seawater preparation.

Plastic item	Color	Surface nature/ texture	Weight (gr) (mean of 3 replicates)	Lenght/widht (cm)	Lenght (cm)	Thickness (cm)	Diameter (cm)	Occurrence of BPA	Occurrence of DEHP
1 Blood bag	Pellucid	±rough	8.62	13.0 × 5.80	-	-	-		
2 Plastic bag	Blue	Smooth	1.57*	10.0 × 16.08	-	-	-		×
1 PVC tube	Grey	Smooth	64.08	-	9.16	-	8.00		×
1 Plexiglass	Clear	Smooth	61.00	-	-	3.00	-	×	

(*: mean of 6 replicates)

Table S2: DEHP and BPA were quantified by isotopic dilution (Internal standard compound DEHP-d4 and BPA-d16, respectively), the validation parameters for chemical analyses are mentioned in the table below:

Compound	Quantification transition (Collision energy) (m/z)	Quantification confirmation (Collision energy) (m/Z)	Retention time (min)	Dwell (ms)	Internal standard compound	Quantification transition (Collision energy) (m/z)	Rtention time (min)	Dwell (ms)
DEHP	149	167	23.98	100	DEHP-d4	153	23.97	100
BPA	227>212.1 (22)	227>132.9 (25)	1.1	120	BPA-d16	241.2>223.2 (20)	1.09	120

Figure S1: Study site with the location of the sampling stations (North of Tunisia, Southern Mediterranean Sea). O: offshore; L: lagoon (Pringault et al., 2016)
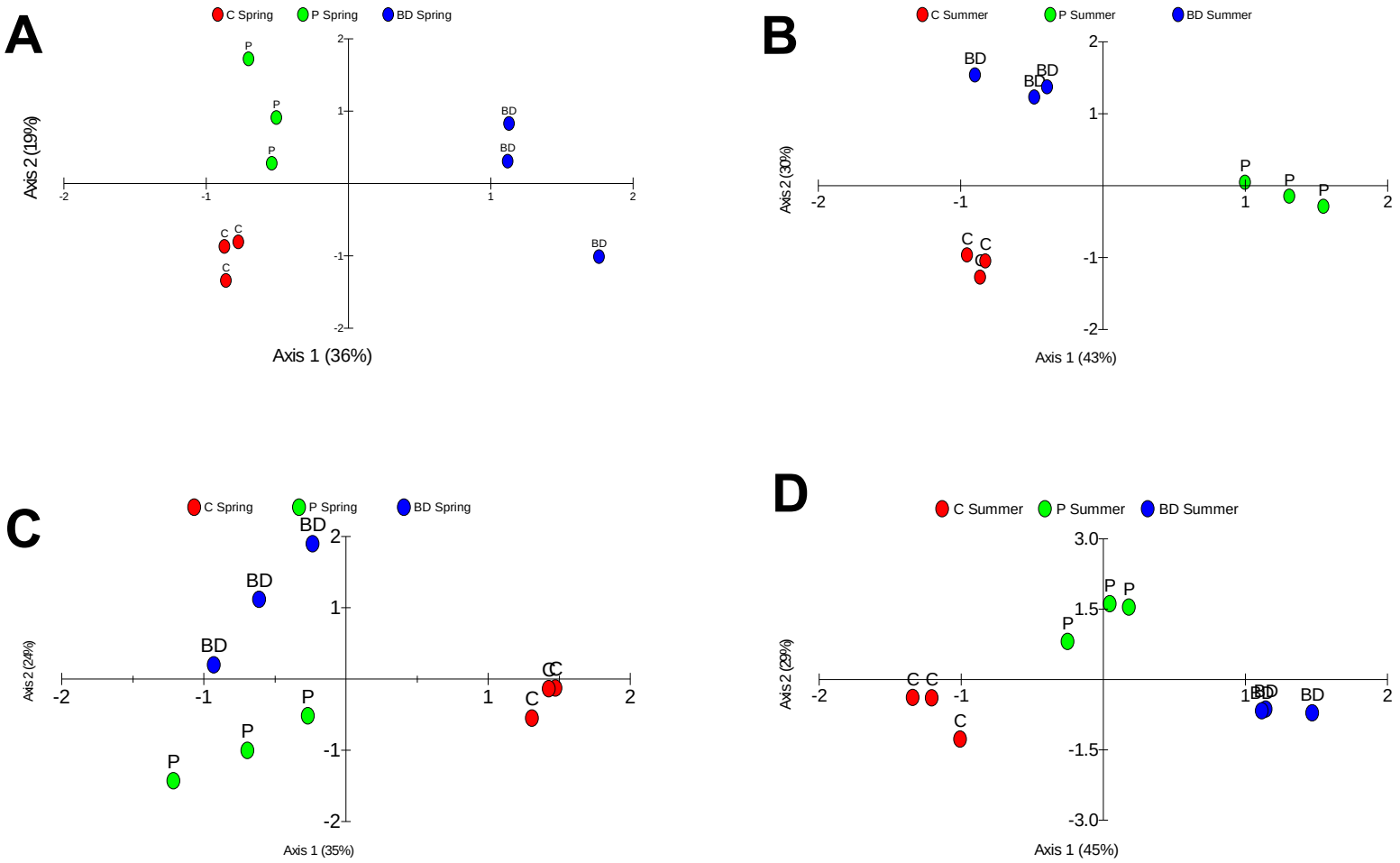
Figure S2: Correspondence analysis obtained after 96 h of incubation from the relative contributions of identified phytoplankton for the offshore waters (in spring panel A and in summer panel B) and the lagoon waters (in spring panel C and in summer panel D). Red circles: controls; Green circles: plastic enriched treatment; Blue circles: pure mixture of BPA and DEHP treatment.