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Rapid responses of pristine marine planktonic communities in experimental approach to diuron and naphthalene (Juan de Nova Island, Western Indian Ocean)

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Abstract. Planktonic communities from Juan de Nova lagoon were submitted to a single exposure of diuron or naphthalene in triplicate outdoor 40-L mesocosms for 5 days. The study followed the temporal changes of the assemblages by determining abundances and taxonomic level of microbial (heterotrophic prokaryotes, picocyanobacteria, heterotrophic protists and phytoplankton) and metazooplankton communities. Exposure to both contaminants did not result in significant changes in phytoplankton abundances. Microscopic identification demonstrated the dominance of Bacillariophyta (mostly *Chaetoceros tenuissimus*) accounting for more than 95% of the microalgae detected in all mesocosms. Using *18S* rRNA metabarcoding, temporal changes in the proportion of eukaryotic operational taxonomic units (OTUs) were highlighted for most taxonomic groups. Diuron had no significant effect on the abundance of eukaryotic picoheterotrophs, ciliates, whereas naphthalene had a significant positive effect on heterotrophic prokaryotes. Metazooplankton was not significantly modified under contaminant exposure, except for copepods nauplii in presence of diuron. Multivariate analysis indicated significant responses of communities linked to the experimental time and conditions. The analysis also revealed a modification of the trophic relationships among communities over time, from a dominant multivorous food web after 48 h towards a reduced microbial loop after 120 h of experiment time.

Keywords: contaminant, taxonomy, metabarcoding, plankton, experiments, lagoon.

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Introduction

Intensive agriculture, domestic waste and industrial activities are the source of many organic and inorganic compounds discharged into coastal marine systems, and can induce detrimental effects on living organisms and ecosystems (Doney 2010). However, our current knowledge on the toxic effect of contaminants on natural assemblages of marine plankton is still limited, owing to the diversity of organisms and the complexity of their physiological responses (Staley *et al.* 2015). A major concern lies in the fact that this diversity of marine organisms could be affected, since pollution pressure is globally increasing worldwide, accounting for a significant part of the human footprint on oceans (Doney 2010; Halpern *et al.* 2019). Because pristine ecosystems are rarefying, assessment of the sensitivity of reference communities to contaminants will be helpful in evidencing and forecasting the pollution-driven changes in biodiversity and community structures that are likely to occur in contaminated areas (Fleeger *et al.* 2003). Coral reefs and atoll lagoons are among the most diversified marine ecosystems but they are also the most threatened by the combined effects of climate change and human activities (Hoegh-Guldberg *et al.* 2017; Hughes *et al.* 2017; Kaimba *et al.* 2019). However, the Iles Eparses (Scattered Islands), which Juan de Nova is part of, located in the Western Indian Ocean around Madagascar, are

almost unperturbed by human action and can be considered as 'pristine' ecosystems (Bouvy *et al.* 2016; Dupuy *et al.* 2016; Quétel *et al.* 2016). The coral-reef lagoons of these islands are, therefore, proposed as reference sites for the general evaluation of the impact of anthropogenic pressures. According to the restrictive regulations of these French overseas territories, anthropogenic pressure on these ecosystems is not expected to increase in the near future because human settlements are prohibited (Quétel *et al.* 2016). Nonetheless, there are residual risks of accidental pollution from ship traffic even around these remote islands, as reported by González *et al.* (2009).

Among threats to marine life, oil tanker wrecks and accidental or deliberate discharges of oil constitute a source of highly toxic polycyclic aromatic hydrocarbons (PAHs), such as pyrene or naphthalene (González *et al.* 2009, 2013). Low molecular-weight PAHs such as naphthalene ($C_{10}H_8$) are the most prone to contaminate open water, owing to higher water solubility (Bera *et al.* 2020). Reported concentrations of naphthalene in seawater are from 0.076 to 7.8 μg L^{-1} according to a recent review (Mojiri *et al.* 2019); thus, this molecule was chosen as the PAH representative for investigating the effect of its toxicity on Juan de Nova planktonic communities. Studies on the toxic effects of PAHs are sometimes contradictory, especially regarding phytoplankton response: (i) toxicity thresholds change depending on phytoplankton origin (Ohwada *et al.* 2003; Sargian *et al.* 2005; Hjorth *et al.* 2007; Hjorth *et al.* 2008), (ii) cell size often appears important in observed sensitivity, whereas mixture toxicity cannot easily be predicted from single-species experiments (Echeveste *et al.* 2010; Ben Othman *et al.* 2012). Differences in sensitivity of taxonomic groups have also been reported (Harrison *et al.* 1986; Hjorth *et al.* 2008). Exposure to PAHs often affects primary producers, but their response is partly determined by the structure and controlling factors of the food web (Hjorth *et al.* 2008). Among microorganisms, numerous taxa are able to degrade PAHs (Ghosal *et al.* 2016), and Garcia *et al.* (1998) highlighted that marine bacteria from polluted environments develop mechanisms to tolerate and even degrade naphthalene as a potential growth substrate.

Pollution of aquatic ecosystems by pesticides is also a major environmental concern (Schwarzenbach *et al.* 2006; OSPAR 2009). Among pesticides, herbicides with photosynthetic inhibitors have been widely used for more than half a century (Wessels and van der Veen 1956). Among these herbicides, diuron has been extensively used in agriculture and gardening (Wauchope 1978), as well as an antifouling biocide in ship paints, and is considered a significant threat to coastal marine life globally (Amara *et al.* 2018). This direct threat to marine life is amplified by the increasing use of diuron to replace banned organotin compounds in antifouling paints (Voulvoulis *et al.* 2002; Okamura *et al.* 2003; Martins *et al.* 2018). Okamura *et al.* (2003) reported mean water concentrations of diuron close to 3 μg L^{-1} in Japanese coastal systems, whereas common levels of contamination are within the nanograms per litre range (e.g. Batista-Andrade *et al.* 2016; Köck-Schulmeyer *et al.* 2019). A legal guideline safety criterion of 1.8 μg L^{-1} was since established by the European Union (Warne *et al.* 2018). Diuron has been shown to result in damages to seagrasses (Wilkinson *et al.* 2017) and coral symbionts (Negri *et al.* 2011), often linked to coastal discharges from rivers contaminated by agricultural drainage (Holmes 2014).

The effects of a single or multiple doses of a given contaminant (PAH or herbicide) have previously been studied on natural phytoplankton assemblages in marine and freshwater environments (Pesce *et al.* 2006; Hjorth *et al.* 2007; González *et al.* 2009; Leboulanger *et al.* 2011; Ben Othman *et al.* 2018). These studies have allowed a better understanding of the effect of toxicants on physiological properties on the scale of a species, population or community, but without accounting for long-term tolerance and adaptation of these organisms to pollution stress. Actually, the observed toxicity could be dependent on the existence of a previous exposure of the phytoplankton community to the tested chemical in a polluted water body, leading to an increased tolerance by selection pressure over years (Larras *et al.* 2016).

The ways that exposure to contaminants, such as diuron or PAHs, affects a plankton system are partly determined by the structure and controlling factors (top-down and bottom-up) of the food webs (González *et al.* 2009). Many studies have demonstrated that phytoplankton is affected by such chemical compounds during short-term (1 week) experimental exposures (Ben Othman *et al.* 2018; Leboulanger *et al.* 2011), with a detrimental effect of contaminants on phytoplankton biomass and activity, which can further result in indirect effects on bacterial and metazooplankton communities (Hjorth *et al.* 2007). Green algae were demonstrated to metabolise PAHs more efficiently than do cyanobacteria, with dioxygenase initiating naphthalene degradation as was similarly reported for bacteria (Warshawsky *et al.* 1995). Reactive oxygen species (ROS) are produced during PAH biodegradation and this large amount of ROS is likely to explain the observed toxicity (Kong *et al.* 2010). Soto *et al.* (1975) reported that a population of the chlorophyte *Chlamydomonas angulosa* in culture was almost eradicated during a 7-day exposure to a naphthalene-saturated medium, but the surviving cells were able to restore growth after PAH dissipation. Both intrinsic metabolic abilities of microalgae (Sakshi and Haritash 2020) and low persistence of PAHs in water due to volatilisation (Bidleman 1988) suggest that microalgae can resist short-term PAH exposure.

The central assumption of the present study, which was conducted between November and December 2013, states that the microbial communities of Juan de Nova lagoon are representative of weakly affected marine ecosystems (also called 'pristine'; Bouvy *et al.* 2016). Consequently, the planktonic communities (picoheterotrophs, picocyanobacteria, nanoflagellates, microalgae, ciliates and metazooplankton) are hypothetically all sensitive to the toxic pollutants, and could be considered as potential reference organisms when studying the susceptibility of coastal tropical ecosystems to pollution. Our experimental approach aimed to evaluate the short-tern responses (5 days) of the different microbial and metazooplanktonic communities sampled in the coastal area of the Juan de Nova Island, during exposure to two model contaminants, namely, diuron as an herbicide and naphthalene as a PAH. A 5-day exposure was chosen to allow direct acute-effect observations, long enough to show indirect effects through trophic relationships and short enough to avoid long-term acclimation of tolerant organisms or selection of potentially resistant ones (Ben Othman *et al.* 2018; Pringault *et al.* 2020). This approach was undertaken using planktonic microcosm experiments under nutrient supply, so as to compare biological responses with direct and indirect effects of the contaminants.

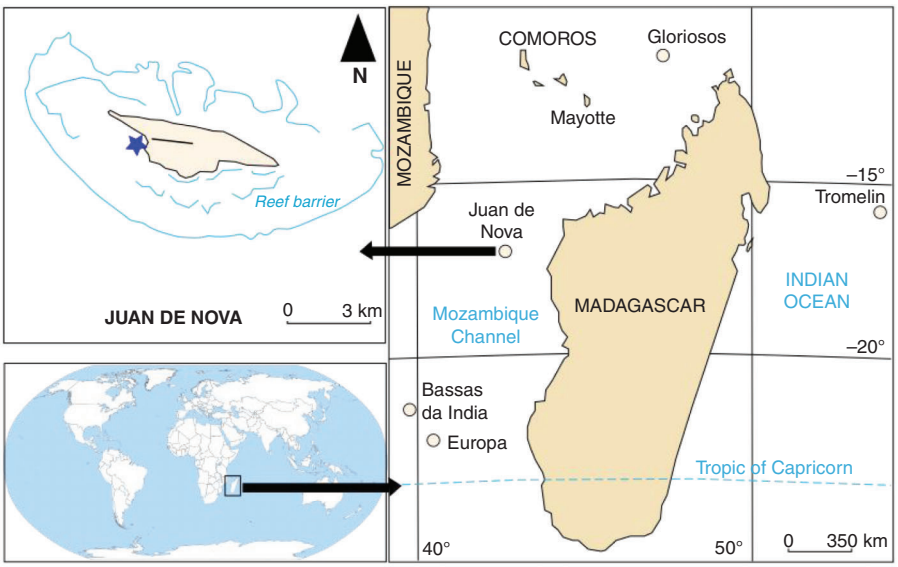

Fig. 1. Location of the Juan de Nova Island in the Mozambique Channel, western Indian Ocean. Sampling location is indicated by a blue star.

Materials and methods

Study site and experimental design

The Iles Eparses are small coral-reef islands located in the Indian Ocean close to Madagascar (from 22°21′S to 12°46′S and 39°44′E to 54°31′E, Fig. 1). Among these islands, Juan de Nova is located in the Mozambique Channel (17°03′S, 42°45′E), 200 km west of Madagascar and 285 km off from East Africa (Fig. 1). The emerged island has a surface of ~5 km^2, and the overall coral-reef structure represents a total area of 250 km^2. The length of the island is 6 km, and the maximum width is 1.7 km (Chabanet and Durville 2015). The island has a 1 km long airstrip for military purposes (permanent settlement of 14 people).

Twelve 40-L mesocosms were constructed using a 200-µm-thick PTFE (polytetrafluoroethylene) liner, previously soaked in 5% hydrochloric acid and thoroughly rinsed using Milli-Q water before use. Polytetrafluoroethylene (PTFE) was chosen to minimise release and adsorption of chemicals and fouling during the experiment (Sargian *et al.* 2005). Each microcosm was fitted within a rectangular openwork PVC box (0.6 × 0.4 × 0.4 m). Three of these mesocosms were used as controls (C), and the other nine held the three experimental treatments in triplicate, with similar nutrient enrichment of 2.5 µM of NH_4^+ (ammonium chloride; Sigma–Aldrich), 2.5 µM of NO_3^- (sodium nitrate; Sigma–Aldrich) and 1 µM of PO_4^{2-} (sodium phosphate monobasic; Sigma–Aldrich), nutrient enrichment alone (E), nutrients plus diuron (1.2 µg L^{-1} nominal concentration) (D), and nutrients plus naphthalene (25 µg L^{-1} nominal concentration) (N). Nutrient (nitrogen and phosphorus) enrichments were repeated after 24 h and 96 h so as to reduce nutrient limitation and to avoid community collapse during incubation, while maintaining the detection of both direct and indirect effects of contaminant toxicity (Hjorth *et al.* 2008; Leboulanger *et al.* 2011). A multiplicative factor was calculated for each variable so as to easily visualise their responses to treatment, considering the concentration ratio between the initial and final concentrations after 120 h (Table 1).

Dissolved organic carbon (DOC) concentration in lagoon waters of Juan de Nova Island were determined, with a mean value of 81.3 ± 5.3 µM (Bouvy *et al.* 2016). As a consequence, using organic solvents according to classical methods in ecotoxicology (Bérard 1996; e.g. 0.05% v/V methanol, ~12 mM) would represent a large amount of DOC that would interfere with potential toxicity responses, especially for heterotrophic bacterioplankton that could potentially grow using methanol as a carbon source (Dinasquet *et al.* 2018). Diuron and naphthalene solutions were therefore prepared by saturation of 0.2 µm filtered seawater stirred for 96 h at room temperature. Solutions were then filtered again through glass-fibre filters (GF/F) to remove undissolved material, and clear diuron and naphthalene stock solutions were stored at 4°C until use, assuming a seawater solubility of 35 mg L^{-1} for diuron (Thomas *et al.* 2001) and 32 mg L^{-1} for naphthalene (Mackay and Shiu 1977).

Using stainless-steel buckets, 480 L of raw lagoon water were collected from the surface during the morning, and gently stirred in a 1000-L polyethylene tank, after filtration through a 200-µm mesh, to remove large grazers, before controlled inoculation of metazooplankton the following day. All mesocosms were placed in an outdoor pool (4-m^3 volume) filled with seawater so as to buffer daily temperature changes. The pool was covered with a screen to reduce high irradiance when the sun was near zenith. Three spherical PAR loggers (MDS-MkV, Alec Electronics, Japan) were randomly placed in the middle of three mesocosms, to record daily scalar irradiance. Oxygen concentrations were monitored using optode loggers inside the mesocosms (HoBo U26-001, Onset, USA), together with temperature (HoBo U22-001, Onset). Each microcosm was equipped with a 60-L min^{-1} aquarium pump to ensure water homogenisation during the incubation. The randomisation of the experimental design included a daily displacement of the

Table 1. Concentrations (mean and s.d.; $n = 3$) of nutrients (PO_4^{3-}, $NO_2^- + NO_3^-$, NH_4^+; µM), particulate organic carbon (POC, µg L^{-1}) and nitrogen (PON, µg L^{-1}), carbon : nitrogen ratio (C : N) and chlorophyll-*a* (Chl-*a* total and <3 µm; µg L^{-1}) during the incubation experiment (0, 48 and 120 h) for the different treatments

	0 h		48 h		120 h		Factor
	Mean	s.d.	Mean	s.d.	Mean	s.d.	
PO_4^{3-} (µM)							
C	0.02	(0.004)	0.04	(0.031)	0.05	(0.031)	2.42
E	0.02	(0.004)	0.59	(0.064)	3.03	(0.418)	159.31
D	0.02	(0.004)	0.37	(0.155)	3.05	(0.284)	160.52
N	0.02	(0.004)	0.51	(0.214)	2.50	(0.088)	131.47
$NO_2^- + NO_3^- = NO_x$ (µM)							
C	0.25	(0.030)	0.07	(0.027)	0.33	(0.197)	1.29
E	0.25	(0.030)	9.30	(0.353)	18.98	(1.806)	75.00
D	0.25	(0.030)	8.94	(0.777)	17.91	(1.050)	70.79
N	0.25	(0.030)	8.98	(5.301)	17.77	(5.613)	70.22
NH_4^+ (µM)							
C	0.05	(0.001)	n.d.	n.d.	1.19	(0.520)	26.00
E	0.05	(0.001)	n.d.	n.d.	8.40	(1.805)	182.63
D	0.05	(0.001)	n.d.	n.d.	11.22	(0.623)	243.80
N	0.05	(0.001)	n.d.	n.d.	3.18	(0.875)	69.17
Chl-*a* (µg L^{-1})							
C	3.10	(0.41)	2.79	(0.52)	1.01	(0.14)	0.32
E	3.10	(0.41)	10.75	(0.61)	5.20	(1.12)	1.67
D	3.10	(0.41)	12.62	(1.46)	5.50	(2.10)	1.77
N	3.10	(0.41)	11.57	(3.48)	8.26	(0.07)	2.66
POC (µg L^{-1})							
C	432.50	(27.40)	387.50	(25.60)	242.40	(23.3)	0.56
E	432.50	(27.40)	482.30	(17.80)	475.00	(56.7)	1.10
D	432.50	(27.40)	505.50	(57.80)	365.70	(33.3)	0.85
N	432.50	(27.40)	486.50	(87.50)	500.30	(14.9)	1.16
PON (µg L^{-1})							
C	53.90	(0.50)	49.60	(6.80)	46.20	(9.2)	0.86
E	53.90	(0.50)	77.20	(6.20)	92.80	(10.2)	1.72
D	53.90	(0.50)	84.60	(8.80)	101.30	(10.9)	1.88
N	53.90	(0.50)	86.70	(14.70)	182.50	(17.4)	3.39
C : N							
C	8.03	(0.44)	7.87	(0.73)	5.38	(1.22)	0.67
E	8.03	(0.44)	6.27	(0.36)	5.13	(0.42)	0.64
D	8.03	(0.44)	5.97	(0.29)	3.63	(0.44)	0.45
N	8.03	(0.44)	5.61	(0.29)	2.76	(0.31)	0.34
Chl-*a* < 3 µm (µg L^{-1})							
C	0.04	(0.04)	0.08	(0.04)	0.15	(0.06)	3.75
E	0.04	(0.04)	2.17	(0.07)	1.11	(0.51)	27.75
D	0.04	(0.04)	2.84	(1.06)	1.24	(0.50)	30.63
N	0.04	(0.04)	2.07	(1.44)	2.90	(1.23)	72.50

tanks within the pool. Diuron stock solution was added at 1.5 mL for each corresponding 40-L mesocosm, whereas naphthalene was added at 30 mL for the same final volume, resulting in nominal concentrations of 1.3 and 24.0 µg L^{-1} for diuron and naphthalene respectively.

To inoculate the metazooplanktonic community, a 200-µm mesh plankton net (40 cm aperture diameter) was towed obliquely for a distance of 30–40 m so as to collect a sample volume corresponding approximately to 4000 L of water, concentrated down to 200 mL. Subsamples (100 mL) were preserved using formalin (5% final concentration) until further examination. Remaining living metazooplankton was then homogeneously distributed in each microcosm, at an initial concentration of individuals equal to 6× the *in situ* density; this step was performed on site using a stereomicroscope.

Subsamples were collected from the mesocosms before any addition of the accommodated fractions (nutrients, contaminants; 0 h), after 48 h and after 120 h to determine chlorophyll-*a* (Chl-*a*) concentrations and other chemical parameters. Additional samples were taken at 24, 72 and 96 h to determine the autotrophic and heterotrophic components (see below); this choice of the number of samples per parameter was guided by the volume of each sample so as to keep at least two-thirds of the total volume at the end of the experiment.

Chemical variables

Dissolved organic carbon (DOC) analyses were performed on 30 mL subsamples collected in pre-combusted (450°C overnight) glass vials, preserved with 35 µL of 85% phosphoric acid. Samples were stored in the dark until analysis by using a Shimadzu TOC VCPH analyser (Rochelle-Newall *et al.* 2008*a*). Samples for dissolved nutrients analysis (NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{2-}-P) were filtered through Whatman GF/F fibreglass filters, stored at –20°C and analysed as described by Strickland and Parsons (1968). Sampled water was filtered through pre-combusted GF/F Whatman filters, which were collected for particulate organic nitrogen (PON) and particulate organic carbon (POC) analyses, completed using a nitrogen carbon analyser after decarbonation through the addition of acid.

Naphthalene concentration in the sterile seawater stock solution was determined using UV absorbance readings at 280–290 nm on a U-3000 spectrophotometer (Hitachi). Effectiveness of the naphthalene dissolution was assessed by monitoring the absorbance at a wavelength of 286 nm (A_{286}) twice a day during dissolution, and comparing readings with seawater without naphthalene. Naphthalene in contaminated mesocosms was quantified after liquid–liquid extraction using gas chromatography–mass spectrometry (GC-MS) according to suitable PAH detection methods in seawater (Pringault *et al.* 2016). The concentration of diuron in the sterile seawater stock solution was determined using liquid chromatography–mass spectrometry (LC-MS) and deuterated atrazine as the internal standard, after solid-phase extraction onto an Oasis HLB cartridge (Pringault *et al.* 2016).

Autotrophic components

Chlorophyll-*a* concentrations (total and below 3-µm fraction) were determined by fluorometry after filtration onto Whatman GF/F fibreglass filters, and directly extracted with methanol (Yentsch and Menzel 1963).

Picophytoplankton samples were fixed with formaldehyde (2% final concentration), stored in liquid nitrogen (–196°C) until analysis with a Facs Aria Flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a HeNe air-cooled laser (633 nm, 20 mW). Cells excited at 633 nm were detected and enumerated according to their forward-angle light scatter (FALS) and right-angle light scatter (RALS) properties, as well as their orange (576/26 nm) and red fluorescence (660/20 nm and 675/20 nm) from phycoerythrin, phycocyanin and chlorophyll pigments respectively. The flow rate was of 40 µL mL^{-1} so as to obtain 200–300 events per second. Fluorescent beads (1–2-µm size) for picophytoplankton were systematically added to each sample. True count beads (Becton Dickinson, San Jose, CA, USA) were added to determine the exact sample volume. List-mode files were analysed using BD FACSDiva software. This method discriminates between various autotrophic groups such as autotrophic picoeukaryotes and picocyanobacteria (*Prochlorococcus* and *Synechococcus*) using their phycoerythrin and phycocyanin content as well as their chlorophyll pigments (Bouvy *et al.* 2016).

For nano-microphytoplankton (called here 'microalgae', larger than 5-µm size), water samples (500 mL) were concentrated to 50 mL by gravity filtration onto a Nuclepore filter (5-µm pore size), and fixed with alkaline lugol iodine (2% final concentration), according to Bouvy *et al.* (2016). Samples were stored in the dark at 4°C until analysis. Microalgae were enumerated using an Utermöhl settling chamber (Hydro-Bios) under an inverted microscope (Olympus IX70), equipped with a digital camera (Motic Moticam Pro).

Heterotrophic components

For heterotrophic prokaryotes (H-Prok: bacteria and archaea), samples were fixed with prefiltered (0.2-µm pore size) buffered formaldehyde (2% final concentration) and stored in liquid nitrogen (–196°C) until analysis. Cells were enumerated by flow cytometry according to the protocol described by Marie *et al.* (1997), slightly modified with the use of a higher fluorochrome concentration (Bouvy *et al.* 2016). First, 1 mL of fixed subsample was incubated with SYBR Green I (Molecular Probes, Eugene, OR, USA), yielding a final concentration of 1/375 (v/v) for 15 min at 4°C in the dark. For each subsample, three replicates were then counted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon laser (488 nm, 15 mW). Stained cells, excited at 488 nm, were enumerated according to their RALS and green fluorescence (FL1) using a 530/30-nm filter with a flow rate of 20 µL mL^{-1} to count 300/500 events per second. These cell parameters were recorded on a 4-decade logarithmic scale mapped onto 1024 channels. Fluorescent beads (1 and 2 µm, Polysciences, Warrington, PA, USA) were systematically added to each sample. Standardised RALS and FL1 values (cell RALS and FL1 divided by 1-µm bead RALS and FL1 respectively) were used to estimate the relative size and nucleic acid content of TP cells respectively (Troussellier *et al.* 1999). True Count beads (Becton Dickinson, San Jose, CA, USA) were added to determine the volume that was required for analysis. List-mode files were analysed using BD Cellquest Pro software (ver. 5.2.1, BD, San Jose, CA, USA, see https://www.bdbiosciences.com).

Heterotrophic prokaryote production (Thym) was estimated using the DNA synthesis rates measured by ^{3}H-methyl thymidine (^{3}H-TdR) incorporation using the microcentrifuge method (Smith and Azam 1992). A sample aliquot (1.4 mL) was added to a sterile polystyrene snap cap tube containing a final saturating concentration of 20 nM of ^{3}H-TdR (specific activity 53 Ci $mmol^{-1}$, Amersham). Triplicate live samples and a control were run for each assay. Abiotic incorporation controls were prepared by adding 70 µL of 100% of trichloroacetic acid (TCA) 15 min before the addition of ^{3}H-TdR. All samples were then incubated in the dark at *in situ* temperature for a short time (no longer than 1 h). Incorporation was terminated by adding 70 µL of 100% TCA. Samples were stored for at least 2 h at 4°C and then centrifuged at 14 000*g* for 15 min. The precipitates were rinsed three times with 5% TCA and once with 70% ethanol and resuspended in 1.5 mL of liquid scintillation cocktail (Ultima Gold LLT, Perkin Elmer), before determining the incorporated radioactivity by liquid scintillation (Beckman LS 6500).

So as to enumerate heterotrophic nanoflagellates (HNF), water samples were fixed with paraformaldehyde (2% final concentration) and stored at 4°C. Then, 25 mL were stained with DAPI (final concentration, 15 mg mL^{-1}) for 15 min, filtered onto a black Nuclepore filter (0.8-µm pore size),

stored at –20°C, and HNF were then counted using an epifluorescence microscope (Nikon Eclipse TE200) under UV excitation (Boenigk *et al.* 2004).

For microzooplankton (ciliate) abundances, water samples (500 mL) were concentrated to 50 mL by gravity filtration onto a Nuclepore filter (5-µm pore size), fixed with alkaline lugol iodine (2% final concentration) according to Bouvy *et al.* (2016), and stored at 4°C in the dark until analysis. Ciliates were enumerated in an Utermöhl settling chamber (Hydro-Bios) using an inverse microscope (Zeiss Axiovert, magnification 400×).

Metazooplankton abundance in the mesocosms was estimated the final day (after 120 h of experiment). The total remaining volume of each microcosm was passed through a 60-µm mesh to collect metazooplankton. Specimens were preserved using 5% final concentration of formalin and counted under a stereomicroscope (Olympus SZX200), using a Dolfuss counting chamber and identified following the methods of Tregouboff and Rose (1957*a*, 1957*b*) and Conway *et al.* (2003).

Metabarcoding of microbial eukaryotes

Water samples were collected (one sample per microcosm) at initial and final (120 h) time, on Millipore polycarbonate filters (0.2-µm pore size). The filters were stored in liquid nitrogen (–196°C) until nucleic acid extraction was performed as described by Lefranc *et al.* (2005). DNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), and extracts were stored at –20°C until polymerase chain reaction (PCR) amplification and sequencing.

A 260 bp long fragment of the V7 region of the *18S* rRNA gene was amplified using general eukaryotic primers 960F (5′-GGCTTAATTTGACTCAACRCG-3′; Gast *et al.* 2004) and NSR1438 (5′-GGGCATCACAGACCTGTTAT-3′; Van de Peer *et al.* 2000). Each PCR was performed in duplicate with a total mix volume of 25 µL, containing 3 µL of 10× NH_4 reaction buffer, 1.2 µL of 50 mM $MgCl_2$, 0.25 µL of BioTaq (Bioline), 0.24 µL of 10 mM dNTP, 0.36 µL of 0.5 µg μL^{-1} bovine serum albumin (BSA) and 1 µL of each primer. The amplification conditions consisted of an initial denaturation at 94°C for 10 min followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min 30 s at 72°C. The amplicons were subjected to a final 10 min extension at 72°C. Amplicons were purified using Illustra GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare Life Sciences, Velizy Villacoublay, France) and quantified using Quant-it PicoGreen kit (Invitrogen, Carlsbad, CA, USA) on a Fluoroskan Ascent FL (ThermoLabsystems, Beverly, MA, USA). Finally, the purified amplicons corresponding to each sample were pooled at equimolar concentrations and sent to Fasteris SA (Geneva, Switzerland) for library preparation and paired-end (2 × 250 bp) sequencing on a MiSeq Illumina instrument (San Diego, CA, USA). The detailed protocols were reported in Capo *et al.* (2016).

The paired-end reads were merged together using UPARSE (ver. 70., see https://www.drive5.com/uparse/; Edgar 2013) tools (option -fastq_mergepairs with a minimal overlap equal to 150 and no mismatch allowed). The raw sequences were submitted to the following three cleaning procedures: (i) no undefined bases (Ns), (ii) a minimum sequence length of 200 bp, and (iii) no sequencing error in the forward and reverse primers. Putative chimeras were detected by UCHIME (see https://www.drive5.com/uchime/uchime_download.html; Edgar *et al.* 2011). The combination of these cleaning procedures with a sequencing depth (2 × 250 bp) covering all the amplicon length (~220 bp without the primers) allowed duplicate sequences of each base and drastically minimised sequencing errors. After this cleaning step and demultiplexing process, the remaining sequences were clustered at a 95% similarity threshold with UPARSE 7.0 option -cluster_fast (Edgar 2013) to obtain the seed operational taxonomic units (OTUs). Delineating the similarity threshold was previously discussed in Capo *et al.* (2016), and limits the artificial inflation of OTU numbers.

The taxonomic affiliation was performed by BLAST against the SSURef SILVA database (see https://www.arb-silva.de/; Pruesse *et al.* 2007) after applying the following selection criteria: length >1200 bp, quality score >75% and a pintail value >50; SILVA database was enriched by aquatic DNA sequences originating from various studies. The taxonomy of an OTU corresponded to the best hit given by the similarity search. If an OTU was associated with several best hits (hits having the same identity), then the affiliation was the common taxonomy of these hits defined as the lowest common ancestor. Operational taxonomic units affiliated to metazoans were removed from these analyses so as to consider only microbial eukaryotes (protist, algal and fungi). Functional trophic groups were attributed to OTUs on the basis of their estimated taxonomy. Most of the information on the trophic classification of microeukaryotes was obtained from specialised literature available for each taxon (Adl *et al.* 2019).

After these different steps (cleaning, clustering and taxonomic affiliation), 2 805 188 quality-filter DNA sequences were clustered in 1622 OTUs classified as microbial eukaryotes. The number of clean DNA reads varied from 58 650 to 145 428 depending on the sample. To perform comparisons between samples, the number of DNA sequences was standardised at 58 650 for each sample.

Data analysis

Chemical variables and microbial components were sampled three times along the experiment (0, 48 and 120 h), and the differences in mean values between treatments were compared using a one-way repeated-measures analysis of variance (ANOVAR) using Statistica software (ver. 7, Statsoft France, see http://www.statsoft.fr/logiciels/logiciels.htm). The analysis compared the means of different treatments (C, E, N and D) using several sampling times, assuming that, in each experimental unit, samples were not independent (Winer 1971). The within-treatment variables were time and the interactions between time and treatment effects. All samples were considered in the analyses except those from Day 0, since no significant difference was detected among mesocosms for any parameters (ANOVA; $P = 0.0000$). To complete this analysis, *post hoc* Scheffé tests were performed to compare the responses among treatments. To test whether ANOVA assumptions were reached we used (i) the Shapiro–Wilk test for distribution normality of residuals and (ii) Bartlett's test for the equality of variances among the treatments we were using. These conditions were always reached when data were log-transformed ($\log(x + 1)$).

So as to perform comparisons among samples, the number of DNA sequences from *18S* metabarcoding analysis was standardised at 58 650 for each sample. Bray–Curtis dissimilarity index was used to compare molecular inventories obtained for each treatment and hierarchical clustering was performed to highlight the differences obtained among treatments.

To better evaluate the relationships between the abiotic (nutrients) and biotic variables (different plankton components) throughout the experiment, principal component analysis (PCA) was performed on the data measured at 48 h and 120 h, using Primer 7 Software. Data at 0 h were not considered in the analysis, being similar for every treatment (ANOVA; $P = 0.0000$). The data were normalised for each variable by subtracting their mean and dividing by their standard deviation. This allowed to derive meaningful distances among samples, using Euclidean distance using PRIMER software.

Results

Variations in physicochemical parameters

Diuron concentrations measured by LC-MS remained similar among the treated mesocosms after 5 days (1.0 ± 0.1 µg L^{-1}), thus revealing a high persistence of the herbicide in the mesocosms. Inversely, naphthalene was not detected by GC-MS after 5 days in any treated mesocosm (initial concentration of 24 µg L^{-1}).

The temperature within the mesocosms varied during the experiment, with a marked day–night cycle ranging from 26.2 to 31.3°C. Salinity did not vary during the experiment and remained close to 35 (see table 1 in Bouvy *et al.* 2016).

The initial nutrient concentrations in the sampled lagoon water were very low for orthophosphate (0.019 ± 0.004 µM), for ammonium (0.046 ± 0.001 µM) and for nitrate plus nitrite (0.253 ± 0.030 µM; Table 1). Additions of nutrients at 24 h and 96 h in E, D and N mesocosms were above natural concentrations (compared to C microcosm). For orthophosphate and nitrate–nitrite concentrations, the difference of concentrations between the initial and final concentrations, after 120 h, clearly increased with values up to 164 and 75× more respectively, and were similar in all the treatments. Ammonium concentrations increased up to 243 times more in D treatment, whereas the increase was lower (69.2×) in N treatment. However, no significant difference (*post hoc* tests, ANOVAR, $P > 0.05$) was observed among E, D and N treatments (Table 3).

Concentrations of particulate organic nitrogen (PON) and particulate organic carbon (POC) were high in all treatments after 120 h of experiment, except in the D treatment with a decrease of POC. The highest increases for POC and PON were observed in N treatment mesocosms (Table 1). The C : N ratio decreased throughout the experiment in all treatments, with the lowest values exhibited in the N treatment.

Picoplankton dynamics

The initial abundance of heterotrophic prokaryotic cells (H-Prok) was $5.19 \times 10^9 \pm 2.69 \times 10^8$ cells L^{-1} and comparable between nutrient alone (E) and control (C) samples, with a 33% loss being observed after 120 h (Fig. 2). The incubation with diuron and nutrient (D) did not cause significant differences in abundance compared with E and C treatments after 120 h (Table 2). In contrast, the addition of naphthalene and nutrient (N) triggered a significant increase in abundance (28% after 120 h), as compared with the nutrient treatment alone (E; Fig. 2, Table 4).

The production of heterotrophic prokaryotes, estimated from thymidine incorporation rates (Thym), was initially 170 pmol L^{-1} h^{-1} and showed a decrease in C treatment (87 pmol L^{-1} h^{-1} after 120 h; Fig. 2). In other treatments with the presence of nutrients (E, D and N mesocosms), a decrease was noted after 48 h; however, in D and N treatments, incorporation rates were low after 120 h, compared with the nutrient-alone treatment (E; Fig. 2). These temporal changes in the presence of nutrients were, nevertheless, not statistically different from one treatment to another ($P > 0.05$; Table 2).

The abundance of picoeukaryotes was low, with initial values being close to 3.6×10^4 cells L^{-1} (Fig. 2). In controls (C), abundances increased at 24 h (3.09× more), and then decreased after 120 h (5.2×10^4 cells L^{-1}). An increase was observed in all other treatments (E, D and N) at 24 h, especially in the E treatment (116× more) as compared with the control (Fig. 2). After 24 h, the abundances decreased until 120 h, without significant difference among the treatments with nutrients (Table 2). The abundance of picocyanobacteria was much higher, with an initial value of 1.94×10^5 cells L^{-1} for *Prochlorococcus* and 2.23×10^7 cells L^{-1} for *Synechococcus*. No significant differences were observed among the treatments for *Prochlorococcus* abundances (Table 2). *Synechococcus* abundances showed a clear increase in all treatments after 24 h of incubation, followed by a decrease until 96 h, before a new increase at 120 h (Fig. 2), especially for N treatment (significant difference with E; Table 2).

Phytoplankton biomass and microalgae dynamics

Significant ($P < 0.05$) increases of total Chl-*a* concentrations were noticed in D and N treatments (Table 2), particularly marked in N treatment with an increase of value from 3.10 ± 0.41 µg L^{-1} at the beginning of the incubation to 8.26 ± 0.07 µg L^{-1} at 120 h (2.66× increase), compared with the other treatments (Tables 1, 2). In controls without added nutrients, the lowest concentrations of Chl-*a* were reported, below the initial concentrations (0.32× less). The fraction <3 µm represented 35% of total Chl-*a* in N treatment, with a 72× increase between the initial and final experiment times (Table 1).

The addition of nutrients during the experiment resulted in a significant ($P < 0.05$) increase in microalgae abundances after 48 h, in the presence or absence of a contaminant (E, D and N treatments, Table 3. Afterwards, the cell densities decreased in all mesocosms, with no statistical difference ($P > 0.05$) being observed among the treatments (Table 2). At time zero, algal communities were characterised by a cellular dominance of Bacillariophyceae (mean of triplicates: 96.99%), whereas Dinophyceae represented only 1.64% of the microalgal community, followed by Cyanobacteria (0.53%) and Cryptophyceae (0.83%; Fig. 3). A shift in algal groups occurred after 120 h, with Bacillariophyceae being negatively affected by contaminant exposure (68.4 and 52.3% for D and N treatments respectively), whereas Crytophyceae increased in abundance concomitantly (18.9 and 28.9% for D and N treatments respectively). The Shannon index *H'* followed an inverse pattern from the one observed for total microalgal densities (Table 3). The initial *H'* value was low (0.233) because of the large dominance of *Chaetoceros tenuissimus*, accounting for more than 95% of the

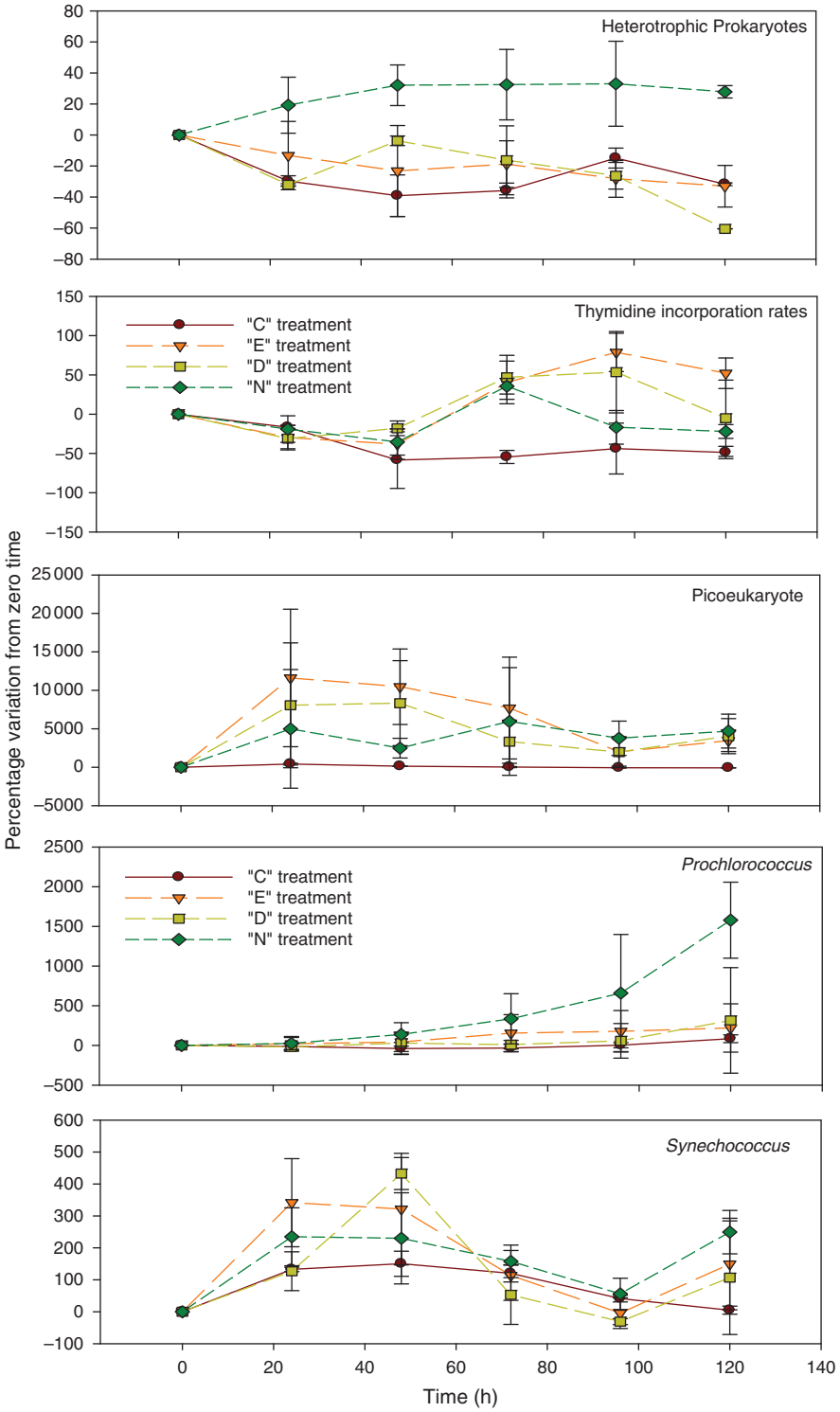

Fig. 2. Temporal variation (expressed as percentage from zero time) of thymidine incorporations rates, and abundances of heterotrophic prokaryotes, picoeucaryotes, and the two picocyanobacteria (*Prochlorococcus* and *Synechococcus*) for the different mesocosm treatments. C, control; E, nutrient enriched; D, nutrient enriched plus diuron; N, nutrient enriched plus naphthalene.

microalgal community, and decreased at 48 h in all experimental conditions. After 120 h, an increase in *H'* values was observed in all treatments (close to 1.2, Table 3). However, no significant differences were observed among the treatments ($P > 0.05$; Table 2). The Bacillariophyceae species *Chaetoceros tenuissimus* was the dominant organism in all treatments, with occurrences

Table 2. Output of one-way repeated-measures ANOVAR for among-treatment effects (C, E, D, N) and within-treatment effect (time, *T*) on chemical and microbiological component variables and of one-way ANOVA for among-treatment effects on zooplankton variables at t_{120}

Significance levels for treatment (Treat), Time and Time × Treat interaction and *post hoc* tests (Fisher LSD) for the differences among treatments are indicated (homogeneous groups with the same letter). Degree of freedom for error is 8. Degrees of freedom for treatment, time and time × treatment interaction are 3, 2, 6 respectively for the variables nutrients, chlorophyll, ciliates and algae. For the other microbial components, degrees of freedom for treatment, time and time × treatment interaction are 3, 5 and 15 respectively. Degree of freedom for treatment is 3 for zooplankton variables. Statistical significances after Bonferroni correction for multiple comparison are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant

Parameter				*Post hoc* for treatment effects			
	Treat	Time	Time × Treat	C	E	D	N
PO_4^{3-} concentration	***	***	***	a	b	bc	c
$NO_3^- + NO_2^-$ concentration	***	***	**	a	b	b	b
NH_4^+ concentration	***	***	**	a	b	b	b
Chlorophyll-*a* amount	***	***	n.s.	a	b	bc	c
H-prokaryotes abundance	**	n.s.	n.s.	a	a	a	b
Picoeukaryotes abundance	***	n.s.	n.s.	a	b	b	b
Prochlorococcus abundance	n.s.	n.s.	n.s.	a	a	a	a
Synechococcus abundance	*	***	n.s.	a	b	a	c
Thymidine incorporation rates	n.s.	*	n.s.	a	b	b	b
Ciliate abundance	*	***	***	a	b	b	b
Aloricates abundance	**	***	***	a	b	b	b
Percentage aloricates	*	*	n.s.	a	b	b	b
Microalgae abundance	**	***	**	a	b	b	b
Shannon index	n.s.	***	n.s.	a	a	a	a
Zooplankton taxa number	n.s.	–	–	a	a	a	a
Zooplankton diversity	n.s.	–	–	a	a	a	a
Zooplankton abundance	n.s.	–	–	a	a	a	a
Percentage copepods abundance	n.s.	–	–	a	a	a	a
Percentage cyclopids abundance	n.s.	–	–	a	a	a	a
Percentage calanoids abundance	n.s.	–	–	a	a	a	a
Percentage harpacticoids abundance	n.s.	–	–	a	a	a	a
Percentage nauplii abundance	**	–	–	a	b	ab	a

Table 3. Abundance of microphytoplankton (cell size >5 µm; 10^6 cells L^{-1}), Shannon index, ciliate abundance (cells L^{-1}) and aloricate percentage of ciliates for the different treatments along the experiment time (h)

Treatments: C, control; E, enriched; D, enriched plus diuron; N, enriched plus naphthalene

Parameter	C treatment		E treatment		D treatment		N treatment	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Algal abundance (10^6 cells L^{-1})								
0 h	2.20	(0.48)	2.20	(0.48)	2.20	(0.48)	2.20	(0.48)
48 h	3.36	(2.76)	23.6	(5.33)	25.7	(7.18)	18.8	(6.74)
120 h	0.27	(0.15)	1.44	(0.42)	0.55	(0.17)	10.5	(11.7)
Shannon index								
0 h	0.233	(0.046)	0.233	(0.046)	0.233	(0.046)	0.233	(0.046)
48 h	0.107	(0.026)	0.038	(0.010)	0.030	(0.010)	0.043	(0.004)
120 h	0.786	(0.405)	0.980	(0.261)	1.278	(0.112)	1.209	(0.288)
Ciliate abundance (cells L^{-1})								
0 h	1104	(235)	1104	(235)	1104	(235)	1104	(235)
48 h	2473	(308)	2293	(1301)	3240	(1242)	2693	(554)
120 h	1420	(134)	4507	(1807)	2100	(1833)	1152	(902)
Aloricate percentage								
0 h	95.8		95.8		95.8		95.8	
48 h	48.2		75		79.4		83	
120 h	57.8		100		99.7		99.1	

being higher than 10%, regardless of the incubation time. *Hemiselmis* sp. of Cryptophyceae and *Protoperidinium quinquecorne* of Dinophyceae were notably abundant (>10%; Fig. 4) after 120 h in the two contaminated treatments (D and N). A centric diatom (*Cerataulina* sp.-like) developed in the enriched (E) and diuron (D) treatments after 120 h (Fig. 4).

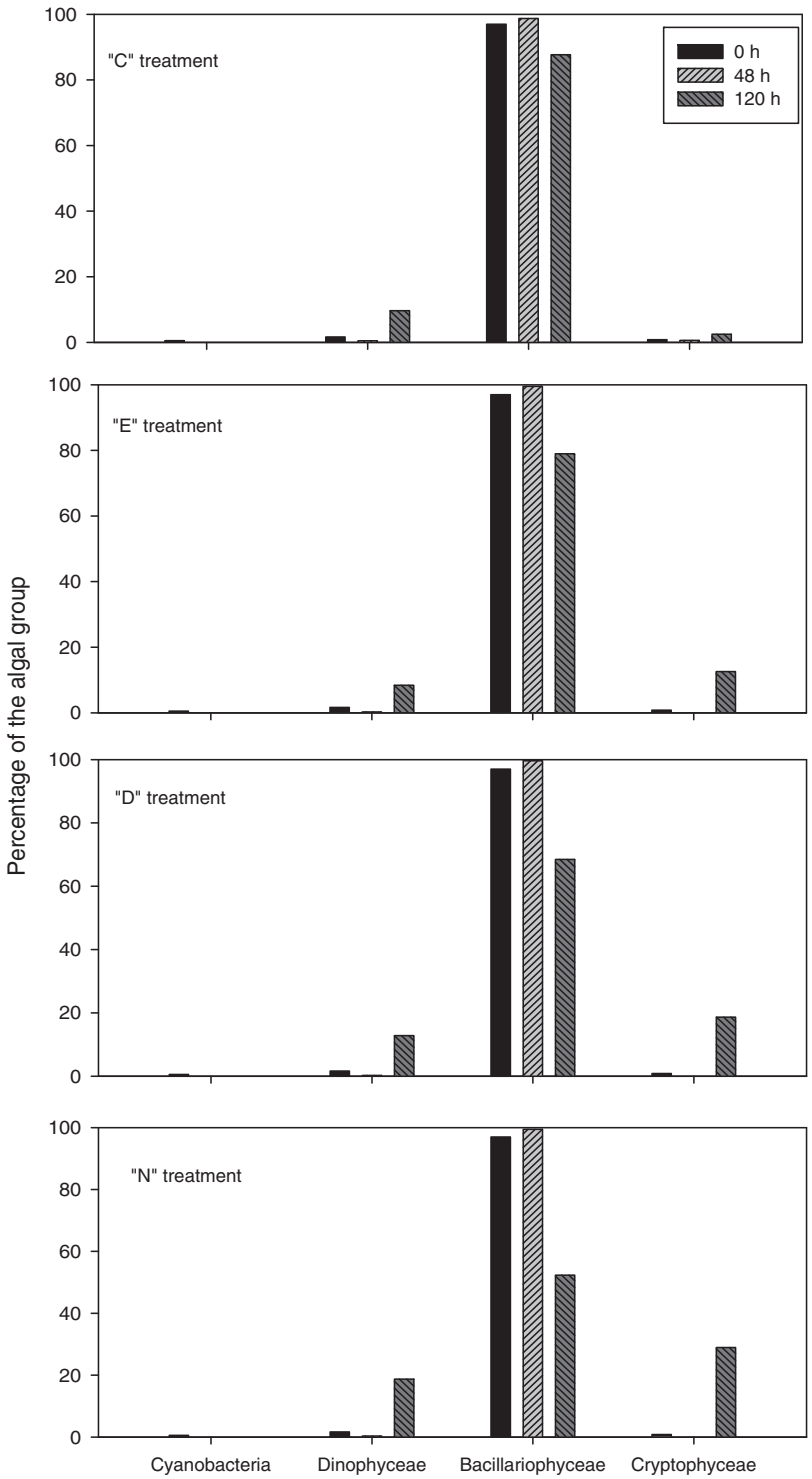

Fig. 3. Percentages of the four main microalgal groups (determined from microscopic counts) during the experimental time (0, 48 and 120 h) for the different mesocosm treatments. C, control; E, nutrient enriched; D, nutrient enriched plus diuron; N, nutrient enriched plus naphthalene.

Protozoa dynamics

Heterotrophic nanoflagellates (HNF) were collected only at two time-points (48 and 120 h) because of required sample conservation. Cell densities of HNF decreased from 48 h onward, starting at 5.1×10^6 ($\pm 2.35 \times 10^5$ cells L^{-1}) and ending at 7.90×10^5 ($\pm 2.96 \times 10^5$ cells L^{-1}) in D treatment, and from 6.2×10^6

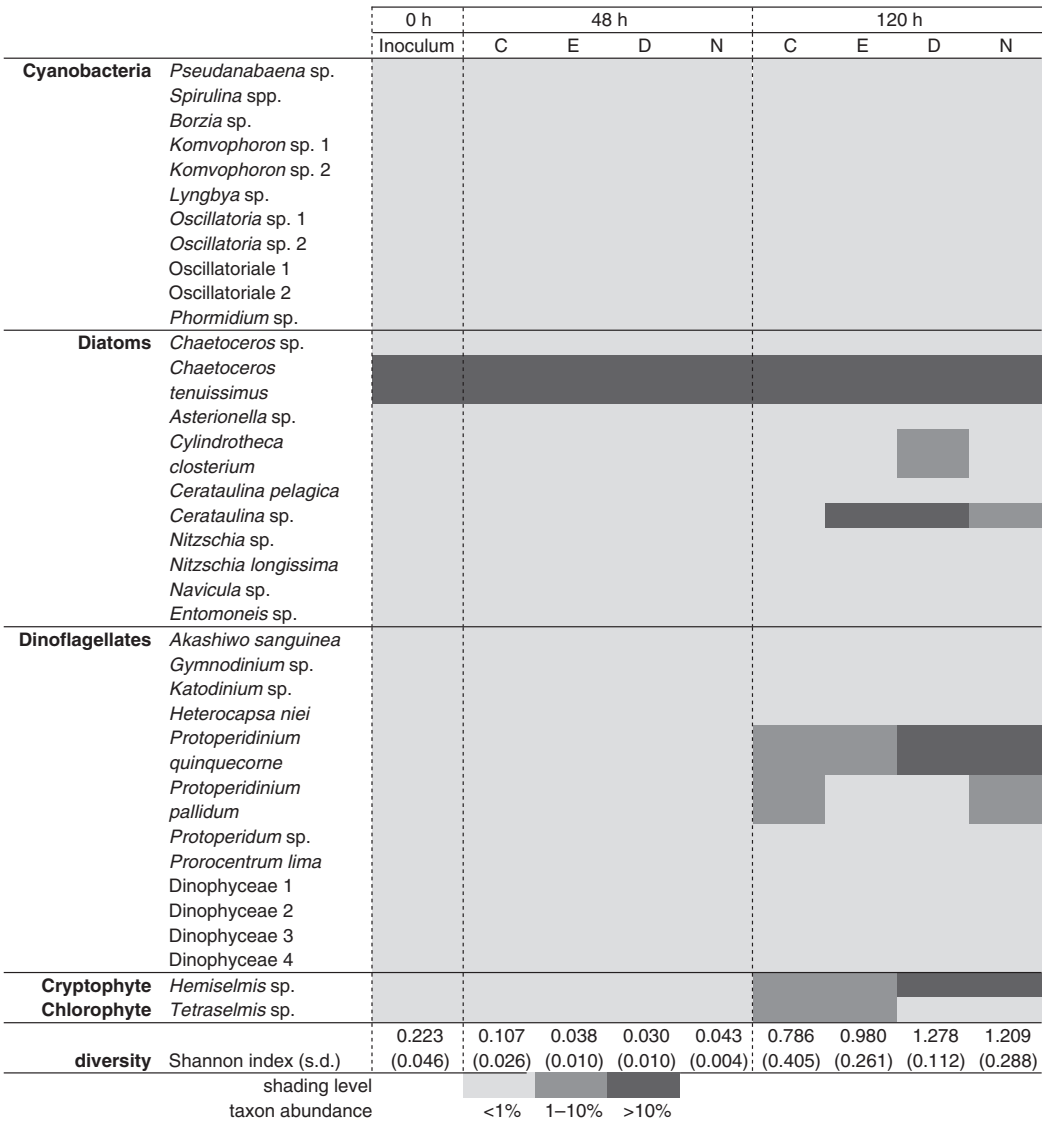

Fig. 4. Heatmap of the contribution (based on triplicates) of microalgal species for the different mesocosm treatments. C, control; E, nutrient enriched; D, nutrient enriched plus diuron; N, nutrient enriched plus naphthalene. Shading in the boxes indicates the percentage of total microalgal density represented by each taxon. Identification was made at the lowest level possible; numbers after genus or class indicate different but unidentified taxa. Shannon indexes are reported (triplicates). Shading legend is indicated at the bottom.

($\pm 3.6 \times 10^5$ cells L^{-1}) to 1.5×10^6 ($\pm 5.67 \times 10^5$ cells L^{-1}) in N treatment. Values in the E treatment were close to those in the D treatment. Significant ($P < 0.05$) differences were noted between the treatments (E, D and N) and the controls (C) after 120 h.

Initial abundance of ciliates was of 1104 individuals L^{-1}, with a majority of aloricate forms (95.8% of total), belonging to the genera *Strombidium* and *Strombidinopsis* (Table 3). Ciliate abundance was noticeably higher in all treatments in the presence of nutrient (significant differences from the control, Table 2), with values up to 4507 individuals L^{-1} after 120 h in the E treatment (Table 2). Ciliate abundances were low in the presence of the contaminant after 120 h, as compared with the enrichment-alone condition (2100 and 1153 individuals L^{-1} in D and N treatments respectively), with there being no significant differences among the treatments ($P > 0.05$, Table 2). Marked differences also occurred in the ciliate community composition, with an increase of loricate forms in the controls (51.64%), with the species *Metacylis jörgensenii* and the genus *Propectella*, as compared with initial samples composed mainly of the genus *Strombidium*. In the other treatments (E, D and N), aloricate forms (see below) represented close to 100% of ciliates after 120 h, with Uronematidae and *Euplotes* spp. (Table 3).

Community structure of microbial eukaryote assemblages (protist, algae and fungi)

In total, 1622 OTUs were retrieved from all the samples analysed (at 0 h and 120 h for the four treatments, in triplicate). Most of these OTUs were represented by a very low number of DNA reads

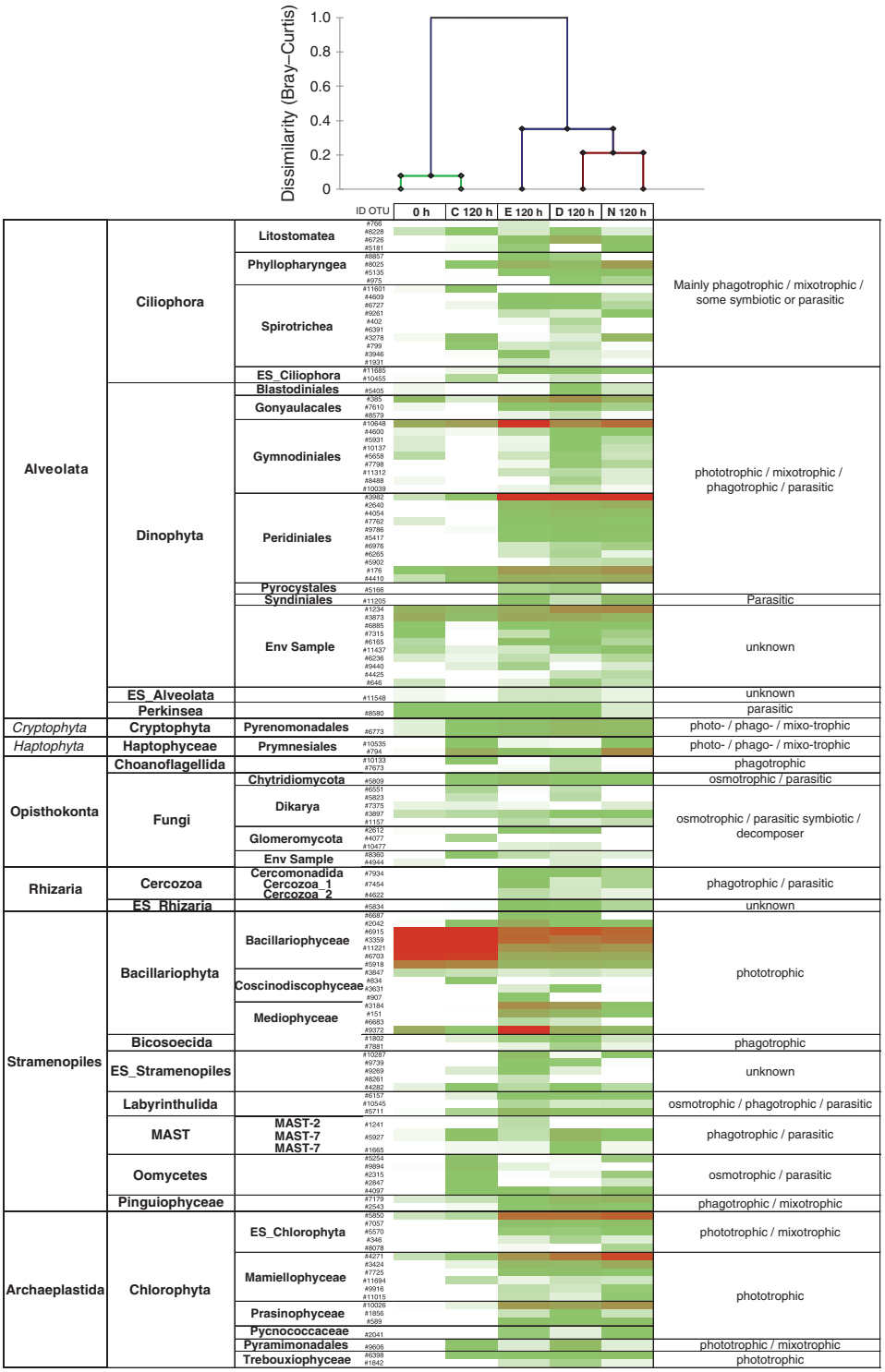

Fig. 5. Structure of microbial eukaryote assemblages (based on *18S* rRNA sequencing): Bray–Curtis dissimilarity and hierarchical clustering tree, showing the relative abundance (heat map) of the dominant operational taxonomic units (OTUs). Main functional groups are mentioned in the right column. 'ES' or 'Env samples' referred to OTUs that were not taxonomically assigned precisely to a genus or a species, and matched to DNA references records from environmental samples. Only the dominant OTUs (accounting for at least 0.1% of the total DNA reads in one of the sample) were considered here.

and were considered 'permanently rare', because they never reached 0.1% of the total amount of DNA reads, regardless of the treatment. We focused the analysis on the 132 remaining OTUs that reached at least the threshold of 0.1% of the total DNA reads in one of the analysed samples. This filtering strategy aimed at removing low-abundance OTUs for which a higher probability of errors of sequencing is recognised (e.g. Bokulich *et al.* 2013), with erroneous reads being associated with diverse but rare events. Some of these OTUs were always dominant (e.g. OTUs #10648 and #176 within Peridiniales (Dinophyta), OTU #6915 within diatoms (Bacillariophyta), OTU #4271 within Mamiellophyceae (Chlorophyta)), but most of them shifted from rare to dominant OTU according to time and treatments. The comparison of the structure of microbial eukaryote assemblages (Bray–Curtis indices and hierarchical clustering tree) showed a low dissimilarity in the controls (C) between initial and final conditions, whereas the other treatments were clearly distinguishable over time (Fig. 5). Marked temporal changes in the proportion of OTUs were detected for all taxonomic groups and all types of functional groups (i.e. phagotrophic, phototrophic, mixotrophic, parasitic, for example).

Among the major rearrangements observed at the final sampling time, we noticed a general decrease in DNA reads associated with pigmented taxa, whereas DNA reads associated with non-pigmented taxa dominated. The proportion of Ciliophora DNA reads clearly increased in all treatments in comparison to initial conditions, with specificities in terms of OTU proportions according to the treatments. Other non-pigmented groups known to be from the phagotrophic taxa exhibited a similar pattern (i.e. increase at the final sampling time in all treatments), such as, for instance, Bicosocieda, MAST-7, and Labyrinthulida.

The proportion of DNA reads affiliated to parasitic groups varied over time and according to the treatment. A general increase of Chytridiomycota, known as diatom parasites, was observed in all treatments at the final sampling time, whereas the relative proportion of Bacillariophyta decreased at the same incubation time (in particular in E, D and N treatments). Other taxonomic groups containing parasites increased at the final timepoint, in particular for the E, D and N treatments; this was the case for Syndiniales and Cercozoa, whereas C treatment was marked by the presence of Oomycetes (known to contain parasitic or osmotrophic taxa). The increase in the proportion of Syndiniales in E, D and N treatments was concomitant with increases in the abundance of their putative hosts (in particular pigmented Dinophyceae).

Among heterotrophic taxa, fungal groups were represented by OTUs belonging not only to Chytridiomycota, but also to Dikarya, with both Ascomycota and Basidiomycota; a few OTUs were also within the group of Glomeromycota. The rearrangement of fungal diversity over time was observed for all those groups. Some OTUs were more particularly represented in contaminated treatments at the end of the experiment (e.g. Ascomycota OTU #3897 increased in N treatments), whereas other OTUs decreased in N treatments (e.g. Glomeromycota OTU #2612).

Marked rearrangements within the dominant pigmented taxa were observed depending on treatments;the proportion of Bacillariophycea remained high at the final timepoint in the control treatment (C), whereas a reduction was noticed with the other treatments, along with an increase of DNA reads assigned to Dinophyceae (Peridiniales in particular) and Chlorophyta (mainly Mamiellophyceae and Prasinophyceae; Fig. 5). Modifications of microbial eukaryotic assemblages were primarily due to the emergence of OTUs that were in low abundance when the experiment began.

Metazooplankton dynamics

Forty metazooplankton taxa were enumerated in the different mesocosms, including 26 copepods, four gelatinous taxa, three other holoplanktonic groups (molluscs, *Argulus* and *Noctiluca*) and seven meroplankton taxa (larvae of gastropods, bivalves, decapods, euphausiids, polychaetes, asteroids and fish eggs). The most abundant copepods were the cyclopoids *Oncaea* spp., *Corycaeus* spp. and *Oithona* spp. Among the other copepods, harpacticoids were mostly represented by species belonging to the genera *Euterpina*, *Clytemnestra*, *Microsetella* and *Tisbe*, whereas the most abundant calanoids belonged to the genera *Paracalanus*, *Clausocalanus*, *Acartia*, *Centropages*, *Temora* and *Pseudodiaptomus* (Table S1 of the Supplementary material).

The mean number of taxa per microcosm (18 ± 2 at the beginning of the experiment) was significantly ($P < 0.05$, Student's *t*-test) decreased in E, D and N treatments at the final sampling time (6.7 ± 0.6, 8.7 ± 2.1 and 10.0 ± 1.0 respectively), but not in the control ($P = 0.101$). Initially, the mean of total zooplankton abundance was 7133 ± 700 individuals m^{-3} in the different mesocosms, and then significantly ($P < 0.01$, Student's *t*-test) decreased down to <3000 individuals m^{-3} in E, D and N treatments (Fig. 6), but did not significantly vary in the control because of high variability among the mesocosms ($P = 0.07$). The percentage of copepod (cyclopids, calanoids and harpacticoids) abundance did not change significantly ($P > 0.05$, Student's *t*-test) between t_0 and t_{120} in any of the treatments, whereas the proportion of nauplii copepods significantly decreased in E and D treatments ($P < 0.05$).

Relationship between abiotic and biotic variables: PCA

The PCA was performed on an independent dataset composed of 13 chemical and biological descriptors measured at 48 and 120 h (Fig. 7). The first two eigenvalues of the PCA analysis accounted for 59% of the total variability. The analysis considered only these two first axes to highlight the relationship among descriptors. Three clusters linked to treatments could be defined. On the first axis (38.3% of total variance), all the samplings performed at 48 h were opposed to the samplings performed at 120 h, whatever the treatment (N, D and E mesocosms). On the contrary, the second axis (20.5% of total variance) showed a defined cluster for all samplings performed in C mesocosms, whatever the incubation time. Samples from E, D and N mesocosms at 120 h were positively correlated with nutrients (PO_4, NO_x, NH_4), and with thymidine production (Thym) and ciliate abundances, to a lesser extent (Fig. 7). Samples from E, D and N mesocosms at 48 h were positively correlated with zooplankton, HNF, microalgae, Chl-*a*, picoeukaryotes and heterotrophic prokaryotes. Spearman correlations (Table 4) highlighted a significant positive correlation among HNF (heterotrophic nanoflagellates), microalgae and zooplankton; these latter three biological descriptors were negatively correlated with the three nutritive descriptors. On the other hand, picoeukaryotes and Thym were positively correlated with two nutritive descriptors (PO_4, NH_4).

Discussion

Methodological considerations

In situ environmental conditions in the inoculum from the Juan de Nova lagoon were characterised by very low nutrient concentrations, consistent with previous observations (Bouvy *et al.* 2016). Without nutrient addition, C treatment exhibited the lowest concentrations of Chl-*a*, below the initial concentration (factor of 0.32), showing a phytoplankton growth limitation by nutritive supply and subsequent biomass decay (Guildford and Hecky 2000). The goal of nutrient enrichment after 24 and 96 h

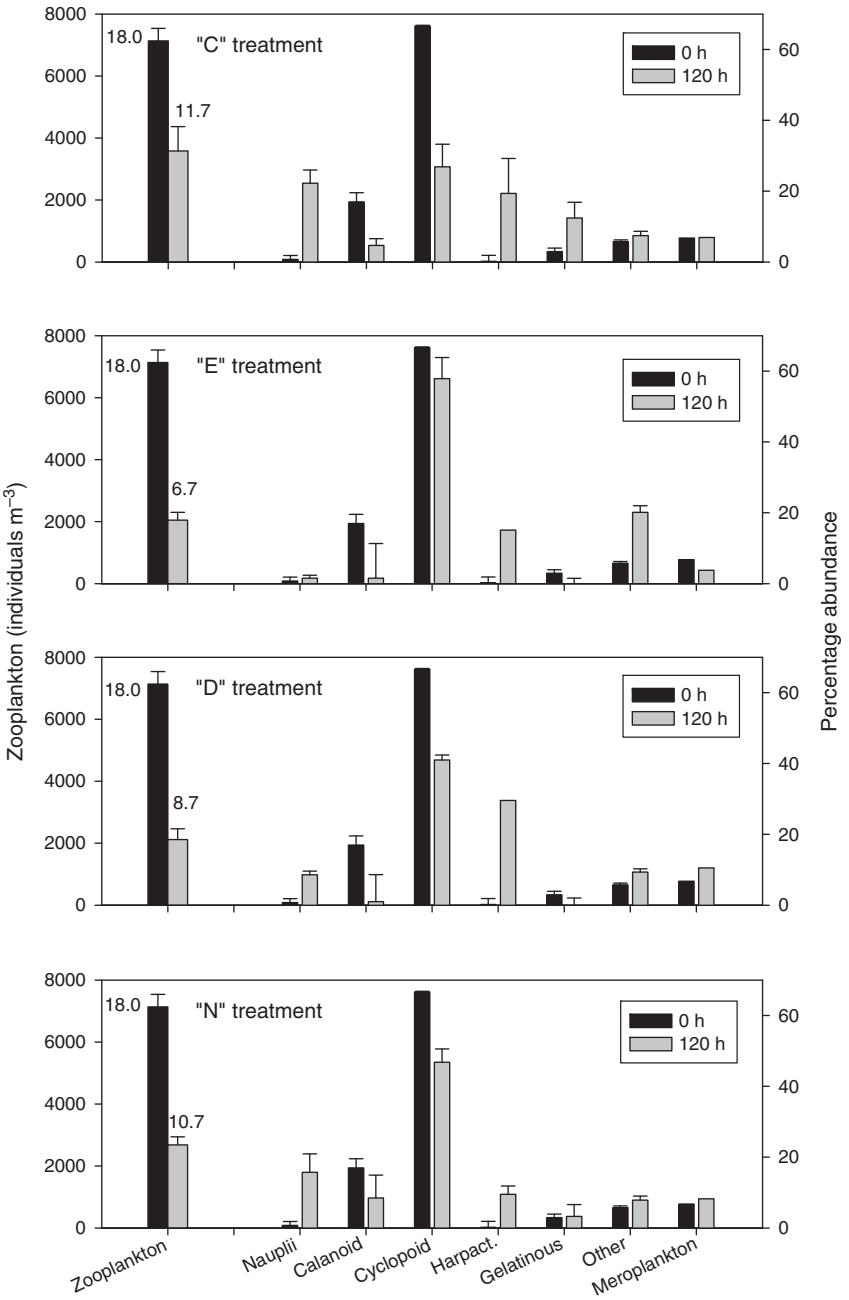

Fig. 6. Total metazooplankton abundance (individuals m^{-3}, left *y*-axis) and percentages of the main zooplankton groups (right *y*-axis) at 0 and 120 h in the different treatments. The numbers close to the bars of zooplankton abundance indicate the mean number of zooplankton taxa per microcosm. C, control; E, nutrient enriched; D, nutrient enriched plus diuron; N, nutrient enriched plus naphthalene.

was to study the effects alone of adding a contaminant (diuron or naphthalene) on microorganisms and not a cumulative effect of the contaminant and a nutrient deficit on microorganisms.

The duration of the experiment (5 days) was long enough to span more than a complete life cycle for organisms, both for smaller microbial organisms and metazooplanktonic grazers (Pagano *et al.* 2000; Leboulanger *et al.* 2011). This duration is a compromise, making it possible to have a response close to that observed in a natural environment, while minimising the effects of confinement, in incubation structures without water renewal (Roman and Rublee 1980). The present experiment was conducted as a short-term estimation (5 days) of the acute toxic effects of the tested contaminants, as compared with a long-term exposure in mesocosms that can detect modifications in biological successions (Hjorth *et al.* 2008; Tadonléké *et al.* 2009; Knauer *et al.* 2010).

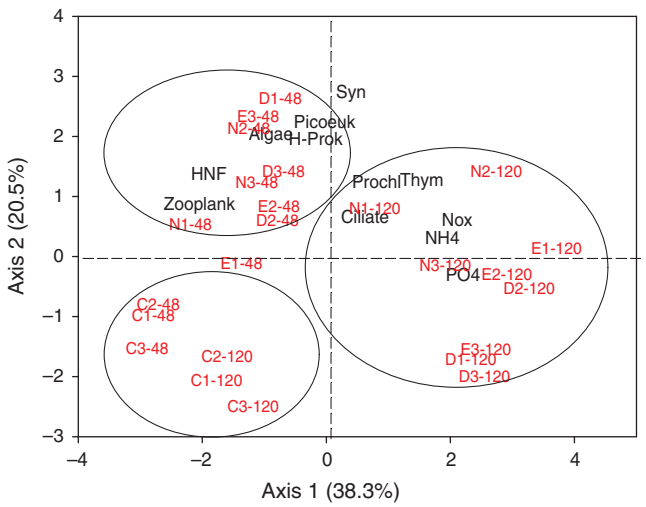

Fig. 7. Principal component analysis (PCA) on the two first axes. Eigenvalues for each axis of the PCA are reported. For each triplicate, two incubation times are reported in the PCA (48 and 120 h). C, control; N, enriched; D, enriched plus diuron; N, enriched plus naphthalene. Abbreviations of the descriptors are s in Table 4.

Nutrient and diuron effects

Contaminant addition (D and N treatments) resulted in a significant increase in chlorophyll biomass after 48 h, as compared with the C and E treatments (Table 2), followed by a net decrease after 120 h, suggesting a direct negative effect of the contaminant. Diuron addition can explain the chlorophyll decrease knowing that phototrophic organisms are sensitive to this herbicide at the tested concentrations during a similar five days exposure (Leboulanger *et al.* 2011). Microalgal concentrations followed the same pattern as described for chlorophyll biomass in the presence of diuron with a decrease after 120 h, suggesting that the increase in chlorophyll concentration of phytoplankton cells observed at 48 h can counteract their decrease in abundance related to herbicide inhibition.

Regarding the heterotrophic compartments, diuron did not cause any significant direct effect on abundance or production of heterotrophic prokaryotes (after 120 h), as supported by the experiments reported by Leboulanger *et al.* (2011) for tropical planktonic bacterioplankton in reservoirs. Diuron addition provoked an initial growth phase of protozoa (HNF and ciliates) at 48 h, followed by an obvious decline after 120 h. Staley *et al.* (2015) showed inconsistent direct effects on protozoans in the presence of herbicides, and concluded that indirect effects of these compounds, particularly on heterotrophic predators, may outweigh direct negative effects. No firm conclusions can be drawn with regard to the potential impact of diuron on the HNF compartment because a similar pattern of increase in abundance of these organisms was observed in the E and D groups. Persistent ciliates represent more opportunistic taxa (i.e. *Euplotes* sp. and Uronemadidae) that are bacterivorous and prefer organic matter-rich systems; their presence may also be explained by the death of

Table 4. Spearman correlation coefficient between abiotic and biotic variables measured in all treatments at 0 h (t_0), 48 h (t_{48}) and 120 h (t_{120}) incubation times

Significant values ($P < 0.05$) are noted in bold. PO_4, orthophosphate; NO_x, oxidised nitrogen (nitrate + nitrite); NH_4, ammonium; Chl-*a*, chlorophyll-*a*; HNF, heterotrophic nanoflagellate; H-Prok, heterotrophic prokaryotes; Picoeuk, picoeukaryotes; Prochl, *Prochlorococcus*; Syn, *Synechococcus*; Thym, thymidine incorporation rates; Algae, microalgae; Zooplank, zooplankton

Parameter	PO_4	NO_x	NH_4	Chl-*a*	HNF	H-Prok	Picoeuk	Prochl	Syn	Thym	Ciliates	Algae	Zooplank
PO_4	1.000												
NO_x	**0.942**	1.000											
NH_4	**0.805**	**0.757**	1.000										
Chl-*a*	0.168	0.225	0.277	1.000									
HNF	**–0.610**	**–0.574**	**–0.542**	**0.692**	1.000								
H-Prok	–0.121	0.007	–0.327	0.348	**0.447**	1.000							
Picoeuk	**0.467**	0.370	**0.489**	0.617	0.337	0.003	1.000						
Prochl	0.060	0.086	0.157	0.119	0.082	**0.430**	–0.079	1.000					
Syn	0.115	0.185	0.097	**0.593**	0.276	**0.412**	**0.508**	–0.011	1.000				
Thym	**0.450**	**0.474**	**0.465**	0.234	–0.302	0.197	0.306	0.382	**0.419**	1.000			
Ciliates	0.291	0.208	0.340	0.224	0.061	–0.256	0.344	–0.037	0.068	0.368	1.000		
Algae	–0.084	–0.122	0.073	**0.795**	**0.747**	0.203	**0.634**	0.097	**0.463**	0.147	0.316	1.000	
Zooplank	**–0.604**	**–0.678**	**–0.543**	0.103	**0.816**	0.095	0.055	–0.075	–0.027	–0.261	0.096	0.423	1.000

different planktonic predators. Indeed, metazooplankton abundance also followed the same tendency owing to a negative effect of diuron, certainly being linked to cascading effects inside the food chain (Leboulanger *et al.* 2011).

Nutrient and naphthalene effects

Naphthalene exposure did not result in visible toxic effects to photoautotrophic communities after 120 h. The effect on total microalgal abundance was not obvious, without significant differences among the E, D and N treatments (Table 2). The unique and clear increase in photoautotrophic microorganisms concerned the picocyanobacteria, especially *Synechococcus* sp. (Table 2, Fig. 2). Other studies demonstrated that picocyanobacteria cells may accumulate hydrocarbons and then make them available to associated hydrocarbon-utilising bacteria (Radwan *et al.* 2005). In this context, naphthalene exposure resulted in positive and significant effects on heterotrophic prokaryote abundance ($P < 0.05$; Table 2), suggesting an increase in global heterotrophic activity (Lebaron *et al.* 2002). Studies on the effect of naphthalene on freshwater and marine ecosystems are scarce, but the precise functions of bacterial strains in these environments are evoked by Jurelevicius *et al.* (2013). These authors concluded that after 32 days of incubation with naphthalene, in total, 41.3% of bacterial isolates from freshwater, marine and hypersaline were grown with this sole carbon source. Lu *et al.* (2011) concluded that heterotrophic microorganisms can break down naphthalene, using it as a resource in aerobic and anaerobic environments, thus making its effects difficult to predict. Temporal changes in prokaryote and picophytoplankton abundances were therefore complex, with simultaneous positive and negative effects. A close interaction between phytoplankton and bacterioplankton communities can exist, which is linked to the exudation of photosynthetic products that can be easily and rapidly assimilated by bacteria; these products are composed of both high and low molecular-weight material (Biddanda and Benner 1997). Therefore, the production of high molecular-weight compounds could stimulate an increase in heterotrophic respiration and production on short time scales (hours to days), as observed in the microcosm addition experiment (Lønborg *et al.* 2016). Hjorth *et al.* (2007) reported planktonic responses (Danish fjord) to pyrene exposure and nutrient enrichment, concluding that there were positive and indirect effects in bacterial communities as a result of nutrient release after algal death. Brito *et al.* (2015) suggested that petroleum mitigation could be stimulated by bacteria that are influenced by the natural conditions of the affected system, such as the availability of other carbon sources that can be degraded more easily than can petroleum. In fact, in certain conditions, bacteria even have the ability to use hydrocarbons, which are typically resistant to biodegradation, as carbon sources (Garcia *et al.* 1998; Lu *et al.* 2011). Nevertheless, further study of the specific degradation mechanisms used by these bacteria in a minimally affected environment is required to advance on isolating and studying the degradation mechanisms.

Effects of nutrient and contaminant on biodiversity

From a microbial biodiversity perspective, the addition of a contaminant can result not only in a decrease of biomass, but also in a modification of the pre-existing community composition (Clements and Rohr 2009). High-throughput sequencing data showed marked modifications in the relative proportions of the taxa (OTUs), with a discrimination of diuron and naphthalene treatments in comparison to controls (Bray–Curtis dissimilarity). Among the pigmented community, the structure of the Bacillariophyta assemblage seemed to be particularly affected by the addition of nutrients and contaminants, with a general decrease in the proportion (in terms of DNA reads) in comparison to Dinophyta (Peridiniales in particular) and Chlorophyta (essentially Mamiellophyceae and Prasinophyceae), which increased.

However, we cannot exclude the role of parasites in these analyses, which may have affected the dynamics of these pigmented groups. A general increase of Chytridiomycota, a parasite of diatoms, was indeed observed in all treatments. The increase of Syndiniales proportions in enriched and contaminated treatments was concomitant with increases in the proportions of their putative hosts, such as pigmented Dinophyta. Syndiniales consisted of a widespread group of obligate parasites with a broad host spectrum ranging from protists to metazoans (Guillou *et al.* 2008). We hypothesise that host–parasite interactions could have been promoted during the experiment because of the confinement of organisms in enclosed mesocosms (Alves-de-Souza *et al.* 2015).

Detected fungi were not only parasites. There is an extensive diversity of fungi that inhabit a large number of marine habitats, such as deep-sea habitats, pelagic waters, coastal regions and hydrothermal-vent ecosystems (Manohar and Raghukumar 2013). Jones *et al.* (2009) reported more than 500 species of marine fungi belonging to Dikarya (Ascomycota and Basidiomycota). The genetic diversity of marine fungi has been studied only in a few coastal areas, coral-reef regions and mangrove areas; however, these molecular studies reported the presence of fungal sequences belonging to Ascomycota, Basidiomycota, and Chytridiomycota, with the detection of some novel environmental clusters (Manohar and Raghukumar 2013). DNA sequences belonging to Glomeromycota and Zygomycota were also found in seawater at depth (Zhang *et al.* 2015).

In our study, we detected mostly Chytridiomycota, Dikarya (Ascomycota and Basidiomycota*)* and Glomeromycota, with some OTUs of known pathogens for various marine species and others being involved in organic matter degradation (osmotrophy, saprotrophy). We cannot exclude that some of the fungal taxa present in the experimental mesocosms may have modified or even degraded contaminants such as naphthalene (in particular some Ascomycota; Simister *et al.* 2015). For instance, we noticed an increase of the relative proportion of OTU #3897 (Ascomycota, Leotiomycota) in the N treatment. On the contrary, other fungal groups such as Glomeromycota were largely reduced in the presence of naphthalene. The role of Glomeromycota in marine systems is poorly known and has rarely been reported on in marine systems (Song *et al.* 2018; Hassett *et al.* 2020); therefore, we cannot confirm that their presence here was not only due to passive dispersal stages from terrestrial origin (most of the Glomeromycota fungi are known to form mycorrhiza). Further ecological, physiological and molecular studies are needed to characterise the functional role of marine fungi.

Microscopic identification confirmed the very large dominance of Bacillariophyta with *Chaetoceros tenuissimus*, accounting for more than 95% of the microalgae community detected in

all mesocosms. Higher values of Shannon index (*H'*) for microalgae were observed after 120 h of incubation, showing an increase of biodiversity compared with initial conditions; however, no significant differences were observed among the treatments (Table 2). Hjorth *et al.* (2008) reported the same conclusion for phytoplankton response to pyrene exposure and nutrient enrichment, with no significant change of communities relative to control. It can be noted that the Cryptophyceae *Hemiselmis* sp. and the Dinophyceae *Protoperidinium quinquecorne* were notably abundant (>10%) after 120 h in the two contaminated treatments (D and N; Fig. 4). Stachowski-Haberkorn *et al.* (2013) demonstrated the spontaneous long-term adaptation of *Tetraselmis suecica*, exposed to diuron (5 μg L^{-1}), with doubling times 2–2.5-fold longer than for control cultures. By contrast, González *et al.* (2009) concluded that the largest fraction of the coastal phytoplankton assemblage, mainly composed of diatoms, was resistant to the PAH concentrations used (8.6 μg L^{-1} of chrysene equivalents).

The response of non-pigmented micro-eukaryotes to our experimental treatments was also detectable for bacterivorous and algivorous groups, and in particular ciliates affected both quantitatively and qualitatively. The shift observed in the relative importance of loricates and aloricates (decrease of loricate forms in contaminated treatments) demonstrated a modification in the dominant trophic groups, i.e. a shift from herbivore to omnivore taxa. Among metazooplankton, copepods nauplii were the most affected in our study, particularly in treatments with diuron. These negative effects were already established for freshwater calanoid and cyclopoid nauplii (Zimba *et al.* 2002; Perschbacher and Ludwig 2004; Leboulanger *et al.* 2011), showing the negative effect of diuron on the copepod larval recruitment. Although no significant effect of treatment was detected in our experiment for gelatinous organisms because of high variability among mesocosms, this group of organisms completely disappeared from the mesocosms with diuron treatment after 120 h, suggesting a negative effect of this molecule. However, data relative to herbicide chemical compound toxicity for marine gelatinous plankton is, to our knowledge, not available.

Trophic switches throughout the experiment

In aquatic pelagic systems, phytoplankton and bacteria represent the most important producers of particulate organic material from inorganic and dissolved organic sources (Azam *et al.* 1983; Berglund *et al.* 2007; Stibor *et al.* 2019). The organic matter can be transferred to higher trophic levels through different food webs, ranging from the microbial loop to the classic herbivore pathways depending on resource availability and the size of the prey (Jessup *et al.* 2004; Berglund *et al.* 2007). The impact of chemical contamination can be observed from a selection of species inside a biological community, but also from a functional point of view such as the stimulation of heterotrophy *v.* autotrophy (Rochelle-Newall *et al.* 2008*b*; Lekunberri *et al.* 2010). Trophic links between biological compartments can be apprehended in our study from the PCA analysis, highlighting some obvious relationships throughout the experiment. The global multivariate analysis showed an effect of time with a modification of the trophic relationship among planktonic communities, from an omnivorous food web at 48 h with a dominance of microalgae, HNF, picoeukaryotes and metazooplankton, to a reduced microbial loop at 120 h with a high activity of heterotrophic prokaryotes (Thym) linked to high concentrations of nutrients. This trophic shift was observed in the E, D and N treatments (Fig. 7), without significant differences among the treatments, suggesting a weak (absence of?) direct and indirect impact of the contaminant on biological communities. Small environmental changes linked to a stressor (nutrient, contaminant, temperature) resulted in modifications of the trophic structure, recently called 'trophic switches' by Stibor *et al.* (2019). In our study, the apparition of parasite groups among the eukaryotes and the modification of the phytoplankton communities with the Cryptophyceae *Hemiselmis* sp. and the Dinophyceae *Protoperidinium quinquecorne* can explain the trophic switch observed at 120 h in N and D treatments. Stibor *et al.* (2019) reported nutrient-dependent trophic switches when size-selective grazers were confronted with nutrient-dependent shifts in food size.

Our results should be taken with caution because the confinement of the organisms within the microcosm experiments could induce modifications in prey–predator interactions (e.g. host–parasite), owing to changes in the enclosed populations. Hence, community dynamics observed in such experiments also depend on the initial state of communities at the start of contaminant exposure. The present work focuses on the plankton community of a pristine ecosystem, Juan de Nova lagoon, with no expected or documented pollution pressure, showing globally no direct or indirect acute effect of the two contaminant models used (diuron and naphthalene) on planktonic communities. However, a modification of the trophic relationships between communities over time can be observed, going from an active microbial network towards reduced heterotrophic systems, and future investigations should be conducted to explore the possible ecological consequences of such a shift.

Further work is needed to better understand the complex but highly relevant question of contaminant mixtures and multiple stressors, and how they directly and indirectly influence aquatic communities. The risk assessment of these pollutants towards these organisms needs further investigation, such as combining innovative molecular ecological methods and *in situ* analyses at the community level.

Contributors

All co-authors participated in study design, sample analysis, and manuscript elaboration; M. Bouvy, C. Dupuy, P. Got and C. Leboulanger performed the field experiment.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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